

Colorimetric Assays for Detection of Cu^{2+} ion using *Punica Granatum* Functionalized Gold and Silver Nanoparticles

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Abstract: Monitoring the levels of potentially toxic metal ions (Cu^{2+}) in aquatic ecosystems is important because of effects of these ions on human health and the environment. Gold nanoparticles (GNPs) and silver nanoparticles (AgNPs) are commonly chosen as sensing materials for their unique size and shape dependent optical properties. In this work, we concentrate on an optical assay of Cu^{2+} ions using functionalized GNPs and AgNPs. The functionalization of nanoparticles has been produced using *Punica granatum* extract. The extract acts as reducing and capping agent for the preparation of nanoparticles. The colorimetric method has been applied using GNPs and AgNPs for detection of Cu^{2+} ions based on changes in absorbance resulting metal ion-induced aggregation of nanoparticles. The enhancement property of GNPs and AgNPs has been employed to study the turn on fluorescence approaches for detection of Cu^{2+} ions. The strong binding of the Cu^{2+} metal ion with chelating ligand facilitates the aggregation of metal nanoparticles.

Keywords: *Punica granatum*, Colorimetric sensor, Assay, Calibration curve.

1 Introduction

Copper is an essential metal ion, which plays significant roles in environmental, biological functions for growth, normal physiological functioning, and maintaining of life. It is a cofactor and a structural component of numerous enzymes and proteins needed in metabolic processes [1]. Copper is widely used in industrial and agricultural sector but it is also highly toxic to organisms, such as certain algae, fungi and many bacteria and viruses [2]. The accumulation of Cu^{2+} in humans leads to many serious conditions, including neurodegenerative diseases and prion diseases [3,4]. High concentration of copper in drinking water is hazard to human health [5]. Some cases of liver damage of children have been proved to be associated with the excessive intake of copper ions [6]. Moreover, high concentration of copper may destroy the biological reprocessing systems in water [7]. Therefore, the fabrication of sensitive and selective methods to monitor Cu^{2+} ions is of great significance. To date, there are many technologies that have been developed to detect Cu^{2+} ion, including organic fluorophore-based assays [8,9], chromogenic sensors [10], quantum-dot-based assays [11], atomic absorption spectroscopy (AAS) [12], inductively coupled plasma mass spectroscopy (ICP-MS) [13], inductively coupled plasma atomic emission spectroscopy (ICP-AES) [14], electrochemical assays [15] and

voltametry [16]. Peptide and oligopeptide modified electrodes have been widely developed in recent years for the detection of metal ions in environmental sample due to their high affinities to different metal ions [17,18]. Self-assembled monolayers of L-cysteine on gold electrode have been reported with a lower detection limit of metal ions [19]. Although the sensitivities of these methods for Cu^{2+} detection are generally high, most of these methods need expensive, specialized instruments thereby becomes time-consuming which greatly limited their applications in wider range. Therefore, it is still in search for developing simple and efficient Cu^{2+} sensor especially for field work.

The colour changes associated with the aggregation of metal nanoparticles has led to the development of a number of assays for a variety of target species [20-23]. Colorimetric methods can be convenient and attractive in many applications because they can be easily monitored with the naked eye [24,25]. Recently gold nanoparticles (GNPs) are becoming increasingly attractive in the fabrication of optical sensors, devices as well as in bio-diagnostics and medical therapeutics due to their excellent chemical and physical properties [26,27]. They have strong surface plasmon resonance (SPR) absorption in the visible wavelength range with extremely high extinction

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coefficients. One attractive property of GNPs is that their SPR frequency will be changed dramatically when the distance between GNPs varied. Therefore, the analytic-induced aggregation of GNPs offered a facile colorimetric approach for the simple visual detection of nucleic acids [28,29], proteins [30], small molecules [31,32] and metal ions [33].

Inspired by these aforesaid assays, we report in this contribution a simple and highly specific green method using *Punica granatum* functionalized gold nanoparticles (GNPs) and silver nanoparticles (AgNPs) based on colorimetric assay for detection of Cu^{2+} ions. The *Punica granatum* capped gold nanoparticles has been used as the chromophores.

2 Material and Methods

2.1 Material

Chemicals used in the experiment are mostly analytical reagent (A.R) grade or of the highest purity available. Silver nitrate (AgNO_3) and chloroauric acid (HAuCl_4) with >99.0% purity are purchased from Sigma Aldrich Pvt. Ltd. used as received without further purification. The used metal $\text{Cu}(\text{NO}_3)_2$ were purchased from Beijing Chemical Reagent Company. Distilled water used in all experiment is obtained with the three stage Millipore Direct-Q 3uv purification system.

2.2 Preparation of *Punica granatum* extract

The *Punica granatum* was collected from local market and washed with double distilled de-ionized water. The skin was removed and collects the peels of *Punica granatum*. 10 g of these were squeezed to get *Punica granatum* juice. Then it was diluted two times using double distilled de-ionized water and filtered to get aqueous extract of *Punica granatum*.

2.3 Synthesis of Gold and Silver Nanoparticles

Gold nanoparticle was produced by reduction of chloroauric acid solution by using the diluted extract of *Punica granatum*. 20 ml of *Punica granatum* extract was added to the 4 ml of 0.003(M) HAuCl_4 solution and then 0.01(M) NaOH solution was added drop wise to make alkaline medium. Further the mixture was heated at 85°C for ten minutes to obtain gold sol. The greenish color solution gradually changes to deep violet coloration. This indicates the reduction of Au (III) to Au (0). Similarly, for the synthesis of silver nanoparticle, 20 ml of *Punica granatum* extract was added to 2.5 ml of 0.003 (M) AgNO_3 solution followed by drop wise addition of NaOH solution. It was heated for twenty minutes at 85°C . The color of the solution gradually changed from greenish to deep yellow. The UV-VIS spectroscopic study further confirmed the formation of GNPs and AgNPs.

2.4 Colorimetric Assay of Copper (II) Ion Detection based on Gold and Silver Nanoparticles

The as prepared functionalized GNPs and AgNPs sol were diluted to three times by distilled water. The resulting nanomolar concentration of the nanoparticles sol was used for Cu^{2+} ion detection. Various concentrations of Cu^{2+} were prepared (25-100 μM) from stock solution by dilution to test the sensitivity limit of the GNPs produced. The colorimetric detection of aqueous Cu^{2+} was studied at room temperature. Typically, 5 mL of GNPs solution was added to 150 μL of Cu^{2+} solution having different concentrations and stirred at room temperature. The violet color subsequently changes to purple. The intensity of purple colour gradually increased with increase of Cu^{2+} concentration. Same procedure was followed for Cu^{2+} ion detection using functionalized AgNPs. The yellow color subsequently changed to bluish color.

2.5 Reverse the colorimetric response

The chelation/aggregation process was found to be reversible via addition of a strong metal ion chelator such as EDTA. The purple to violet color change can be reversed by increasing concentration of EDTA (0.001 M to 0.007 M), a good Cu^{2+} ion extracting agent from.

2.6 Characterization

The absorbance spectra of the GNPs and AgNPs were analysed by using a 'SHIMADZU' UV 1800 spectrophotometer and TEM images were taken using JEOL-JEM 2100 high resolution transmission electron microscope (HRTEM). Samples for the TEM studies were prepared by placing a drop of the aqueous suspension of particles on carbon-coated copper grids followed by solvent evaporation under vacuum. The crystalline nature of the GNPs and AgNPs was examined using X' Pert Pro X-ray diffractometer operated at a voltage of 40 kV and a current of 30 mA with $\text{Cu K}\alpha$ radiation. The FTIR measurements were analyzed by using Perkin Elmer Spectrum Version 02 spectrometer.

3 Results and discussion

Bio-synthesis of nanoparticles using plant extracts are in trend now-a-days. The development of biological process for the synthesis of nanoparticles is evolving as an important branch of nanotechnology. The present study deals with the synthesis of gold and silver nanoparticles using aqueous *Punica granatum* extract. *Punica granatum* extract is primarily composed of a variety of alkaloids and polyphenols. The term phenolic compound embraces a wide range of plant substances that bear in common an aromatic ring with one or more hydroxyl substituents. The antioxidant activity of phenolic compounds is mainly due to

their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. The active constituent that appears to be responsible for its multiple health benefits is Ellagic acid. Ellagic acid is a naturally occurring phenolic compound found in several fruits and nuts. *Punica granatum* extract has demonstrated a variety of beneficial functions including antioxidant and anti-viral activity. Ellagic acid effectively protects cells from damaging free radicals. Additional phenolic compounds found in pomegranate known as anthocyanidins (also well-known scavengers of free radicals) combine synergistically with Ellagic acid to greatly augment pomegranate's potency as an antioxidant.

Antioxidant action of phenolic compounds is due to their high tendency to chelate Cu^{2+} ion. Phenolics possess hydroxyl and carboxyl groups, which act as complexing and stabilizing agent besides its reducing property. A composite from this functionalized gold nanoparticle and Cu^{2+} ion have been employed for quantitative detection of Cu^{2+} ion. The probable mechanism for reduction of metal ion is shown in Figure 1 as scheme.

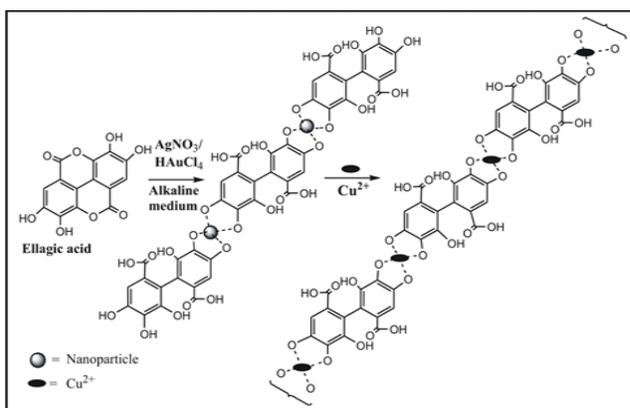


Fig. 1 General structure of Ellagic acid and reaction pathway, aggregation and chelation of nanoparticles.

3.1 UV-Vis spectral studies

The produced *P. granatum* functionalized GNPs (Figure 2A) and AgNPs (Figure 3A) were exhibited deep violet and deep yellow color in water, respectively. A smooth and narrow absorption band for GNPs and AgNPs were observed at 520 nm and 400 nm, respectively. It is expected that *P. granatum* capped gold and silver nanoparticles should have much better colloidal stability due to chelating ability of poly phenol groups present in Ellagic acid in the extract. Several concentrations of $\text{Cu}(\text{NO}_3)_2$ solution (0 to 100 μM) was added in the functionalized GNPs and corresponding changes of absorption coefficients were observed. Copper (II) ions can be quantities based on its aggregation effects on gold nanoparticles. The aggregation can be monitored visually through the change in color and spectrophotometrically through the change in the absorption spectra [34]. It was noted that with increasing

concentration of Cu^{2+} , the surface plasmon band originally centred at 520 nm decreases dramatically as a new absorption band between 620 nm to 660 nm progresses (Figure 2A). Aggregation of the nanoparticles by addition of Cu^{2+} ion yields a shift in Plasmon band energy and a substantial increase in long-wavelength, result in a violet to purple color change for GNPs [35]. The chelation/aggregation process was found to be reversible via addition of a strong metal ion chelator such as EDTA [36]. The purple to violet color change can be reversed by increasing concentration of EDTA, a good Cu^{2+} ion extracting agent (Figure 2B). With the colorimetric experiment, addition of EDTA fully reverses the response, as expected if Cu^{2+} chelation is responsible for particle aggregation.

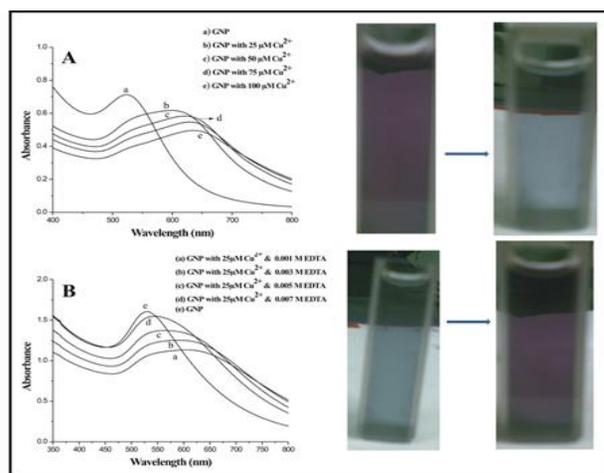


Fig. 2 UV-Visible spectra of (A) GNPs with varying concentrations of Cu^{2+} ion at different ranges (inset: digital photographic image) (B) reversing the process by increasing concentration of EDTA, a good Cu^{2+} ion extracting agent.

Several concentrations of $\text{Cu}(\text{NO}_3)_2$ solution (0 to 75 μM) was added in the functionalized AgNPs and corresponding changes of absorption coefficients were observed. As soon as $\text{Cu}(\text{NO}_3)_2$ solution was added in it, a new peak at around 720 nm was found in addition to the peak at 400 nm. More interestingly, with the increase of concentration of Cu^{2+} ion in solution the absorption coefficient of 400 nm peak decreased whereas the absorption coefficient of 720 nm peak increased gradually (Figure 3A). The SPR of functionalized AgNPs at 400 nm and 720 nm were related to the quantities of dispersed and aggregated AgNPs, respectively. Thus we used the ratio of the absorbance value of 720/400 ($E_{720/400}$) to express the molar ratio of aggregated and dispersed phase in the sol.

A linear relationship between $E_{720/400}$ and concentration of Cu^{2+} was established from the calibration curve (Figure 3B). This calibration curve can help one to detect and estimate quantitatively the presence of Cu^{2+} in a sample at μM level. These findings encourage us to suggest that this method can be used for detecting the Cu^{2+} in solution with a

minimum detectable limit of 25 μM . Morphology changes or complex formation of the metal is evidenced from the appearance of a peak at around 720 nm [37].

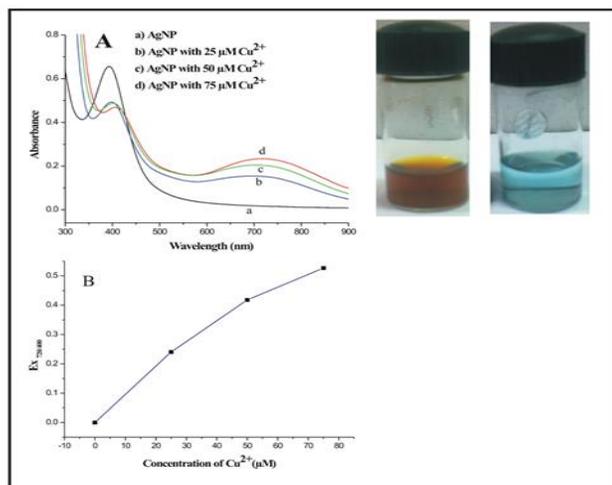


Fig. 3 UV-Visible spectra of (A) AgNPs with varying concentrations of Cu^{2+} ion at different ranges (inset: digital photographic image) (B) calibration curve between absorption ratios ($E_{720/400}$) versus concentration of Cu^{2+} ion.

3.2 TEM studies

Figure 4 shows the TEM images of *P. granatum* functionalized GNPs. The particles are mostly spherical and their sizes varied from 5 to 20 nm. Being a good capping agent *P. granatum* induces rapid nucleation of gold nanoparticles occurred and hence homogeneous monodispersed behaviour was observed in TEM image (Figure 4A and 4B). Selected area diffraction (SAED) pattern (Figure 4C) illustrates the crystalline nature of the nanoparticles.

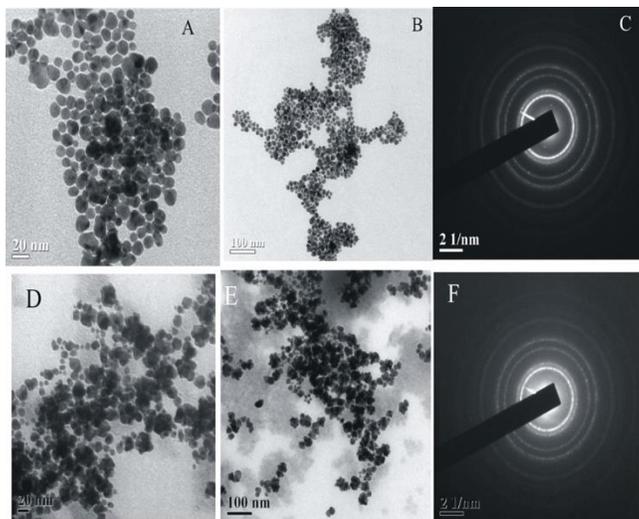


Fig. 4 TEM micrographs (A, B) & SAED(C) pattern of GNPs and TEM micrographs (D,E) & SAED(F) pattern Cu-complex of GNPs

The TEM images of Cu-complex of gold nanoparticles are shown in Figure 4 (D, E). Due to the presence of both oxygen rich functional groups (such as -COOH, -OH), and hydrophobic aliphatic and aromatic groups in *P. granatum*, it is capable to act as a chelating ligand. The strong binding of the Cu^{2+} metal ion with chelating ligand facilitates the aggregation of GNPs in random way and thereby morphology of the nanoparticle changed enormously which is shown in TEM micrograph (Figure 4). Linking of spherical particles was observed and the ultimate production of cage like structure (Figure 4) was visible in the TEM images. These observations may be explained by considering the removal of *P. granatum* from the surface of nanoparticles to make the surface charged which helps to aggregation of nanoparticles in random fashion to facilitate the morphology change.

Instead of spherical the rod like structures were obtained for silver nanoparticles (Figure 5). To explain this observation we may consider that the removal of *P. granatum* from the composite makes the surface charged which prevents the aggregation of nanoparticles in random fashion and facilitate the rod like structure formation by linear aggregation.

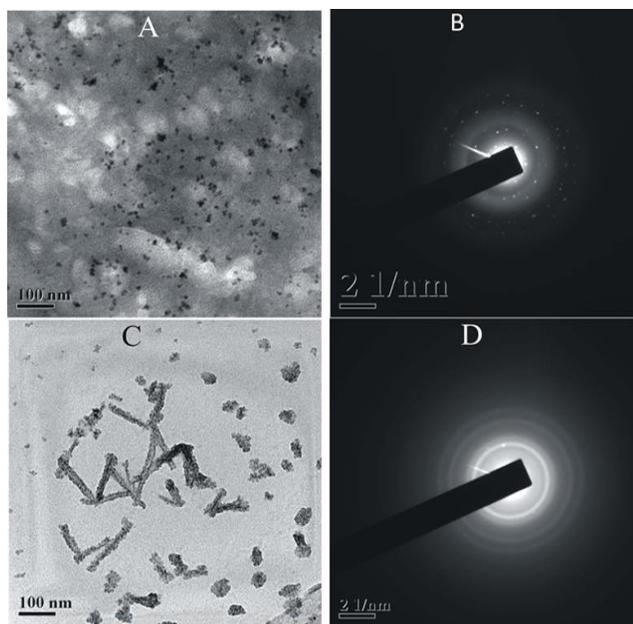


Fig. 5 TEM micrographs (A) & SAED pattern (B) of AgNPs and corresponding Cu-complex with AgNPs (C & D).

3.3 FTIR spectra studies

FTIR analysis was performed to identify the bio-molecules localized on the surface and responsible for the reduction of silver solution as well as Cu-complex formation. Representative FTIR spectra of Ellagic acid (*Punica granatum*), synthesized AgNPs and Cu-complex of AgNP are shown in Figure 6. Bands attributable to the

$\nu_{\text{assym}}(\text{COO}^-)$ and $\nu_{\text{sym}}(\text{COO}^-)$ modes of carboxylate groups present in Ellagic acid (*Punica granatum*) appeared at 1639 and 1385 cm^{-1} with comparable intensities (Figure 6A). When AgNPs were prepared in presence of Ellagic acid (*Punica granatum*), the respective peaks shift to 1636 and 1413 cm^{-1} due to reduction of silver nitrate solution, the former being less intense than the latter which is shown in Figure 6B. Finally when Cu^{2+} ion was added to the above composite, the corresponding $\nu_{\text{sym}}(\text{COO}^-)$ modes of carboxylate group further shifted to the 1630 and 1390 & 1365 cm^{-1} (Figure 6C). The change of frequencies of carboxylate band can only be explained considering the stable inner complex formation between Cu^{2+} ion and Ellagic acid (*Punica granatum*).

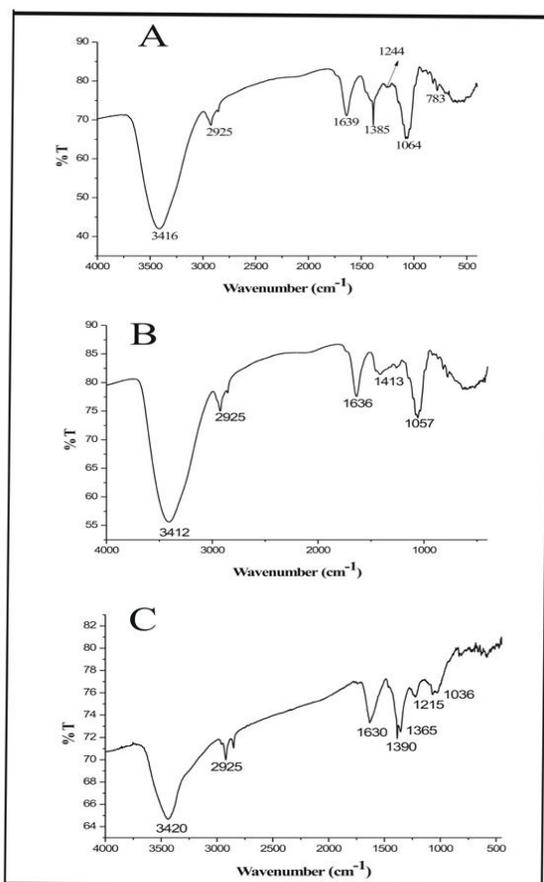


Fig. 6. FTIR spectra of (A) Ellagic acid (*Punicagranatum*), (B) AgNP and (C) Cu-complex of AgNP.

3.4 XRD studies

The XRD analysis was performed to confirm the crystalline nature of biologically synthesized AgNPs. Various Bragg's diffraction patterns were clearly visible the synthesized AgNPs, GNP and copper complex of AgNPs are shown in Figure 7. The face centered cubic (fcc) structure of the synthesized AgNPs having sharp peaks at 32.32, 38.20°, 44.42°, 64.64° and 77.78° indicated the presence of corresponding (111), (200), (220), (311) and (222) planes, respectively (Figure 7A). Which suggest that the

synthesized AgNPs and GNPs are fcc structure and essentially crystalline in nature. XRD of copper complex of AgNP nanocomposite in presence of Ellagic acid confirmed that Cu existed in the form of ions in the complex (Figure 7B & 7C). Literature values available for Cu^{2+} -complexes are in conformity with our findings. XRD of AgNPs in presence of Cu^{2+} ion shows peaks at 12.45°, 24.95°, 28.61°, 29.71°, 41.61°, 46.87°, 55.58°, 58.58°, 67.19°, 81.40°, and 86.90° in addition to AgNP nanocomposite peaks at 32.71°, 38.07°, 44.42°, 64.59°, 77.80° [38,39].

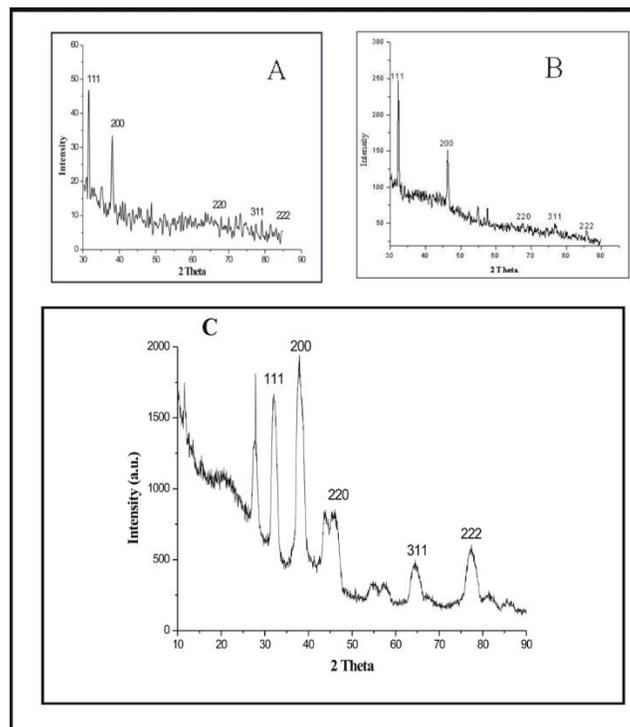


Fig. 7. XRD pattern of (A) AgNP nanocomposite and (B,C) Cu-complex of AgNP nanocomposite with different magnification.

3.5 Fluorescence studies

A turn off approach of fluorescent sensor has been developed for the sensing of Cu^{2+} ions in aqueous solution based on Cu^{2+} ions decreasing of Ellagic acid molecules on the surfaces of functionalised AuNP. Ellagic acid molecules are fluorescent in bulk solution, but its fluorescence diminishes by more than two times when adsorbed onto AuNP surfaces (Figure 8). The luminescence quenching occurred through aggregation induced by Cu^{2+} -hydroxyl bonding and strong Cu-Au metalophilic interactions. In an attempt to detect and estimate Cu^{2+} ions present in tap water various solutions having concentrations between 0 to 75 μM were prepared and the fluorescence spectra were drawn. It is observed that this probe is capable to detect Cu^{2+} ions present at concentrations as low as 25 μM upon excitation at 380 nm. With the increase of Cu^{2+} ion the intensity of 460 nm peak slightly decreases up to the concentration of 75 μM . It is also to be noted that this probe

is unable to detect the metal ion concentration lower than 25 μM .

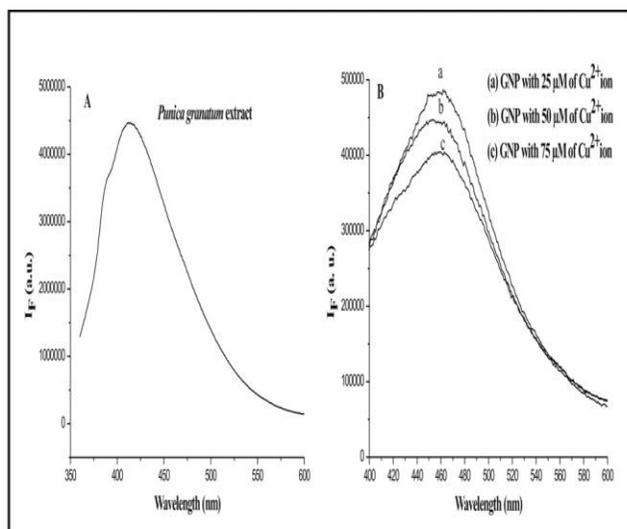


Fig. 8. Fluorescence response of (A) *Punicagranatum* extract (B) a) *P. granatum* capped GNPs, upon addition of Cu^{2+} ions b) 25 and c) 50 μM .

4 Conclusion

A simple, cost-effective and sensitive colorimetric method has been developed using GNP-based and AgNP-based colorimetric sensor for detection of Cu^{2+} ions based on changes in absorbance resulting from metal ion-induced aggregation of nanoparticles or direct deposition of metal ion (Cu^{2+}) onto nanoparticles. The strong binding of the Cu^{2+} metal ion with chelating ligand facilitates the aggregation of GNP in random way and thereby morphology of the nanoparticle changed enormously. Linking of spherical particles was observed and the ultimate production of cage like structure.

The SPR of functionalized AgNPs at 400 nm and 720 nm were related to the quantities of dispersed and aggregated of AgNPs respectively. Thus we used the ratio of the absorbance value of 720/400 ($E_{720/400}$) to express the molar ratio of aggregated and dispersed phase in the sol. Instead of spherical the rod like structures were obtained for silver nanoparticles. The enhancement property of GNPs has been employed to study the turn on fluorescence approaches for detection of Cu^{2+} ions. Due to the presence of both oxygen rich functional groups (such as $-\text{COOH}$, $-\text{OH}$), and hydrophobic aliphatic and aromatic groups in *P. granatum*, it is capable to act as a chelating ligand.

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