Supplementary materials for the manuscript **entitled "Immunodipstick based gold nanosensor for vitamin B_{12} in fruit and energy drinks"** Manuscript ID AY-ART-11-2012-026320.R2 for **revision** to Journal **Analytical Methods.**

2. Experimental Section

Apparatus

Spectral analysis of GNPs was recorded in the range from 300 to 800nm using spectrophotometer UV-1601 (Shimadzu, Japan), and a characteristic surface plasmon resonance (SPR) peak was noted. Particle size characterization of synthesized GNPs was recorded using Transmission Electron Microscopy (TEM) (Jeol 2100, USA).Crystal characterization of synthesized GNPs powder was recorded in the range from 30 to 80 degree (2θ)using Philips PW 1140 diffractometer (Bragg-Branto to Geometry,Brucker, USA).The characteristic peaks were noted and compared with standard peaks. Fourier-transform infrared (FTIR) spectra, measured by KBr pellets containing GNPs, were obtained in the range of 4000–400 cm^{-1}using Avatar 360 FTIR spectrometer (Nicolet Instrument Corp., Madison, MI). Fluorimetric assay was done with the help of spectrofluorometer RF-5301 PC (Shimadzu, Japan). ELISA was performed in microtitre plate (Tarsons, India) and ELISA-based analysis was carried out using a Versa Max tunable micro-plate reader (Molecular Devices, USA). Agarose gel electrophoresis was performed using Genei apparatus (Genei, Banglore, India).

GNPs Synthesis

An aqueous solution of monodisperse quasi-spherical GNPs was prepared by modified Turkevitch et al., method. [21,22] A total of 45 mL of Milli-Q water was taken in a reaction flask and refluxed for 10 min. Addition of mixture of 5 mL of 0.1% tetrachloroauricacid ($HAuCl_4$), 2 mL of 1% trisodium citrate and 42.5 µL of silver nitrate drop by drop in to reaction flask after incubating for 5 min. The reduction of gold metal ions (Au^{3+}) to yield GNPs (Au^{o}) was confirmed by the appearance of dark cherry red color. Colloidal GNPs were stored at 4°C. Colloidal gold solution was centrifuged then dried and subjected to characterization using various physico-chemical techniques.

Bioconjugation of GNPs with proteins

GNPs synthesized as above were conjugated with vitamin B_{12} antibodies (IgY) by reacting 10 mL colloidal gold solution of pH 9.0 with 400 µg of IgY. IgY was added drop by drop with gentle stirring. After overnight incubation at 4°C the mixture was centrifuged at 10,000 rpm for 30 min at 4ºC. The pellet was resuspended in minimum amount of storage buffer (1mM phosphate buffer of pH 7.4 with 0.05% Tween-20). The binding of GNPs to IgY was confirmed by the absorption spectrum analysis of the pure GNPs and GNPs conjugated IgY in the range of 300–700 nm.

Agarose gel electrophoresis

To find out the bioconjugation efficiency of GNPs with proteins (BSA and IgY) agarose gel electrophoresis was performed. A total of 50µL of concentrated GNPs, GNP-BSA and GNP-IgY were loaded in wells of 1% agarose gel in 1X tris-borate-EDTA buffer and ran for 2-3 h with a power supply of 80 V. Gel images were captured on a SONY digital camera (Coolpix) equipped with a 6 mega pixel CCD chip and transferred to computer using standard camera software.

Flocculation assay

Flocculation assay was done to determine and optimize the minimum amount of IgY needed to stabilize the GNPs. Varying concentrations of IgY were prepared from the stock solution. To 1 ml of colloidal gold solution 100 µL of IgY dilution was added and incubated for 15 min. Flocculation was induced by adding 100µL of 10% NaCl and the absorbance was scanned from 400 to 800 nm. The amount of IgY required to prevent flocculation was found at the point where absorbance is nearly constant with increasing concentrations of IgY. To determine the amount of IgY bound to GNPs different concentrations of IgY was prepared and absorbance was measured for each dilution at 280 nm. One mililiter of colloidal gold solution was added to each dilution and incubated with mild shaking at RT for 2 h. After incubation the mixture was centrifuged at 12,000 rpm for 30 min and the supernatant was collected separately. Absorbance of the

collected supernatant was measured at 280 nm and subtracted from the initial absorbance to find the amount of IgY bound to GNPs.

Critical flocculation concentration (CFC)

CFC of GNP-IgY was found by adding 100 µL of increasing concentrations of NaCl (0.01-1 M) to 1 ml of bioconjugates (GNP-IgY). The mixture was incubated at RT for 1 h with mild shaking. Aggregation was assessed by monitoring changes in the characteristic GNPs plasmon frequency. The threshold NaCl concentration that caused the aggregation of particles in colloidal gold solution was determined as CFC.

Fluorimetric assay

Spectrofluorimetric assay was done to determine the binding constant of IgY to GNPs. 100 µg/ml of IgY solution was mixed with different concentrations of GNPs and the mixture was incubated at RT for 2 hrs. Fluorescence of bioconjugate (GNP-IgY) solution was measured between 300 and 500 nm with an excitation at 290 nm. Binding constant was calculated as reported previously. [23]

Fourier-transform infrared spectroscopy (FTIR)

FTIR spectra of IgY as well as GNPS-IgY were measured using Avatar 360 FTIR spectrometer in KBr. The IgY and GNPs-IgY were dried in lyophilizer prior to their use for FTIR analysis. Spectra was obtained at RT.

Enzyme-linked Immunosorbent Assay (ELISA) for vitamin B_{12} determination

ELISA was done as reported in our previous publication and absorbance was measured at 405 nm in an ELISA microplate reader.[20]

3. Results and discussion

Preparation

During the preparation of colloidal gold, tetrachloroauric acid was reduced due to the transfer of electrons from the carboxyl group of tri-sodium citrate and Au^{3+} ion leads to the formation of Au^{0}. This metallic gold then nucleates and grows to form GNPs, and is subsequently capped and stabilized by the citrate ions. The concentration of tri-sodium citrate used in its synthesis dictates the size of GNPs and reduces gold chloride to GNPs.[24] The change in color of the gold colloid is shown in Fig. S1. The color of the solution changes from pale yellow to metallic cherry red color. Minute concentration of silver nitrate was added in order to get uniform nucleation during the preparation of GNPs.

Spectrophotometry

The size and concentration of GNPs were determined using the UV-vis spectra of GNPs.[25] Average size and concentration of the gold sol were found to be 15 nm and 4.19×10^{-9} M. Size and shape of the resulted GNPs were further confirmed using TEM micrograph of Fig.1 with diameter of about < 20 nm and provided strong evidence of formation of uniform quasi-spherical GNPs during synthesis. The surface plasmon resonance (SPR) peak of freshly synthesized GNPs was observed at 504 nm as shown in Fig. 1.

The size and concentration of GNPs were calculated using the UV-vis spectra of GNPs as shown below.[25]

From the spectrophotometric data the following values were obtained,

$$\boldsymbol{\lambda_{SPR} = 504nm}$$

$$\boldsymbol{A_{450} = 0.914}$$

$$\boldsymbol{A_{SPR} = 1.495}$$

Diameter of the GNPs = $(\boldsymbol{A_{SPR} / A_{450}})$

The ratio of absorbance of GNPs at the SPR peak (A_{SPR}) to the absorbance at 450 nm (A_{450}) was calculated and found to be 1.6356 which corresponds to a GNPs size (diameter) of 14-16 nm, so average size of 15 nm is taken for further studies. The concentration of the GNPs was calculated using the formula

$$C = (A_{450}/\varepsilon_{450})$$

where, ε_{450} is the molar decadic extinction coefficient at 450 nm. And the average value of ε_{450} was found to be 2.203 x 10^8 M^{-1} cm^{-1}. By substituting the value in the above equation we get the concentration of the gold sol as 4.19 x 10^{-9} M. Average size and concentration of the gold sol were found to be 15 nm and 4.19 x 10^{-9} M. Size and shape of the resulted GNPs were further confirmed using TEM micrograph as shown in inset of Fig. 1 with diameter of about < 20 nm and provided strong evidence of formation of uniform quasi-spherical GNPs during synthesis. The surface plasmon resonance (SPR) peak of freshly synthesized GNPs was observed at 504nm as shown in Fig. 1.

X-ray diffraction (XRD)

The precipitate obtained after centrifugation of colloidal gold was confirmed for its crystalline nature using XRD as shown in Fig. S2. Several peaks are observed, these being at 38.2°, 44.5°, 64.7°and 77.6°, which correspond to the {111}, {200}, {220} and {311} facets of the face-centred cubic (FCC) crystal structure of gold, respectively. The observation of diffraction peaks for the GNPs indicates that these are crystalline in this size range, while its broadening is related to the particles in the nanometer size regime. Similar observation was made by few groups.[26, 27]

Bioconjugation of GNPs with proteins

Spectral analysis

The synthesized GNPs were capped with citrate layer, which imparts them a net negative charge that can be targeted for bioconjugation based on charge-coupled

interactions as shown in scheme-S1. Electrostatic interactions of GNPs are possible with antibodies.[28] As the isoelectric point of antibodies is around pH 9.0 where they exist in zwitter-ion conditions, the positive amino terminals of IgY were targeted for conjugation with GNPs by adjusting the pH of GNPs to pH 9.0 with 0.1M K_2CO_3. Further, the conjugation of GNPs to IgY was confirmed by the spectrum analysis of the pure and conjugated GNPs in the range of 300–700 nm and observed a red shift in the peak of about 6nm from 504 to 510 nm as shown in Fig.1. This shift is due to the conjugation of double bonds lowering the energy required for the electron transitions and hence causing an increase in the wavelength. The shift in absorption peak is an evidence for the confirmation of GNPs bioconjugation with IgY. It was reported that nanoparticles can show shift in their absorption as a result of bioconjugation due to the change in their overall structure and spatial closeness of biomolecules and nanoparticles.[29]

Agarose gel electrophoresis

Agarose gel electrophoresis was done to confirm the bioconjugation of GNPs with IgY and BSA. The bands in the 1% agarose gel showed a marked difference in the movement of bioconjugates. GNPs are poorly negatively charged molecules so remain static in the well-1 and well-4 against the electrophoretic separation. Whereas the bioconjugates were strongly charged due to conjugation with proteins and showed marked difference in the movement in the gel according to the molecular weight of the protein. GNP-IgY (well 2 and well 5) moved half the way through the gel in comparison with GNP-BSA (Well 3 and well 6) as shown in Fig. S3. The molecular weight of IgY is 190 KDa and BSA is 66 KDa.

Flocculation assay

Concentration, pH and appropriate functional group are very critical parameters for bioconjugation of any biomolecules to nanoparticles.[29] To determine the minimum amount of IgY required for nanoparticles stabilization, flocculation assay was done for varying IgY concentration for citric acid-reduced GNPs. Fig.S4 Inset shows the absorption spectrum of different concentrations of IgY (µg) bioconjugated with per mL of

GNPs. The stability of GNPs can be maintained by the adsorption of IgY on it preventing the salt-induced aggregation. Most of the GNPs get aggregated at IgY concentration less than 30µg indicating that the lower concentration of IgY are not enough to shield the GNPs. Whereas, at IgY concentration more than 30µg GNPs are not getting aggregated indicating the complete shielding of GNPs. Thus, among all the concentration of IgY 30µg was selected as optimum because visually and graphically distinct color change was observed. The color change from red to blue of the GNPs is mainly due to varying interparticle plasmon coupling during aggregation and resulting in plasmon band shift. The full saturation of the colloidal gold surface increases the chance of an antibody–antigen interaction after collision with the protein and improves the stability of colloidal particles by shielding its surface against coagulation.[30] Fig.S4 shows the absorption spectra for IgY containing solutions before and after reaction with the GNPs. Fig. S4 showed the absorption spectra for IgY containing solutions before and after reaction with the GNPs. At low IgY concentration, the absorbance of the supernatant of the centrifuged IgY solution after treating with colloidal GNPs essentially decreases to zero. Only after saturation of the GNPs surfaces with the IgY molecules, the noticeable absorbance of supernatant is observed. For a fixed IgY concentration (1 mg/ml), the amount of IgY adsorbed on GNPs was found to be 30 µg /ml. The amount of IgY molecules per ml of GNPs (15 nm) were calculated by measuring the difference of absorbance of the IgY solution before and after incubation with GNPs.

Critical flocculation concentration

GNPs are highly polarizable materials with a large Hamaker constant.[31] They are prone to aggregation in high ionic strength solutions in which van der waals attraction is stronger than the electrostatic and steric repulsion provided by surface-bound ligand. For confirmation of stability of GNP-IgY complex, CFC was determined, as the threshold concentration of the electrolyte (NaCl) in the colloidal gold that caused rapid aggregation of particles. The CFC is indicated by a large decrease and/or red shift of the maxima as a result of aggregation of GNPs. A CFC of 1M of NaCl was determined for the citrate-capped GNPs as shown in Fig. S5. This confirmed the good stability of

citrate-capped GNPs toward electrolyte (NaCl)-induced aggregation. The CFC value provides a critical parameter for the development and functioning of dipstick while analyzing vitamin B_{12} in food samples.

Determination of Binding constant (Kb) of GNP-IgY by Fluorimetric assay

Specific non-covalent binding of nanoparticles to biological macromolecules, such as proteins and other molecules due to quenching of fluorescence intensity of tryptophan residues of protein molecules enabled the determination of binding constant (K_b) of proteins (IgY) to GNPs. Fig. S6 shows the relative fluorescence intensity of tryptophan residues of IgY with different concentrations of GNPs. The fluorimetric assay has been chosen due to the high intensity of the technique in probing the intrinsic fluorescence of the tryptophan residue of IgY molecules, which is quenched by the binding of GNPs to the specific sites.[23] This provides a strategy to investigate the interaction between GNPs and IgY through the evaluation of specific parameters that clearly describe the binding process as the binding constant (K_b). IgY caused a linear reduction in the addition of increased concentrations of GNPs.

Binding constant (K_b) was determined using the fluorimetric assay. Binding constant is useful in determination of stability of the bioconjugate. The tryptophan residue fluorescence intensity (F) scales with the GNPs concentration [GNP] through $(F_0\text{-}F)/(F\text{-}F_{sat}) = ([GNP]/K_{diss})^n$. The binding constant Kb was obtained by plotting log $(F_0\text{-}F)/(F\text{-}F_{sat})$ versus log[GNP], where F_0 and F_{sat} are the relative fluorescence intensities of the IgY alone and the IgY saturated with GNPs, respectively. The value of log [GNP] at $\log(F_0\text{-}F)/(F\text{-}F_{sat}) = 0$ equals to the logarithm of the dissociation constant (K_{diss}). The reciprocal of (K_{diss}) is the binding constant Kb. Fluorescence intensity data corresponding to nanoparticles with a core size of roughly 15 nm is shown in inset of Fig.S7 (See supplementary material), represent the best fit in the data using $(F_0\text{-}F)/(F\text{-}F_{sat}) = ([GNP]/K_{diss})^n$. The value of K_b obtained from the fitting of GNP-IgY was equal to 2.535×10^8. This showed good binding of IgY with GNP and provided an idea about the bioconjugation stability.

Fourier-transform infrared spectroscopy.

GNPs are good adsorbents for biomolecules such as proteins and antibodies. The citrate-capped GNPs were used to bind IgY to make stable GNPs-IgY complexes. The adsorption is established through electrostatic interaction between the surface-terminated anionic groups ($-COO^-$) on the nanoparticles and the positively charged amino groups ($-NH_3^+$) of the lysine residue of the protein. Apart from the electrostatic interaction, ionic/hydrogen bonding between the $–NH_3^+$ and $–COO^-$ functionalized surface is also possible. The typical binding energies for such bonds are in the range of 1-10 kcal $mole^{-1}$. It is therefore obvious that lysine rich proteins of immunoglobulin (IgY antibodies) are highly attractive toward the citrate-capped GNPs. Fig. S7 (See supplementary material) shows the IR spectroscopic measurements of IgY and the bioconjugated GNPs, respectively, showed bands at 3700-3500 cm^{-1} from the amide N-H stretch, 3500-3300 cm^{-1} from the amine stretch, 3550-3200 broad peak from the O-H stretch, 3100-3010 cm^{-1} from the alkenyl C-H stretch, 3000-2500 cm^{-1} from the carboxylic acid O-H stretch, 2260-2100 cm^{-1} from the alkynyl C≡C stretch, 1700-1500 cm^{-1} from the aromatic C=C bending, 1250-925 cm^{-1} from the C-O-C asymmetric and symmetric vibrations of phospholipids and 860-680 cm^{-1} from the aromatic C-H bending.[32,33] However, significant difference was observed between the unconjugated and conjugated IgY due to electrostatic interactions between the various functional groups of IgY and GNPs. From FTIR analysis the newly formed bonds between the IgY and GNPs-IgY was observed.

Enzyme-linked Immunosorbent assay

The vitamin B_{12}-binding capacity of the antibody was estimated in our previous report by the direct ELISA with limit of detection (LOD) 10 ng/ml.[20]

Legends of Supplementary Schemes and figures as supplementary materials.

Scheme. S1. Scheme showing the nucleation of GNP and its electrostatic conjugation with IgY.

Fig.S1. Change in color of the gold sol during preparation.

Fig.S2. XRD pattern of precipitate obtained from centrifuging colloidal gold thus formed.

Fig.S3. Agarose gel electrophoresis band of GNP and bioconjugates.

Fig.S4. The graph shows the amount of IgY molecules adsorb on per ml of gold particle (~15 nm) was calculated by measuring the difference of absorbance of the IgY solution before and after incubation with GNPs.Inset shows the flocculation assay determining the amount of IgY (µg mL^{-1})required to stabilize the GNPs.

Fig.S5. UV–vis spectra of GNPs (~15 nm) incubated with varying concentrations of NaCl. The CFC is indicated by a large decrease and red shift of the plasmon absorption band.

Fig. S6. Graph showing the quenching of fluorescence of IgY by GNP. Inset shows the double logarithmic plot of the quenching of the fluorescence by GNPs for the calculation of binding constant (*K*b).

Fig.S7. FTIR absorbance spectra for IgY and bioconjugate (IgY-GNP). FTIR spectra were acquired at room temperature.

Scheme. S1.

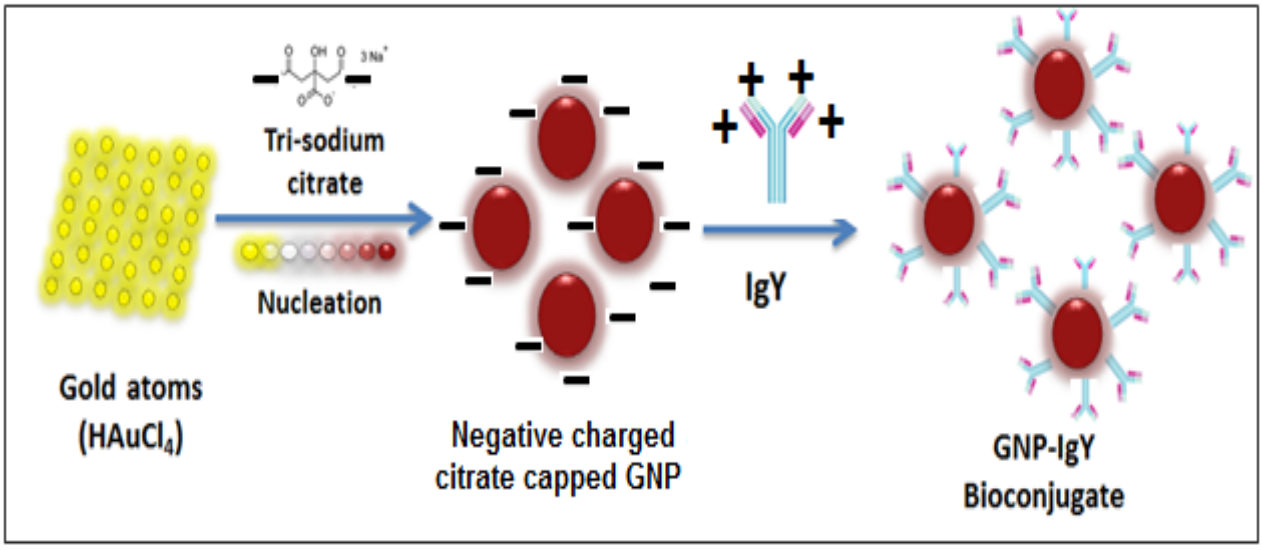

Fig.S1.

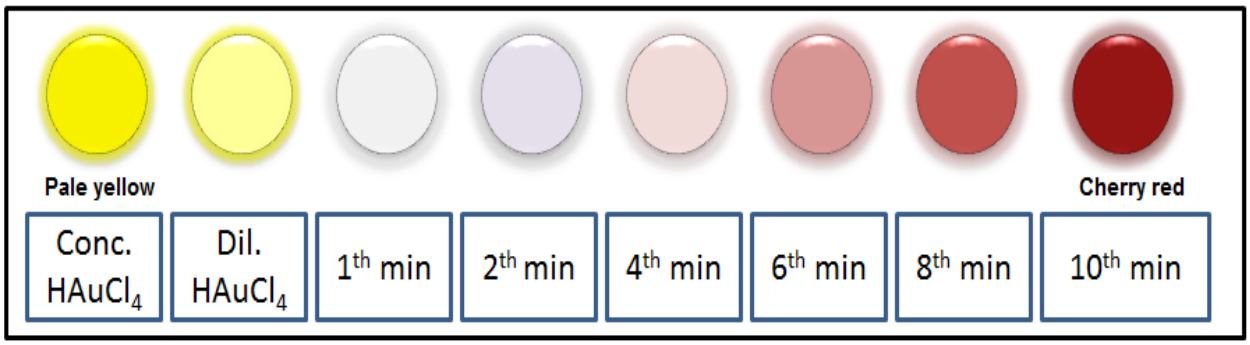

Fig.S2.

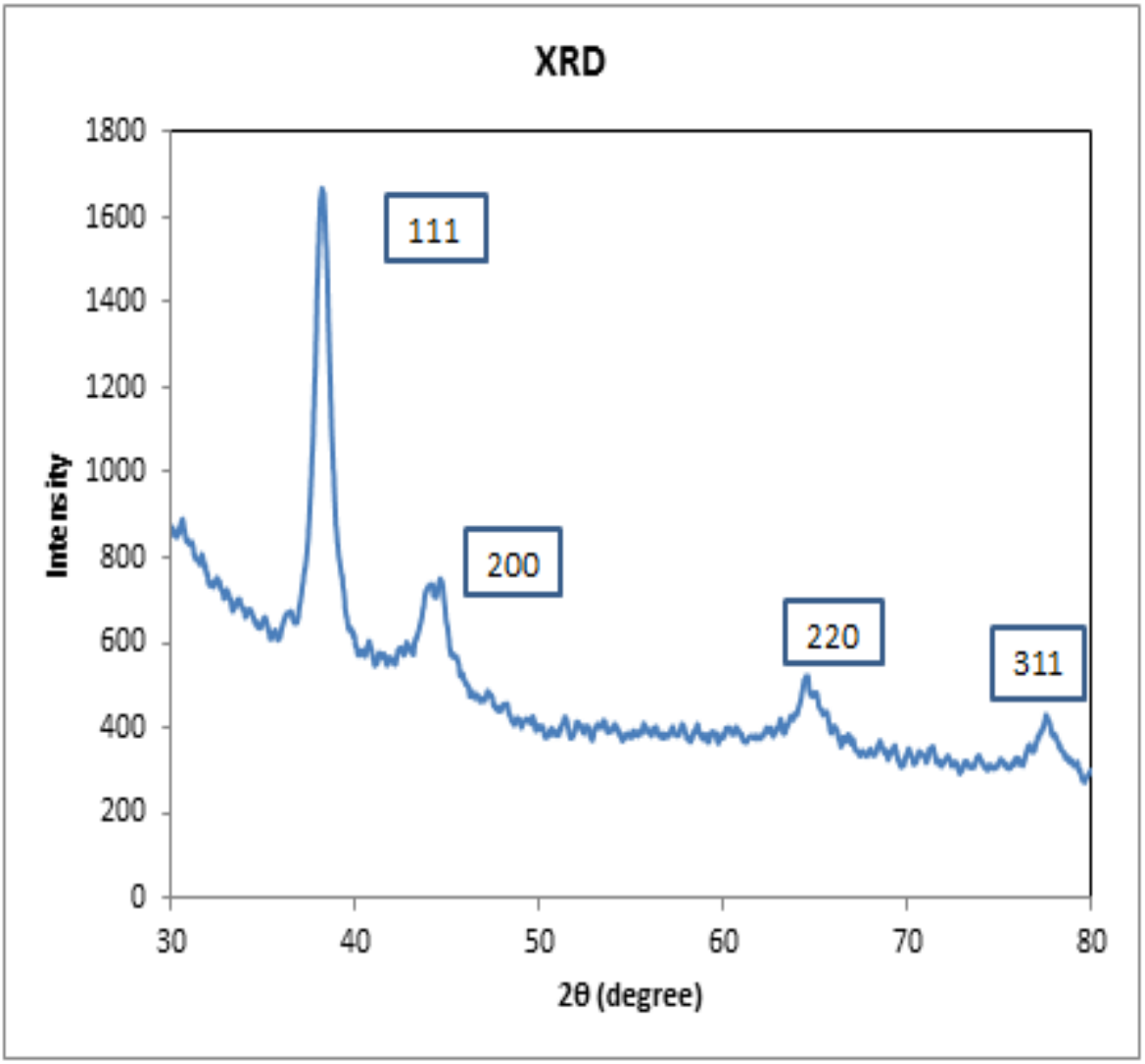

Fig. S3.

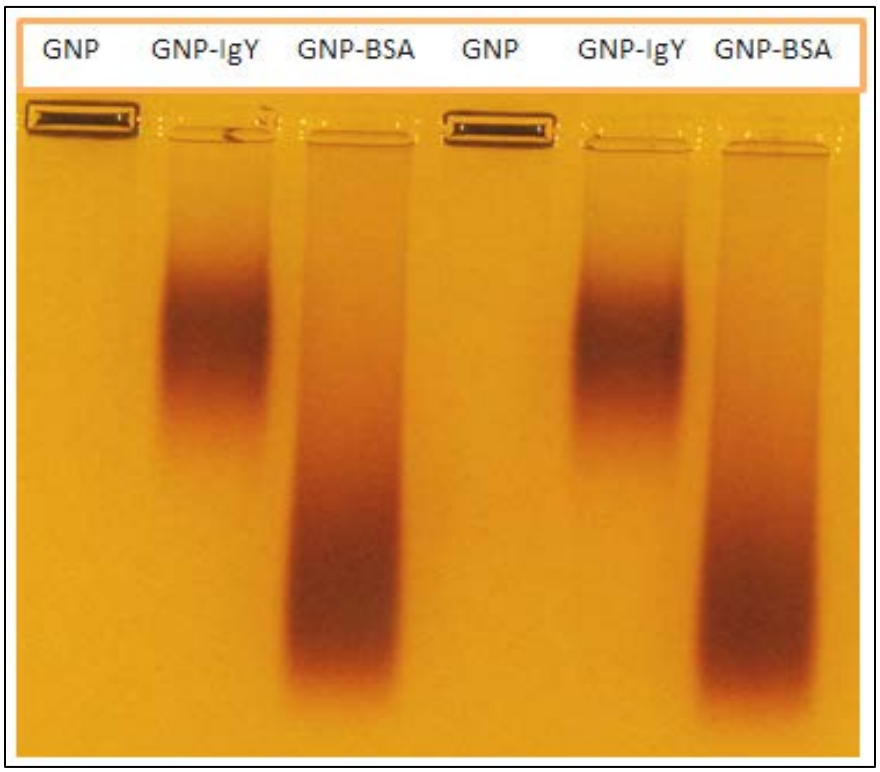

Fig. S4.

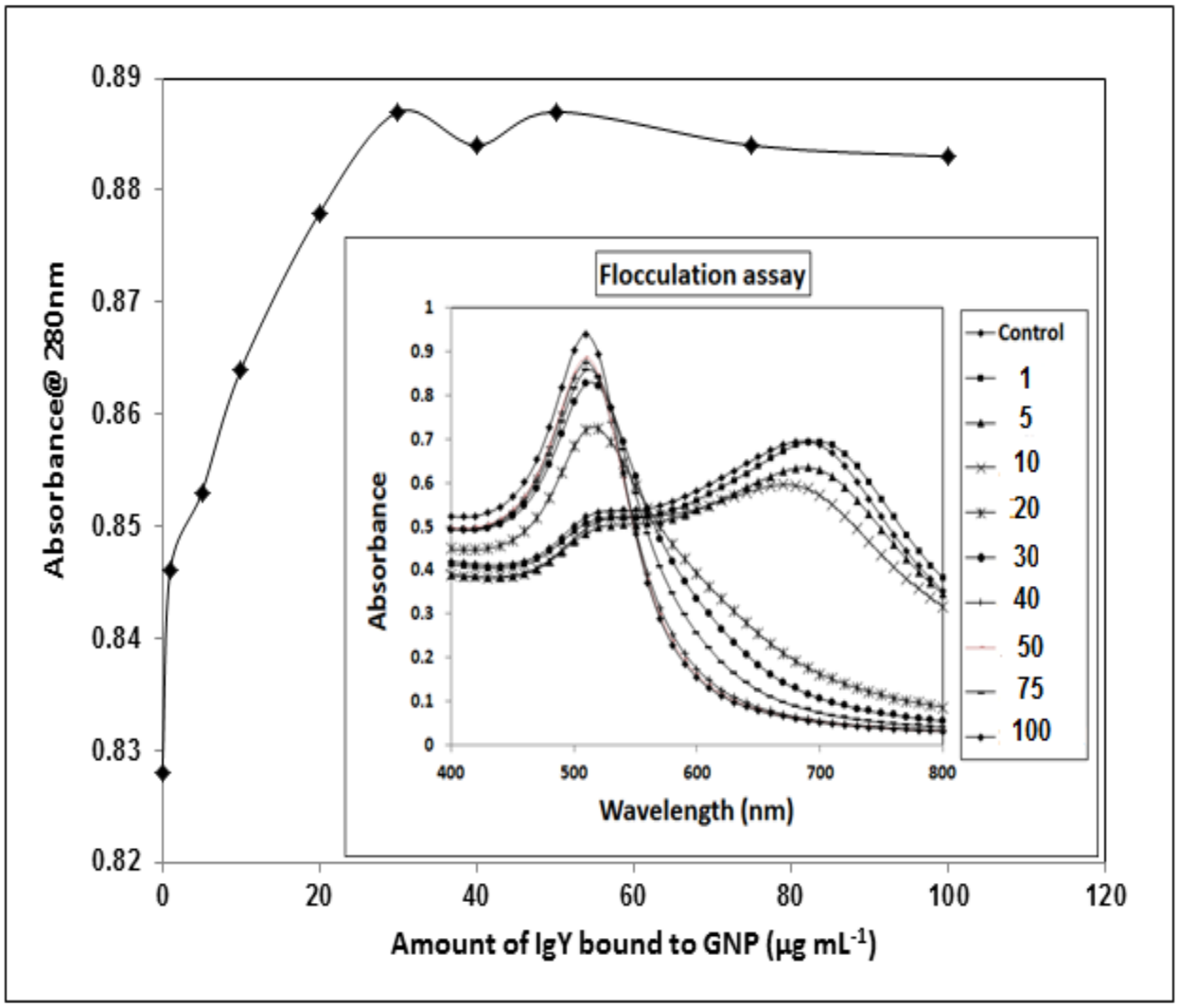

Fig. S5.

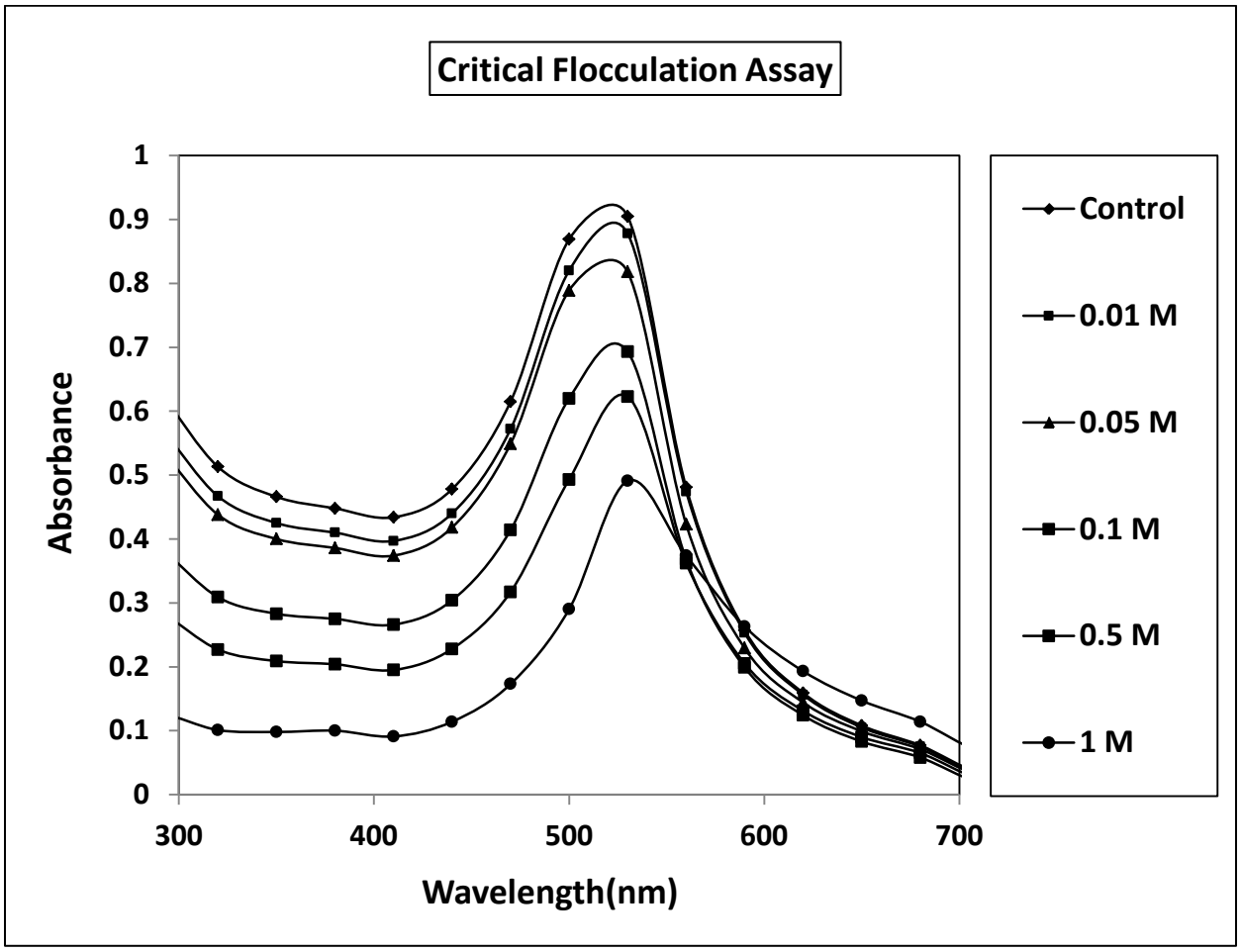

Fig. S6.

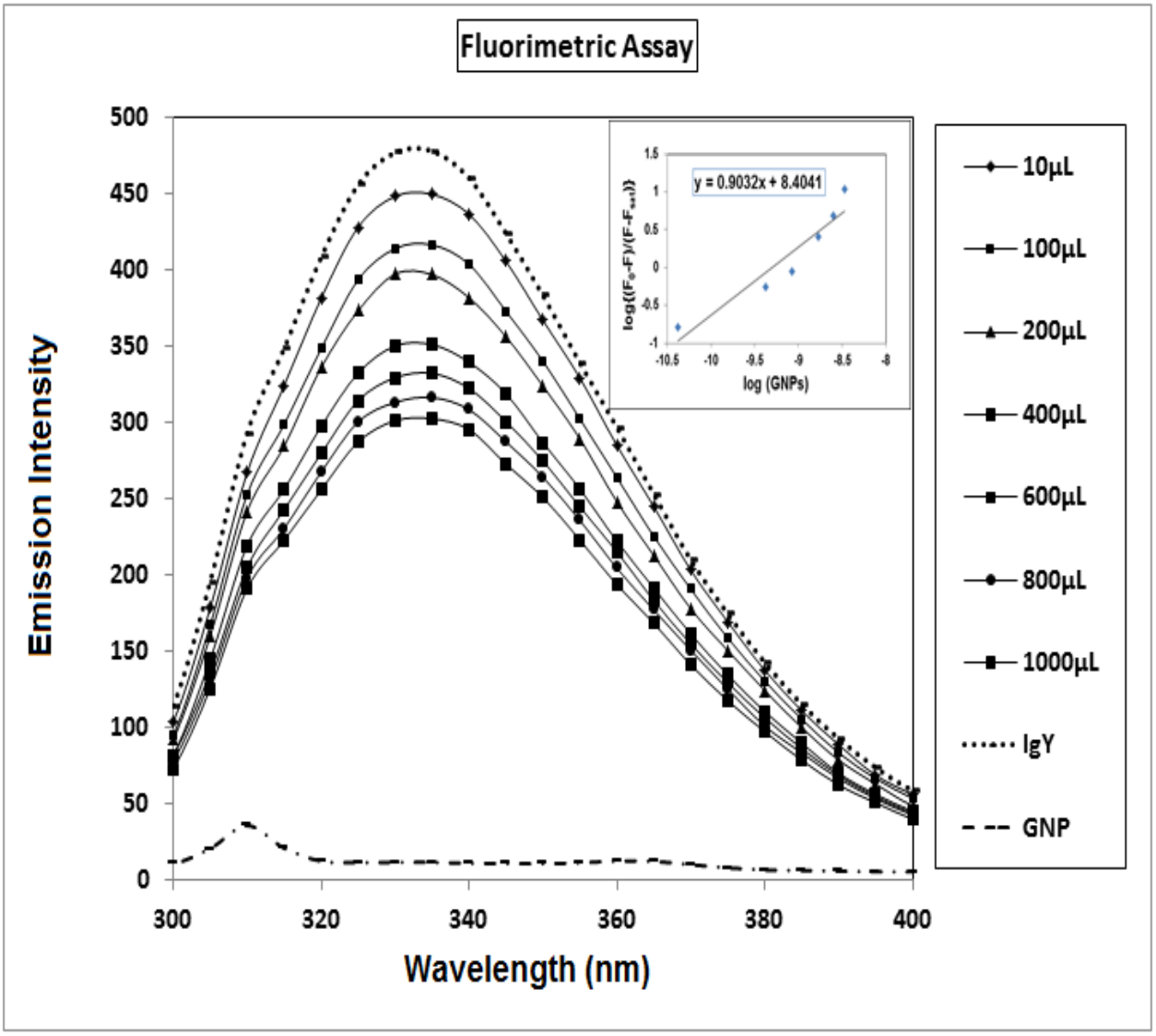

Fig. S7.