Interaction of thiolated amino acids and peptide onto the gold nanoparticle surface: Radical scavenging activity

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The interaction of gold nanoparticles (AuNPs) with thiolated amino acid, L-cysteine, and peptide, glutathione, is reported. Additionally, thioglycolic acid has also been employed to study the effect of other functional groups such as amine on interparticle interaction mechanism. The functionalized AuNPs have been characterized through UV-visible spectroscopy, FTIR analysis and transmission electron microscopy. The plasmon-plasmon interactions amongst the functionalized AuNPs are found to be dependent on ligand concentration, pH and salt environment. The assembly of AuNPs mediated by L-Cys and GSH has been monitored by the evolution of the surface plasmon resonance absorption. The optical spectra of AuNPs is shifted to the red region, indicating dipole-dipole interactions in the AuNPs assembly. The self-assembly is more pronounced in the case of amino acid and tripeptide, due to the zwitterion-type electrostatic interactions between amino acid groups of L-Cys/GSH bound to different NPs. Furthermore, the scavenging behavior of the studied thiolated ligands and the corresponding AuNPs has been studied by using 1,1-diphenyl 2-picryl hydrazil radical. AuNPs significantly reduces the radical scavenging ability of thiolated antioxidants.

Keywords: Interparticle interactions, Plasmon-plasmon interactions, Zwitterion-type electrostatic interactions, Self-assembly, Nanoparticles, Gold nanoparticles, Amino acids, Thiolated amino acids, Peptides, Radical scavenging, Scavenging

Since the discovery of noble metal nanoparticles (NPs) in 1857 by Faraday1, it has found importance in all aspects of science, particularly in nanotechnology2, 3. Among the noble metal nanocrystals, gold is superior to the other elements in terms of biocompatibility, due to its inertness in physiological environment and nontoxic nature4. One of the most significant issues in this field is the conjugation of AuNPs with biomolecules (mostly amino acids and proteins). Bioconjugation of AuNPs provides appropriate functions for various biological applications, such as selective targeting of specific cells. In order to attach the biomolecules on to the AuNP surface, the ligands on the AuNPs surface are exchanged by alkyl thiols that can strongly bind to the proteins5, 6. Another way to prepare bioconjugated AuNPs is the incorporation of biomolecules as surfactants during the particle synthesis7-12. In this case, proteins can be adsorbed on the AuNPs surface either by electrostatic interaction or by Au-S coordination with cysteine moieties13-15. Besides the interaction, proteins can also behave as reducing agents, owing to hydroxyl and thiol groups involved in the amino acid chains9, 11, 12.

Macromolecules of biological origin are considered to be nature’s innate nano-creations15. Recently, the macromolecule assisted synthesis of NPs has been reported in literature16-18, where they act as both reducing and capping agents; the most studied system in this category is AuNPs. In fact, practically every known class of biomolecules (proteins19, 20, peptides21, 22, sugars23, 24, amino acids25-27 and even plant extracts28, 29) have been shown to reduce chloroaurate ions to AuNPs, but the mechanism behind most of these cases is still to be revealed. In many instances, this reductive synthesis leads to in situ biomolecule capped AuNPs that retain the bioactivity of the capping molecule largely17. In view of the above, herein we have selected two of the most studied essential biomolecules, i.e., L-cysteine (L-Cys) and glutathione (GSH). L-Cys is an amino acid with a thiol (-SH) group which is one of the most important part in various proteinous structures and acts as a support to hold the quaternary structure together. GSH is a tripeptide, which comprises three different amino acids out of which cysteine is one of the constituents. GSH is the principle antioxidant found
in mammals and acts as an important physiological defense against oxidative damage. GSH is a redox reactive molecule, which has diverse physiological functions, such as detoxification, conservation of crucial thiol status, antioxidant activity, and regulation of cellular growth and death\(^3\). The dysregulation in cellular GSH level may cause aging\(^3\) and has several biological implications\(^3\), e.g., cholestasis\(^3\), diabetes\(^3\), endotoxemia\(^3\) and drug-resistant tumors\(^3\). In general, reactive oxygen species (ROS) are reduced by cellular GSH which is itself oxidized to GSSG, which is again reduced back to GSH by GSH reductase. L-Cys is a precursor of GSH, which is also believed to play a crucial role in regulating the cellular redox reactions to limit cellular damage due to ROS\(^3\). Therefore, it is important to explore the interaction of L-Cys and GSH with AuNPs. Amine (−NH\(_2\)) and thiol (−SH) groups of L-Cys and GSH may work as a surface regulator during NPs synthesis\(^3\). The unique characteristics of AuNPs includes the optical properties and surface binding affinity of AuNPs towards thiold molecules, provide an intriguing opportunity to develop nanoprobe.

The present study aims to gain insights into the assembly formation of AuNPs and interparticle interactions in the presence of L-Cys and GHS. Additionally, thioglycolic acid (TGA) is also employed to study the effect of other functional groups such as amine (−NH\(_2\)) on interparticle interaction mechanism. Furthermore, we have also investigated the effects of AuNPs on the ability of L-Cys, GSH and TGA to quench the ROS and DPPH radical.

Materials and Methods

Hydrogen tetrachloroaurate(III) (HAuCl\(_4\).3H\(_2\)O, 99%), trisodium citrate dihydrate (HOC(COONa)\(_2\).2H\(_2\)O), thioglycolic acid (HSCH\(_2\)COOH, 98%), L-cysteine hydrochloride monohydrate (98%) and all other chemicals used were of analytical grade. Sodium borohydride and all other chemicals used were of analytical grade. All the experiments were performed with Millipore water.

Synthesis of AuNPs and its interaction with thiolated ligand

AuNPs were synthesized by the Martins method\(^4\). For a typical preparation of AuNPs, 0.25 mM of HAuCl\(_4\).3H\(_2\)O dissolved in 25.0 mL millipore water was added to 0.25 mM trisodium citrate with continuous stirring to make a homogenous solution. A freshly prepared 0.1 \(M\) aqueous sodium borohydride solution was added dropwise to acquire the desired concentration. In our experiment, we maintained the concentration of NaBH\(_4\) at 1.0 mM in the aqueous solution. The solution turned dark ruby red immediately, which confirmed the formation of AuNPs. Different concentrations of HAuCl\(_4\).3H\(_2\)O (Supplementary Data, Fig. S1) were employed to optimize the formation of AuNPs, which was monitored by UV-visible spectra.

The functionalisation of AuNPs by thiolated molecule was achieved by ligand exchange process from already prepared citrate capped AuNPs. In a typical procedure, varying concentrations of thiolated ligands (TGA, L-Cys and GSH) were used to study the Au-S interactions. To 5.0 mL of previously synthesized citrate stabilized AuNP, thiol ligands were added maintaining the desired concentration of thiols. The ratio of the concentrations of HAuCl\(_4\) to thiol ratio was kept in the range of 1:1–1:5. The immediate color change from ruby red to blue violet indicates the ligand exchange process and was monitored by the shift in the absorption spectra. UV-visible spectra were recorded using ThermoScientific Evolution-300 spectrophotometer operating at a resolution of 2 nm. The FTIR spectra of ligands and thiol functionalized AuNPs were recorded on a Shimadzu IR Affinity FTIR spectrometer. Transmission electron microscope (TEM) measurements were obtained on a Jeol JEM-2100F instrument operated at the accelerating voltage of 200 kV. Samples were prepared by placing a drop of the thiol stabilized AuNPs solution on carbon-coated TEM grids.

DPPH radical scavenging assay

The DPPH\(^*\) scavenging activity was studied according to the method of Zhao et al.\(^4\). The antioxidant activity of thiol and AuNPs was evaluated by monitoring its quenching ability against synthetically stable DPPH radical. The thiol conjugated AuNPs (1.5 mL, 0.25 mM) was added to 1.5 mL of 0.25 mM DPPH in ethanol. The solution was then mixed and kept in the dark for 40 minutes at 37 °C and the change in color was observed spectrophotometrically at 517 nm. The percentage of DPPH\(^*\) scavenging activity was calculated using Eq. (1).

\[
\% \text{RSA} = \left( \frac{A_{\text{DPPH}} - A_{\text{DPPH}}} {A_{\text{DPPH}}} \right) 
\]

…(1)
where $A_S$ is the absorbance of the DPPH solution with NPs and $A_{DPPH}$ is the absorbance of the DPPH solution without NPs\textsuperscript{41}. Ascorbic acid was used as standard antioxidant agent.

Results and Discussion

Thiol-containing biomolecules have high affinity for AuNPs surface, which can be well explained by the hard-soft acid-base (HSAB) principle\textsuperscript{42}. The theory implies that a soft acid has stronger affinity for a softer base compared to a hard base. Metals with the zero oxidation state (elemental form) have proved to be a softer acid than the other ions with positive oxidation state.\textsuperscript{43} Generally, softer ligands are believed to interact with the metal surface via their lone pair of electrons present on their head groups. Ligands having amine and alcohol groups interact poorly with gold surface and conserve their electronic properties. On the other hand, thiol groups robustly interact with the surface of AuNPs and bring about significant charge redistribution. Besides the electronic interaction, thiolated molecule may covalently bind with the surface gold atoms by back $\sigma$-bonding commencing from the sulfur atom, which results in large shifting of the SPR band as expected by Mie theory\textsuperscript{42}. Thiol-containing biomolecules, i.e., L-Cys and GSH displace the citrate shell of AuNPs and stimulate the aggregation process. As shown in Fig. 1, a new absorption band of AuNPs appears at longer wavelength (600/650 nm) when L-Cys/GSH (thiolates) are mixed with AuNPs. Furthermore, the microscopic image of AuNPs-S conjugates evidently shows the self-assembly of AuNPs with short interparticle space via the interaction with L-Cys/GSH. On comparing the UV-visible spectra of AuNPs with that of AuNPs-S conjugates in Fig. 1, it can be concluded that the assembly of AuNPs-S and the interparticle plasmons coupling leads to the increase in size of AuNPs, which results in the bathocromic shift (red shift with $\Delta \lambda = 80/135$ nm L-Cys/GSH). The assembly is due to the electrostatic interaction or hydrogen binding between non-covalently adsorbed thiolated biomolecule. The aggregation of AuNPs-S decreases the interparticle distance, and when the distance reaches below the average particle diameter, the color of the gold sol

Fig. 1 − Effect of pH on plasmon resonance absorption. [(a) L-Cys-AuNPs (1:1); (b) GSH-AuNPs (1:1); (c) TGA-AuNPs (1:5); (d) citrate-AuNPs].
turns blue, which causes the red shift in the corresponding absorption spectra. Furthermore, the band shift and broadening is also due to the electric dipole-dipole interaction and coupling between the adjacent particles.

Due to the presence of various functional groups such as carboxyl, hydroxyl and amine in L-Cys and GSH, pH is another critical parameter that plays a key role in the interaction between AuNPs and L-Cys as well as GSH. Therefore, the effect of solution pH was studied over the pH range of 2.0–11.0. The absorption spectra of L-Cys-AuNPs conjugates strongly depends on the pH of the solution and attains maximum intensity at pH 6.0 (Fig. 1a). The SPR band intensity of citrate capped AuNPs increases slowly with increasing pH of the solution. The ionization of the –COOH group of citric acid present on the NPs surface is induced by increasing the pH, which results in increase in negative charge of the COO⁻ group. It is reported that the electrostatic binding between the –NH₃⁺ and COO⁻ groups of citrate becomes stronger and prominent with increasing pH. Therefore, the SPR band intensity of L-Cys-AuNPs conjugates increases with pH. Citric acid has two pKₐ: pK₂ at 3.128 and pK₃ at 4.761, which shows that the ionization of citric acid is almost complete at pH 5.0, after the release of the second hydrogen at pK₂ and the electrostatic binding attains the maximum. Therefore, the SPR band intensity of L-Cys-AuNPs conjugates achieves maximum intensity at pH 5.0. However, the ionic strength of solution also rises with increasing pH value. The rise in the ionic strength is due to the increase in the concentrations of Na⁺ and Ac⁻, which can change the mode of electrostatic binding. Therefore, the SPR band intensity is found to decrease after the pH 5.0. These results signify that high ionic strength can wipe out the electrostatic binding between the –NH₃⁺ and COO⁻ groups of citrate on AuNPs surface.

Figure 1a represents the absorption spectra of L-Cys-AuNPs conjugates at different pH values. The SPR band with maximum intensity at 532 nm is seen at pH 6.0 and above pH 6.0, the intensity of the SPR band (pH 7.0–11.0) decreases with a slight blue shift (Δλ = 7.0 nm). The presence of a new band at 600 nm is observed at pH 4.0, while the red shift become more dominant as pH decreases further. As stated above, the occurrence of a new band at longer wavelength indicates the formation of AuNPs aggregates due to the electrostatic interaction of the –COO⁻ and –NH₃⁺ (Scheme 1). Microscopic images also infer that the addition of L-Cys to the citrate stabilized gold dispersion results in the formation of ligand mediated aggregates, which is also confirmed by the color change of the sample (from wine red to blue). These investigational results imply that most of the citrate molecules on the AuNPs surface are substituted by L-Cys molecule resulting the formation of a “double organic layer” structure (Scheme 2)⁴⁵.

Similarly, in GSH, there almost no shift in the SPR band is observed until the pH value is lowered to 5.0, indicating no aggregation until pH 5.0. At lower pH, the NPs aggregate due to decrease in the energy barrier by both lowering the surface potential of the particles as well as increasing the ionic strength. The aggregation of NPs due to the introduction of GSH depends upon the solution pH as in the case of L-Cys. Formation of NPs aggregates with GSH is observed at pH 5.0, which is marked by the color change from red to blue. GSH is a tripeptide, which consists of three amino acids, viz., glutamic acid, cysteine and glycine, and hence its binding mode with AuNPs surface is rather sensitive to solution pH. In GSH, various potential binding sites are present, which offer a variety of possible binding modes with AuNPs. The accessibility of these prospective anchoring points for binding with the AuNPs surface depends essentially

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**Scheme 1**

Schematic view of the head-to-head zwitterion-type electrostatic interactions (i.e., –CH(NH₃⁺)-CO₂⁻ –***O⁻C(NH₃⁺)-HC-) between amino acid groups of L-Cys/GSH at AuNP surface

**Scheme 2**

Formation of electrical double layer structure after addition of L-Cys/GSH to citrate-stabilized gold dispersion.
on the pH of the medium. The anchoring sites in GSH are the two carboxylic groups (–COOH) in the glycine residue and glutamic acid moiety, three amine groups (–NH₂) and one thiol group (–SH) in the cysteine residue. The pKₐ values of the two carboxylic groups⁴⁶ (–COOH) in GSH are at around 2.56 and 3.50, while that of the thiol group (–SH) of cysteine residue⁴⁷ is at 9.42. However, the binding mode predicted based on pKₐ is not always accurate because the acid base character of certain groups can alter the binding ability with the metal surface as well as with the medium. The GSH attaches with the surface of AuNPs via –SH as well as –NH₂ anchoring points and causes aggregation of AuNPs. At lower pH < 5.0, GSH exists in its zwitterionic form, which activates linking of the NPs via α-amines; while linking via –COOH groups is prohibited due to the undissociated carboxylic groups. On the other hand, at intermediate or higher pH, ionization of the –COOH hinders the binding of AuNPs through the α-amine group, resulting in little or no cross-linking. The pH dependent optical spectra of GSH-AuNPs conjugates at a particular concentration of GSH solution (0.25 mM) are shown in Fig. 1b. Red shift in the SPR band with decreasing pH is observed. As stated above, no spectral shift occurs until pH 7.0. However, as the pH reaches 6.0, a shoulder appears in the absorption band. At pH 5.0, a new SPR band appears at 650 nm. As the pH decreases further, the intensity of SPR band decreases with red shift in SPR band (pH 2.0). As discussed in the previous section, the decrease in pH shifts the dipole plasmon resonance to longer wavelength. At lower pH, thiol group (–SH) of the cysteine residue and the primary amine group (–NH₂) of the glutamic acid moiety bind efficiently with the AuNPs to form aggregates; this is also supported by the FTIR spectral data.

Similar experiments have also been performed with TGA for the assessment of the proposed mechanism for the L-Cys and GSH mediated assembly of AuNPs. The effect of pH on TGA-AuNP conjugates in the range of pH 2-11, is illustrated in Fig. 1c. The results indicate that no red shift occurs by the addition of TGA except at very low pH 2.0. The red shift at pH 2.0 can be attributed to the hydrogen bonding between the –COOH groups. Based on the spectral studies, comparison was made between TGA with L-Cys and GSH; all these molecules have a thiol (–SH) group but TGA has no amino group (–NH₂). There are only covalent bonds between TGA and AuNPs and no electrostatic interactions between them. Thus, TGA cannot cause self-assembly of AuNPs. However, by adjusting the pH to an acidic environment (< 2.0) at which the carboxylic group exists in their protonated form, the aggregation could be induced due to hydrogen bonding between them. Similarly, no aggregation is observed in the absence of thiolated molecule (L-Cys and GSH) in citrate capped AuNPs at lower pH (Fig. 1d). According to the above findings, it is evident that the thiol (–SH) via covalent bond and the amino group (–NH₂) through electrostatic interaction induce the self-assembly of AuNPs.

We have further investigated the nature of the self-assembly of AuNPs with variation in concentrations of the TGA, L-Cys and GSH. The absorption spectra of the AuNPs aggregates at varying concentration ratios of L-Cys or GSH:AuNPs at pH 5 is shown in Fig. 2. Variation in reagent concentrations can shift the dipole plasmon substantially. However, the shape of the NPs aggregate also plays a key role in deciding the band position. The concentration of reagent definitely influences the shape as well as the size of the aggregates. The breadth of the dipole plasmon depends upon the polydispersity in size and shape of the AuNPs aggregates. We observed that addition of 0.25 mM of L-Cys (1:1) in the citrate-stabilized AuNPs shifts the SPR band from 515 nm to 620 nm (Δλ = 105 nm) (Fig. 2a, curve 2). Further increase in L-Cys concentration results in decrease in the intensity of SPR band without any further red shift. Similarly in the case of GSH-AuNP conjugates, addition of 0.25 mM of GSH (1:1) results in formation of a new SPR band at 655 nm (Fig. 2b, curve 2). Curves 3-6 in Fig. 2b represent the SPR bands, indicating that the intensity of SPR band of GSH-AuNP conjugates at pH 5.0 decreases with increasing concentration. However, the TGA-AuNPs show the same trend at lower pH (Supplementary Data, Fig. S2). Additionally, we have also investigated the effect of salt environment on the interparticle interaction since ion strength plays a crucial role in the aggregation process. This effect can be ascribed to the ability of strong electrolytes to constrict the aroused electrical double-layer (Scheme 1) from the stabilizing agent. We observed that presence of NaCl enhances the rate of aggregation (Supplementary Data, Fig. S3).

Interactions of TGA, L-Cys and GSH with AuNPs have been studied by FTIR spectral analysis. FTIR spectra of TGA consist of a strong and intense band due to –SH stretching, vibration at 2325 cm⁻¹ (which is absent in TGA-AuNPs), another band at 1637 cm⁻¹...
corresponding to -COOH group and a strong band at 3000 cm\(^{-1}\) attributed to –OH vibration (Supplementary Data, Fig. S4). The amino acid L-Cys (H\(_2\)N-CH\((\text{CH}_2\text{SH})\)-COOH) plays an important role in defining the tertiary structure of proteins through disulfide. The strong –SH vibration band at 2375 cm\(^{-1}\) is clearly seen in the free L-Cys molecule, which vanishes on coordination with AuNPs (Fig. 3a). This is strong evidence of surface binding of L-Cys to the gold surface via thiolate linkage. A broad band at 3150–2875 cm\(^{-1}\) and a weak band at 2800 cm\(^{-1}\) attributed to NH\(_2\) and OH stretching which is reduced in L-Cys-AuNPs, indicates the participation of this group in aggregation. The FTIR spectra of GSH shows a strong band at 2475 cm\(^{-1}\) corresponding to the –SH stretching vibrations; this band is absent in the spectra of GSH-AuNPs, indicating deprotonation and coordination of the thiol group (Fig. 3b). In GSH, the band at 1709 cm\(^{-1}\) is attributed to the -COOH group of the glycine residue, which is reduced in GSH-AuNPs. This indicates interaction of -COOH with metal ions. The key factor for this binding is the pH of the solution (8.45 for synthesized NPs), which affects the protonation or deprotonation of the -COOH group. The deprotonated -COOH group binds to the NPs. In the GSH-AuNPs complex, the doublet peaks of GSH for the symmetric stretching vibrations of the –NH\(_2\) group appearing at 3242 cm\(^{-1}\) and 3340 cm\(^{-1}\), merge into a single band at 3345 cm\(^{-1}\). The -N-H stretching frequency in the 3125–2900 cm\(^{-1}\) region corresponding to the free ligand (which is hydrogen bonded to zwitterions -OOC-C-NH\(^{3+}\) of the amino acid) does not show any considerable shift in GSH-AuNPs; this indicates non-participation of the N-H group. These free groups aggregate the GSH-AuNPs in the solution.

The size and morphology of AuNPs synthesized in the presence of different thiolated ligand have been analyzed with the help of TEM images. It can be seen that well-
defined NPs of variable shapes are formed. For TGA-AuNPs, no well-defined NPs are observed (Fig. 4a). However, HRTEM image and their corresponding SAED pattern reveal the presence of TGA-AuNPs with average size 15±5 nm of different shapes. SAED pattern reveals that TGA-AuNPs have face centered cubic structure with interplaner distance 0.274 nm, corresponding to the \( d_{111} = 111 \). The TEM images of GSH and L-Cys-AuNPs and their corresponding HRTEM and SAED patterns are shown in Fig. 4(b) and Fig. 4(c). Interestingly, L-Cys and GSH-AuNPs are of similar dimension and size (size distribution histogram is shown in the inset of their corresponding TEM image). The SAED pattern consists of many diffused rings, which indicate the polycrystallinity of AuNPs. These rings can be indexed as 111 and 200. The interplaner distance is found to be 0.286 nm, corresponding to the first diffused ring (principle plane) indexed as 111, suggesting face centered cubic (FCC) structure of AuNPs.

**In vitro antioxidant activity**

DPPH• is a stable nitrogen centered organic free radical which has been used for evaluation of the antioxidant capacity. The DPPH antioxidant capacity of AuNPs in presence of thiolated ligands was monitored with time by UV-visible spectral data. The %DPPH inhibition of AuNPs in presence of thiolated ligands was found to increase monotonically with time (Fig. 5). The
% inhibition depends on the interaction of thiolated ligand with AuNPs, which indirectly depends on the nature and polarity of ligands. L-Cys is more polar than TGA, therefore it easily aggregates via zwiterionic type electrostatic interaction phenomena. According to this phenomena, L-Cys-AuNPs (36.31%) shows the minimum %inhibition, and GSH-AuNPs (42.89%) shows the maximum %inhibition of their stabilizing property while that of TGA-AuNPs is intermediate (38.53%). The inhibition of L-Cys-AuNPs and GSH-AuNPs is due to its strong covalent interaction with AuNPs, respective to its smaller size.

Conclusions
Interaction of AuNPs with thiolated molecules, viz., TA, L-Cys and GSH, have been investigated by UV-visible spectrophotometer, FTIR analysis and TEM images. The assembly of AuNPs can be induced by the thiolated amino acid L-Cys and peptide GSH, which possess an additional thiol functional group besides the α-amine. The assembly of AuNPs mediated by L-Cys/GSH in solution is primarily due to the zwiterion-type interparticle interactions (-CO₂⁺⁺⁺⁺NH₃⁺) which is sensitive to the pH of the solution. Spectroscopic studies indicate that the assembly of AuNPs in presence of thiolated ligand depends on the nature, pH and concentration of ligand. Spectral evidence suggests that the self-assembly was not observed in the presence of TGA due to the absence of amino group (-NH₂). This suggests that only covalent bond exists between TGA and AuNPs, and there is no electrostatic interaction between them. Similarly, the assembly was also dependent on the salt (NaCl) environment, reflecting an effective screening of the diffuse layer around the initial citrate-capped nanoparticles that decreases the barrier to the L-Cys/GSH adsorption onto the surface, and around the subsequent L-Cys/GSH functionalized NPs that facilitate the zwiterion-type electrostatic interactions between L-Cys/GSH bound to different AuNPs. The current study also shows that the antioxidant activity of the physiological important antioxidant GSH and L-Cys decreases in the presence of AuNPs due to Au-S interaction.

Supplementary Data
Supplementary data associated with this article, viz., concentration versus absorbance plot for citrate capped AuNPs, concentration dependent UV-visible spectra of TGA-AuNPs, UV-visible spectra of GSH-AuNPs in presence of salt and FTIR spectra of TGA and TGA-AuNPs are available in the electronic form at http://www.niscair.res.in/jinfo/ijca/IJCA_54A(10)1206-1214_SupplData.pdf.

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