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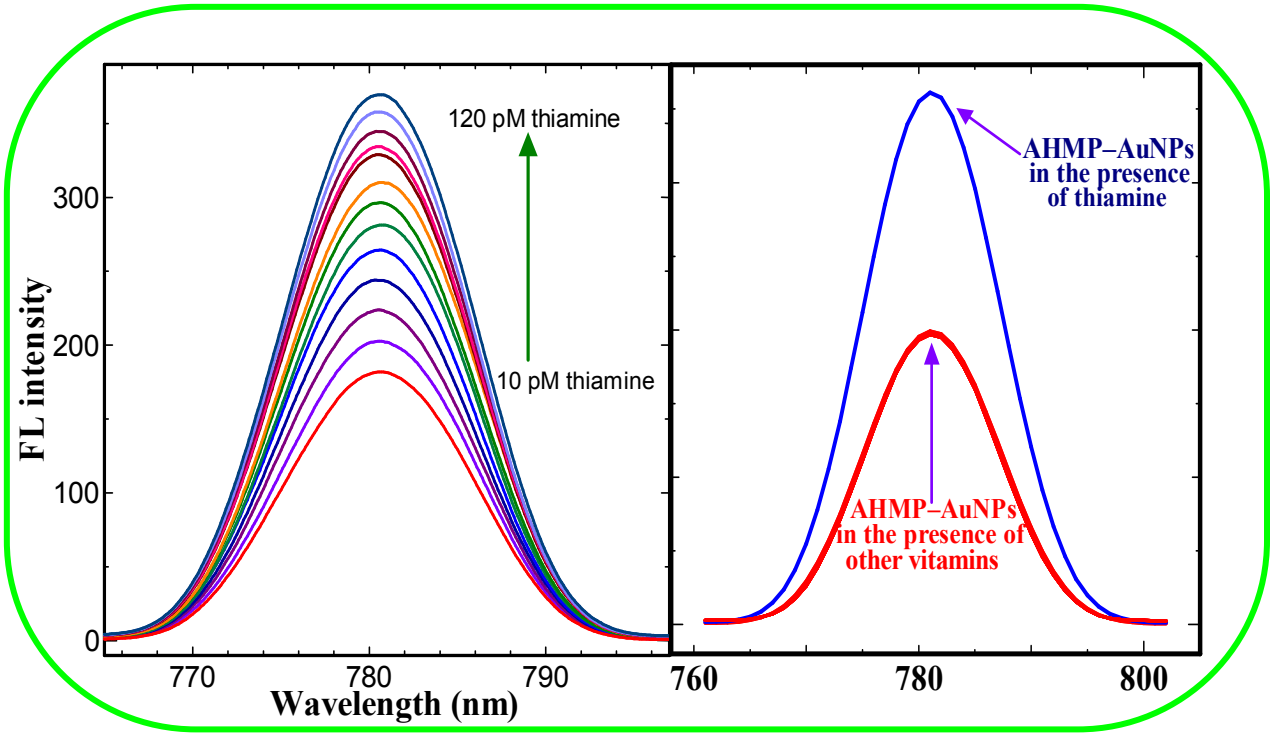
Graphical abstract

This work describes the spectrofluorimetric determination of thiamine in the presence of vitamin B complexes using 4-amino-6-hydroxy-2-mercaptopyrimidine as fluorophore. The detection limit was found to be 6.8 fM L^{-1}(S/N=3).



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ARTICLE

Sensitive and highly selective determination of vitamin B1 in the presence of other vitamin B complexes using functionalized gold nanoparticles as fluorophore

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The present work describes highly selective and sensitive determination of vitamin B1 (thiamine) using 4-amino-6-hydroxy-2-mercaptopyrimidine capped gold nanoparticles (AHMP-AuNPs) by spectrofluorimetry. The AHMP-AuNPs were synthesized by wet chemical method and were characterized by HR-TEM, XRD, UV-visible, zeta potential and spectrofluorimetry. They show emission maximum at 781 nm while exciting at 520 nm and a large stock shift (261 nm) with a narrow emission profile and good photostability. While adding 0.15 μM thiamine, the red color solution of AHMP-AuNPs changes to purple and the absorbance at 520 was decreased. This is due to the aggregation of AHMP-AuNPs and it was confirmed by HR-TEM. No change in absorbance was observed in the UV-visible spectra for AHMP-AuNPs in the presence of less than micromolar concentration of thiamine. On the other hand, the emission intensity of AHMP-AuNPs was enhanced even in the presence of picomolar concentration of thiamine. Based on the enhancement of emission intensity, the concentration of thiamine was determined. Interestingly, no change in the emission intensity was observed while adding even milli molar concentration of other vitamin B complexes. The present fluorophore showed an extreme selectivity towards the determination of thiamine in the presence of 10,000 fold common interferents including vitamin B2, B3, B6, B9 and vitamin C while the presence of cysteine and glutathionine interferes for the determination of thiamine. A good linearity was observed from 10 to 120 × 10^{-12} M thiamine and a detection limit was found to be 6.8 fM/L (S/N = 3). The present method was successfully used for the determination of thiamine in human blood serum samples.

Introduction

Thiamine (also called Vitamin B1) is a well known water soluble vitamin of B complex. It consists of an aminopyrimidine ring and a thiazole ring with methyl and hydroxyethyl side chain linked by a methylene bridge (Chart S1; ESI (structure A)). Thiamine is a biologically and pharmaceutically important compound and it is an essential vitamin in the human metabolism and proper functioning of the brain, muscle, heart and cardiovascular systems of the body. It is also used in the synthesis of neurotransmitter acetylation and gamma amino butyric acid. The coenzyme in metabolism of amino acids and sugar is thiamine pyrophosphate which is the best characterized form. This is the only synthesis in bacteria, fungi and plants and therefore it is an essential nutrient in human diet. Besides, it acts as a catalyst in the main reaction which converts blood sugar into energy.[1,2,3]

The average daily intake of thiamine is 0.5-1 mg. When the daily intake of thiamine is less than this, it is readily absorbed in the small intestine via an active carrier mediated transport system. If larger amounts are consumed passive diffusion occurs. Nowadays, thiamine is routinely prescribed to young adults and pregnant women.[4,5] The risk of thiamine deficiency is increased among people subsisting on white rice or highly refined carbohydrates (such as polished rice, white flour, white sugar) and alcoholics. Thiamine deficiency, which can be defined as decrease of blood thiamine concentration is associated with diabetes as well as with decreased activity of the thiamine-dependent enzyme transketolase and the severe thiamine deficiency has also been linked to beriberi (a chronic neurological and cardiovascular disease) and Wernick-Korsakoff syndrome (caused brain abnormalities).[6,7] In addition, a non-co-factor role of thiamine in the physiologic

maintenance of the internal milieu is well established. Thus, availability of thiamine is a prerequisite for normal cellular functioning. Any disturbance in thiamine levels can result in severe neurological deficits, with the most common cause of this problem being inadequate uptake or absorption of thiamine, combined with chronic alcohol consumption. Thus, development of a cheap, fast, accurate and precise method for the determination of thiamine in food and pharmaceutical preparations is highly essential.

Metal nanoparticles have received significant interest in the past decade as active component for widespread applications in the form of biomedicine, biosensors and drug delivery systems.[8,9,10] Especially, gold nanoparticles (AuNPs) have received much attention because of their shape and size dependant optical properties and quantum confinement effects.[11] Several methods have been used for the determination of thiamine which include spectrophotometry,[12,13] spectrofluorimetry,[14,15,16,17] high performance liquid chromatography,[18,19,20] chemiluminescence,[21] capillary electrophoresis[22,23] and electrochemical analysis[24,25] Among them, the spectrofluorimetry method has several advantages over other methods which include high sensitivity, selectivity and reproducibility. Even though, the reported papers were detected micro and nanomolar level of thiamine, they failed to achieve high selectivity. Thus, the objective of the present study is to determine thiamine with high selectivity and sensitivity.

Recently, our research group has synthesized AuNPs capped with different functional groups.[26,27,28] We found that the determination of a particular analyte in the presence of other analytes depends on the type of functional groups present on the surface of AuNPs. For example, 2,5-dimercapto-1,3,4-thiadiazole (DMT) capped AuNPs showed extreme selectivity towards Hg(II) even in the presence of 50,000-fold higher concentration of other metal ions[27] whereas 3-amino-5-mercapto-1,2,4-triazole (AMTr) capped AuNPs showed selectivity towards melamine[28]. In the present study, 4-amino-6-hydroxy-2-mercaptopyrimidine (AHMP) (Chart S1; ESI (structure B)) capped AuNPs are synthesized and utilized for the selective determination of thiamine in the presence of other vitamin complexes. 2-Mercaptopyrimidine (2-Mpy) derivatives have distinct properties in contrast to normal aromatic thiols such as thiophenol.[29] Compared to 2-Mpy, AHMP contains an additional amine group which can also prevent the AuNPs from aggregation by electrostatic repulsion. The AHMP-AuNPs show the emission maximum at 781 nm while exciting at 520 nm. The absorption intensity of AHMP-AuNPs was decreased while adding 0.15 µM thiamine and the wine red color of AHMP-AuNPs was changed to purple. Interestingly, the emission intensity of AHMP-AuNPs was enhanced even in the presence of picomolar concentration of thiamine. Based on the enhancement of emission intensity, the concentration of thiamine was determined. The detection limit was found to be 6.8 fM/L (S/N=3). Further, 10,000 fold common interferents including other vitamin B complexes do not interfere for the determination of thiamine. This method was successfully applied to determine thiamine in human blood serum samples.

Materials and Methods

Chemicals

Hydrogen tetrachloroauarate trihydrate ($HAuCl_4.3H_2O$), 6-amino-6-hydroxy-2-mercaptopyrimidine (AHMP), thiamine hydrochloride (vitamin B2), niacin (vitamin B3), pyridoxine hydrochloride (vitamin B6), folic acid (vitamin B9), Vitamin B12 and ascorbic acid (vitamin C) were purchased from Sigma-Aldrich and were used as received. Sodium borohydride and mercury nitrate were purchased from Merck and were used as received. All other chemicals used in these experiments were of analytical grade and used directly without further purification. Millipore water (18 MΩ cm) was used to prepare all the solutions.

Instrumentation

Fluorescence spectral measurements were performed on a JASCO FP-6500 spectrofluorimeter equipped with a xenon discharge lamp and a 1 cm quartz cell at room temperature. Absorption spectra were measured by using a JASCO V-550 UV-visible spectrophotometer. HR-TEM images of AuNPs were obtained from a JEOL JEM 2100 operating at 200 kV. For TEM measurements, the sample was prepared by dropping 2 µl of a colloidal solution onto a carbon-coated copper grid. A large volume (500 ml) of AHMP-AuNPs was synthesized and centrifuged and the particles were separated. Then, repeatedly washed with water and dried in vacuum. The dried AuNPs powder was used for XRD measurements. X-ray diffraction analysis was carried out with a Rigaku X-ray diffraction unit using Ni-filtered Cu Kα (λ = 1.5406) radiation. The zeta potential measurements were performed on Zetasizer Nano S90 (Malvern). Millipore Milli-Q (18 MΩ cm) water was prepared by using Direct-Q Millipore (Cylus Laboratory Equipment, France).

Synthesis of AHMP-AuNPs

All glassware used in the preparation of colloidal AuNPs was cleaned with freshly prepared aqua regia and rinsed thoroughly with water. Briefly, 0.5 ml of $HAuCl_4.3H_2O$ (31.7 mM) and 0.25 ml of 1 mM AHMP solution were added into 23.5 ml of water. Then, 2 ml of freshly prepared ice cold $NaBH_4$ (0.125%) was added with constant stirring for 20 min. The color of the solution was changed into wine red immediately after the final addition of $NaBH_4$, indicating the formation of AuNPs. The synthesized AHMP-AuNPs were stored in a bottle at 4^0C and they remained stable for several months.

Results and discussion

Spectral and zeta potential studies of AHMP-AuNPs

The AuNPs have unique optical properties in the visible region because of the surface plasmon oscillation of free

electrons.[30,31] The formation of AHMP-AuNPs was monitored by UV-visible spectroscopy (Fig. S1; ESI). The AHMP in water shows an absorption maximum at 275 nm with a very weak shoulder band at 245 nm (curve a) due to the existence of thione-thiol equilibrium in water. The $HAuCl_4$ (0.317 mM) in water shows an absorption maximum at 291 nm (curve b). The addition of AHMP solution to $HAuCl_4$ does not affect the absorption maximum but the intensity of the absorption was increased which may due to the formation of complex between AHMP and $HAuCl_4$ (curve c). When 2 ml of 0.125% of $NaBH_4$ was slowly added to a mixture of AHMP and $HAuCl_4$ solution, the yellow color solution was slowly changed into a wine red color indicating the formation of AuNPs, which shows a new absorption peak at 520 nm (curve d), corresponding to the surface plasmon resonance (SPR) band. The observed SPR band at 520 nm confirms the successful formation of AHMP-AuNPs. Fig. S2A; ESI shows the UV-visible spectrum of AHMP-AuNPs. It shows the absorption maximum at 520 nm. The AHMP-AuNPs exhibit an emission maximum at 781 nm while exciting at 520 nm, (Fig. S2B; ESI). They show a large stock shift (λ_{em} - λ_{ex} = 261 nm). The obtained narrow emission profile and large stock shift indicate that AHMP-AuNPs are highly fluorescent.[27,32] Further, we have monitored the stability of AuNPs by UV-visible spectroscopy. Fig. S3; ESI shows the UV-visible spectra of freshly prepared and five month aged AHMP-AuNPs. In contrast to the UV-visible spectrum of freshly prepared AHMP-AuNPs, no appreciable spectral changes were observed for five month aged AHMP-AuNPs. Further, the wine red color of the solution remains same (inset Fig. S3; ESI).

The zeta potential measurement for AHMP-AuNPs was measured. It shows a value of -30.1 mV (Fig. S4A; ESI). It is expected that the thiol group of AHMP is chemisorbed on the surface of AuNPs whereas amine and hydroxyl groups available in AHMP are free from binding. The presence of lone pair electrons of amine groups predominantly stabilized the AuNPs.

HR-TEM and XRD studies

The size and morphology of the AHMP-AuNPs were characterized by HR-TEM. The HR-TEM images taken at different magnifications are shown in Fig. 1. Fig. 1A illustrates that they are roughly spherical in shape and the high magnification image depicts that AHMP-AuNPs are in spherical shape with a size of ~11 nm (Fig. 1B). Selected area electron diffraction pattern (inset Fig. 1A) exhibits the crystalline nature of the AHMP-AuNPs.[33]

The crystalline nature of AHMP-AuNPs was further confirmed by XRD analysis (Fig. S5; ESI). It illustrates the diffraction features appearing at 38.17°, 44.36°, 64.59° and 77.57° corresponding to (111), (200), (220) and (311) planes, respectively. The peak corresponding to the (111) plane is more intense than that from the other planes. The ratio between the intensity of the (200) and (111) diffraction peaks was much lower, suggesting that the (111) plane is a predominant orientation. The width of the (111) peak was employed to calculate the average crystalline size of the AHMP-AuNPs using the Scherrer equation.[34] The calculated average size of the AuNPs is ~11 nm, which closely matches with the particle size obtained from the HR-TEM images.

Determination of concentration of AuNPs, molar extinction co-efficient and band gap energy of AHMP-AuNPs

The concentration of AHMP-AuNPs was calculated based on the reported procedure[35] and was found to be 1.9 µM. The molar extinction co-efficient of AHMP-AuNPs was calculated[36] using the following equation.

$$\varepsilon = A / b\,c \quad (1)$$

where 'ε' is the molar extinction co-efficient, 'A' is the absorbance and 'b' is the path length of sample and 'c' is the concentration of AuNPs. The molar extinction co-efficient was found to be 7.1×10^5 M^{-1} cm^{-1}. The obtained value suggests that the synthesized AuNPs having good photochemical properties.[36]

The band gap energy of AHMP-AuNPs was calculated [37] by using the following equation.

$$\text{Band gap energy } (E) = hc / \lambda \quad (2)$$

where 'h' is the planck constant and 'c' is the speed of light and 'λ' is the absorption maximum. The band gap energy of AHMP-AuNPs was found to be 2.38 eV. We have also estimated the band gap energy of the AHMP-AuNPs by DFT and obtained the value of 2.499 eV (Table S1; ESI). It closely matches with the value calculated from the planck equation. The obtained value suggested that AHMP-AuNPs are semiconducting nature.[37]

Spectrophotometric determination of thiamine

Fig. 2 shows the UV-visible spectra of AHMP-AuNPs in the presence of different concentrations of thiamine. They show the SPR band at 520 nm. While adding 0.15 µM thiamine, the wine red color of AHMP-AuNPs was slightly changed into purple and the absorbance was decreased with a small red shift (~1 nm). While increasing the concentration of thiamine from 0.30 to 1.05 µM (curve b-h), the absorbance intensity was decreased with a red shift. The wine red color was completely changed into purple when 1.20 µM thiamine was added and absorption peak was red shifted to 690 nm (curve i, inset (ii-B) photograph). Interestingly, an isosbestic point was appeared at 560 nm (Inset (i)), indicating a neat conversion of AHMP-AuNPs into complexed thiamine-AuNPs. Further increasing the concentration of thiamine up to 1.80 µM, the purple color of AuNPs was completely disappeared and a precipitate was settled down at the bottom of the quartz cell (Inset (ii-C) photograph) and the SPR band was decreased without any significant shift at 690 nm (Fig S6; ESI (curve j-m)). These results are in good agreement with Mie theory.[38] According to Mie theory, when the distance between two nanoparticles become smaller than the sum of their radii, the SPR band displays a red shift, broadening and decreasing in intensity. The observed spectral and color changes were attributed to the aggregation of AHMP-AuNPs. The red shift in the SPR band was ascribed to the near-field coupling that occurs when the

interparticle distance decreases. Since thiamine having positive charge, it electrostatically interacts with the negatively charged AHMP-AuNPs and decreases the interparticle distance due to charge screening effect. The aggregation of AHMP-AuNPs by thiamine was confirmed by HR-TEM. The HR-TEM images of AHMP-AuNPs in the presence of 1.20 µM thiamine show the aggregated structure (Fig. 3B). The strong interaction between AHMP-AuNPs and thiamine was confirmed by zeta potential studies. Fig. S4B; ESI shows the zeta potential of AHMP-AuNPs in the presence of 1.20 µM thiamine, and it shows value of +2.7 mV in contrast to -30.1 mV in the absence of thiamine. The zeta potential study shows the neutralization of surface charge by the positively charged thiamine with AuNPs causes aggregation.

The absorption spectra of AHMP-AuNPs in the presence of nanomolar concentrations of thiamine do not show any significant changes at 520 nm unlike the addition of micromolar concentrations of thiamine. Further, no visible color change was also observed after the addition of nanomolar melamine to AHMP-AuNPs solution. These results indicate that spectrophotometric method is not suitable to determine thiamine at trace levels using AHMP-AuNPs.

Spectrofluorimetric determination of thiamine

The emission spectrum of AHMP-AuNPs was recorded by varying the excitation wavelength from 450-600 nm (Fig. S7; ESI). The emission intensity position does not vary while varying the excitation wavelength. On the other hand, the emission intensity increases while increasing the excitation wavelength and reaches maximum at 520 nm and after that it decreases. Thus, we have chosen the excitation wavelength of 520 nm for emission studies. Fig.4 shows the excitation (A) and emission (B) spectra of AHMP-AuNPs in the presence of various concentrations of thiamine. The emission and excitation spectral results showed that the enhancement of intensity is directly proportional to the concentration of thiamine. Thus, AHMP-AuNPs have a well defined excitation and emission spectra with a narrow profile and large stock shift. The excitation spectrum shows the excitation maximum at 520 nm while emitting at 781 nm (curve a). After the addition of 10 pM thiamine, the emission intensity of AHMP-AuNPs was increased without affecting the wavelength (curve b). While increasing the concentrations of thiamine from 20 to 120 pM (curve c-m), the emission intensities were dramatically increased. The observed emission intensity enhancement can be attributed to photoinduced electron transfer[39]. On the other hand, AHMP-AuNPs aggregation in the presence of thiamine can reduce their vibration and rotation speed. Weakness of the Brownian movement and decrease of collision probability between the AuNPs result in the increase of the external energy rate and this leads to an enhancement of the fluorescence intensity of AHMP-AuNPs[40]. A good linearity was obtained in the emission intensity against 10 to 120 × 10^{-12} M concentrations of thiamine (R^2 = 0.9968). Based on the enhancement of emission intensity, the concentration of thiamine was determined. The limit of detection (LOD) was found to be 6.8 fM/L (S/N = 3). The LOD obtained in the present method was compared with the previous methods and are given in Table S2; ESI. It can be seen from the table, the lowest detection limit was achieved for thiamine using the present method compared to any other methods. Further, the relative quantum yield (ϕF) of AHMP-AuNPs and AHMP-AuNPs with thiamine was calculated using the comparative William's method[41]. The fluorescence quantum yields for AHMP-AuNPs and AHMP-AuNPs with thiamine were found to be 0.6312 and 0.8528, respectively. The obtained enhancement of quantum yield indicates that AHMP-AuNPs with thiamine is more emittive than AHMP-AuNPs.

Binding constant

The binding constant (K_A) and binding site (n) values can be calculated using the double logarithm equation given below[42]

$$\log [F - F_0/ F_0] = \log K_A + n\log[Q] \quad (3)$$

where "F_0" is the fluorescence intensity of AHMP-AuNPs and "F" is the fluorescence intensity of AHMP-AuNPs in the presence of thiamine. K_A is the binding constant and n is the number of binding sites and Q is the concentration of thiamine. K_A and n can be measured from the intercept and slope obtained through plotting log [F - F_0/ F_0] against log [Q] (Fig. S8; ESI). The emission data using in equation (3), was obtained by setting n= 0.8034 and K_A = 6.028 × 10^7 mol^{-1} L. The obtained binding constant value suggests that there is a strong binding force between thiamine and AHMP-AuNPs.

Selective determination of thiamine

The effect of various interferences for the determination of thiamine was investigated. Fig.5 shows the emission spectra of AHMP-AuNPs in the presence of 10,000 fold higher concentrations of (1 mM each) pyridoxine (vitamin B6), riboflavin (vitamin B2), cyanocobalymine (vitamin B12), ascorbic acid (vitamin C), niacin (vitamin B3), folic acid (vitamin B9), Hg^{2+}, Pb^{2+}, Cu^{2+}, Zn^{2+}, and Ca^{2+}. As shown in Fig.5, 10,000 fold higher concentrations of common interferents including Vitamin B derivatives and metal ions does not interfere the determination of 100 nM thiamine. The obtained high selectivity is due to strong electrostatic interactions between thiamine and AHMP-AuNPs. Thiamine has positive charge on nitrogen atom and it also contains thiazole ring. Therefore, strong electrostatic interactions between thiamine and AHMP-AuNPs are possible. On the other hand, such electrostatic interaction is not possible for the other vitamin B derivatives due to the absence of positive charge on nitrogen atom (Chart S1; ESI). Besides, B2 and vitamin B9 contain bulky groups and therefore, the possible steric repulsion inhibits the interaction of them with AHMP-AuNPs. Further, the interference of L-amino acids was investigated in the presence of thiamine. We found that except cysteine all other 19 amino acids do not interfere even in the presence of micromolar concentration. Besides, glutathione also interferes for the determination of thiamine. However, nanomolar concentration of cysteine and glutathione do not interfere for

the determination of 100 pM thiamine. To the best of our knowledge, this is first report for the determination of thiamine in the presence of 10,000 fold higher concentration of common interferences including vitamin B complexes by spectrofluorimetry method.

Practical application

The practical application of the present method was evaluated by determining thiamine in human blood serum samples. The blood serum samples collected from the nearby clinical laboratory were used in this investigation. The standard addition technique was used to examine the recovery of thiamine. 1 mL of the blood serum sample was diluted to 50 mL solution. While adding serum sample into AHMP-AuNPs, the emission intensity was enhanced at 781 nm. To confirm that the observed enhancement is due to thiamine, a known concentration of thiamine (10 nM) was added into the same solution. The enhancement of emission intensity at 781 nm confirmed the presence of thiamine in blood serum sample. The recovery results for the different additions of thiamine in blood serum samples are given in Table S3; ESI. The recovery of 99.4% and 99.6% were obtained and good agreement was obtained between spiked and measured thiamine. These results indicated that the present method could be efficiently used for the determination of thiamine in practical applications. Further, the obtained results closely match with the results obtained from ICEPS method.

Conclusions

The present work demonstrates the highly sensitive and selective determination of thiamine in the presence of other vitamin complexes using AHMP-AuNPs as fluorophore. The AHMP-AuNPs were more stable and highly fluorescent with a large stock shift. The emission intensity of fluorophore was enhanced while adding thiamine even in the picomolar range. Based on the enhancement of emission intensity, the concentration of thiamine was determined. A good linearity was observed from 10 to 120 $\times$ 10^{-12} M thiamine and the limit of detection was found to be 6.8 fM/L (S/N = 3). Further, the selective determination of 100 nM thiamine was accomplished in the presence of 10,000 fold higher concentration of common interferences including other vitamin complexes. The present method was successfully utilized to determine thiamine in human blood serum samples. This is the first report with a high selectivity and low detection limit by any other method for the determination of thiamine.

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Electronic Supplementary Information (ESI) Available:

UV-vis absorption spectra for reaction monitoring and stability of AHMP-AuNPS, zeta potential and X-ray diffraction pattern of AHMP-AuNPS and UV-vis absorption spectra, zeta potential, binding constant of AHMP-AuNPs with thiamine is available in the online version of this article. See DOI: 10.1039/b0000.

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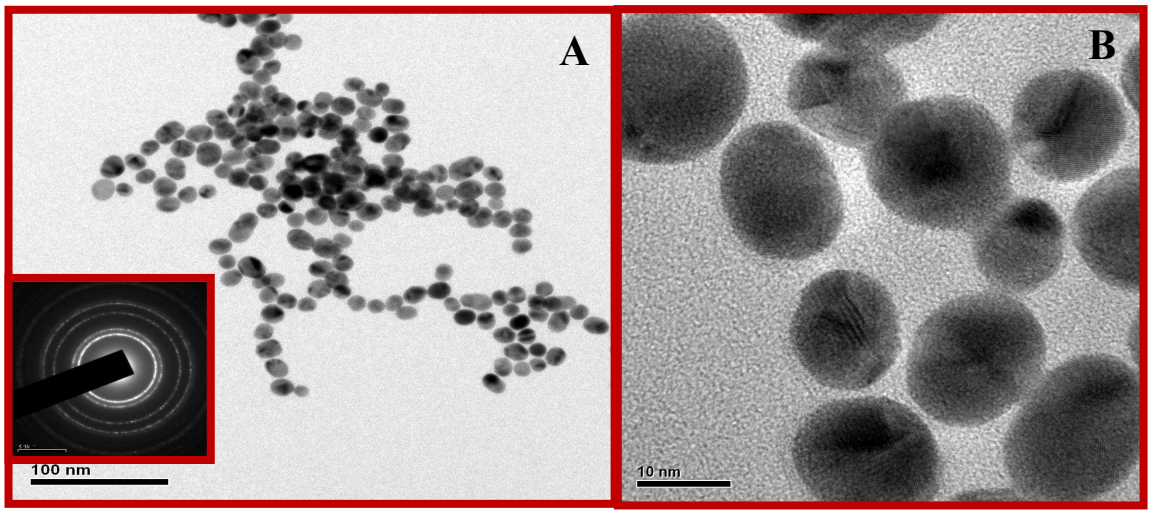

Fig. 1. HR-TEM images of AHMP-AuNPs. (A) low magnification, (B) high magnification. **Inset A:** selected area electron diffraction (SAED) pattern of AHMP-AuNPs.

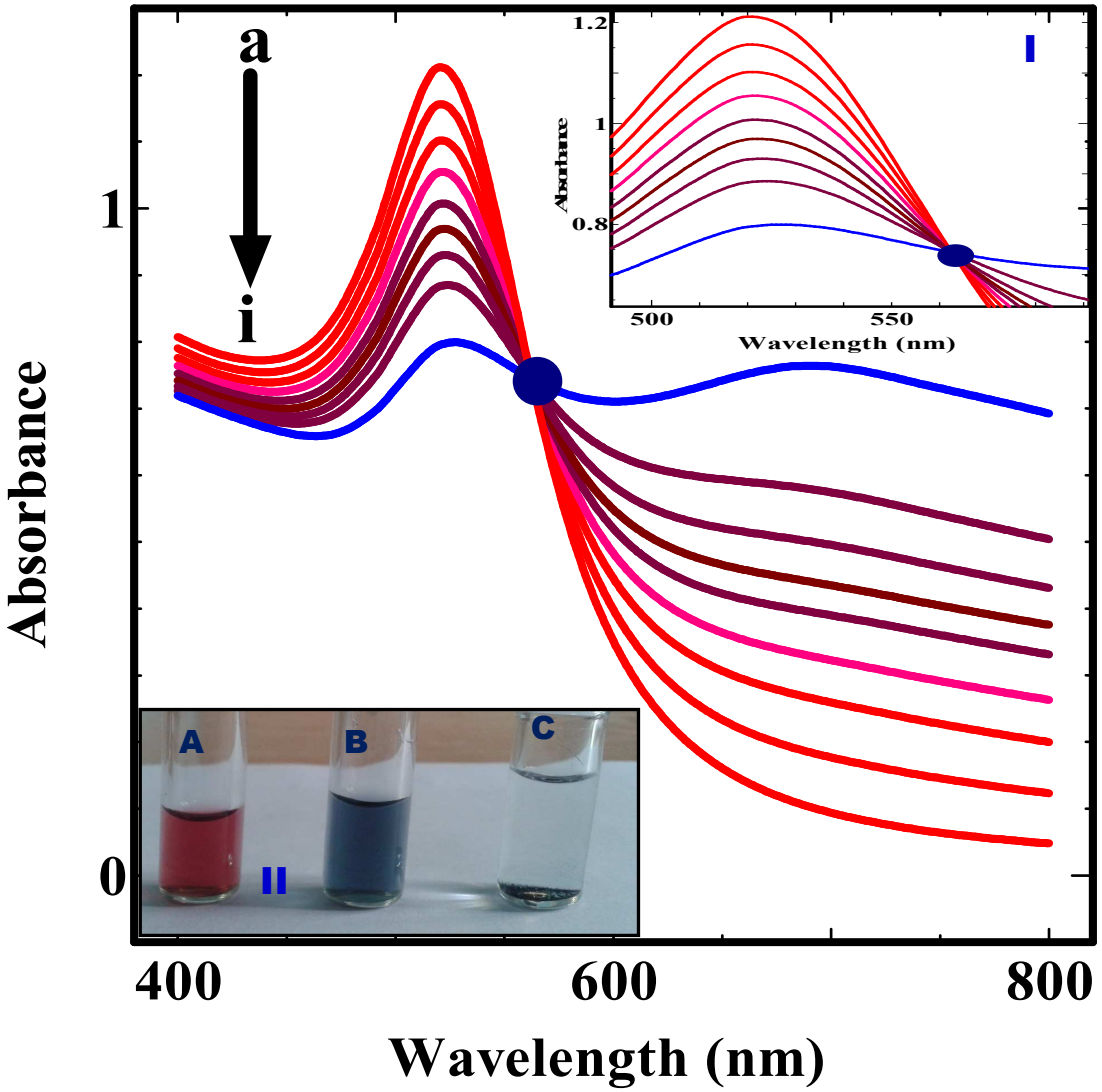

Fig. 2. UV-vis spectra of AHMP-AuNPs in the presence of different concentrations of thiamine: (a) 0, (b) 0.15, (c) 0.30, (d) 0.45, (e) 0.60, (f) 0.75, (g) 0.90, (h) 1.05 and (i) 1.20×10^{-6} M. **Inset**: (i) isosbestic point (ii) photographs of (A) AHMP-AuNPs, after the addition of (B) 1.20×10^{-6} M and (C) 1.80×10^{-6} M thiamine.

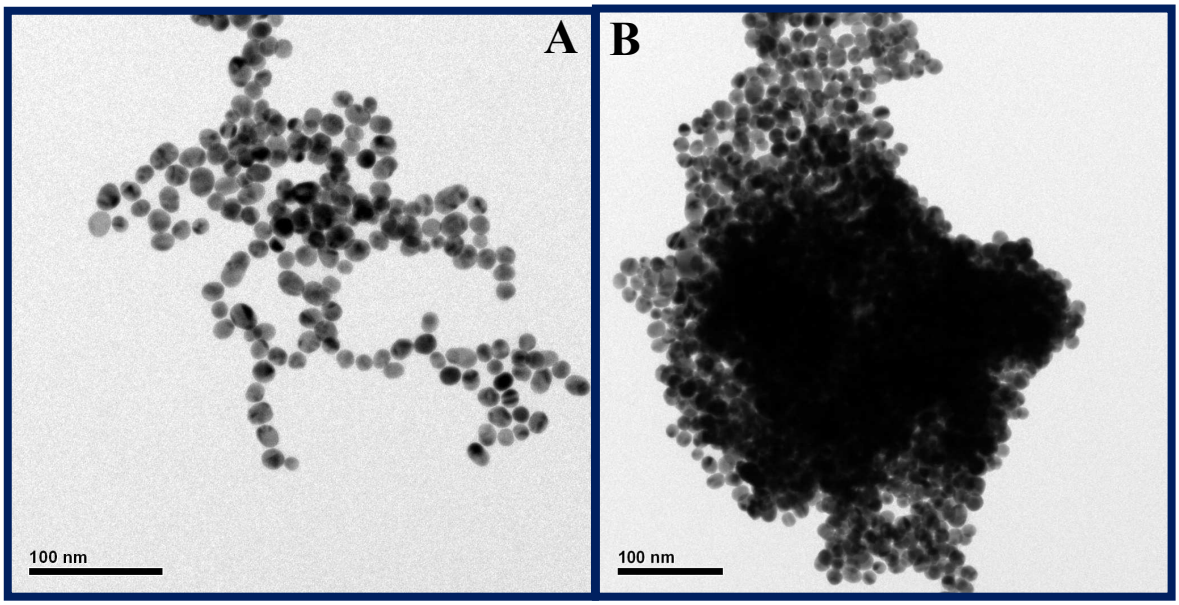

Fig. 3. HR-TEM images of AHMP-AuNPs (A) before and (B) after the addition of 1.20×10^{-6} M thiamine.

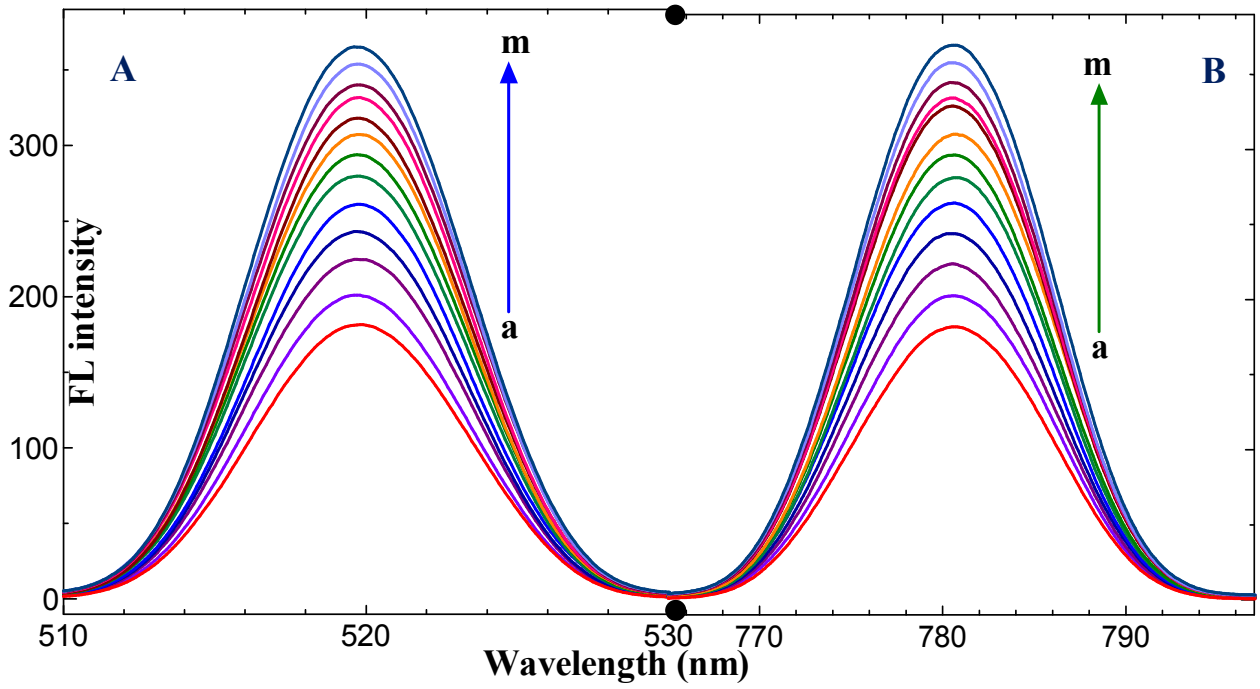

Fig. 4. Excitation (A) and Emission (B) spectra of AHMP-AuNPs after the addition of different concentrations of thiamine: (a) 0, (b) 10, (c) 20, (d) 30, (e) 40, (f) 50, (g) 60, (h) 70, (i) 80, (j) 90, (k) 100, (l) 110 and (m) 120×10^{-12} M (λ_{ex} : 520 nm ; λ_{em} : 781 nm).

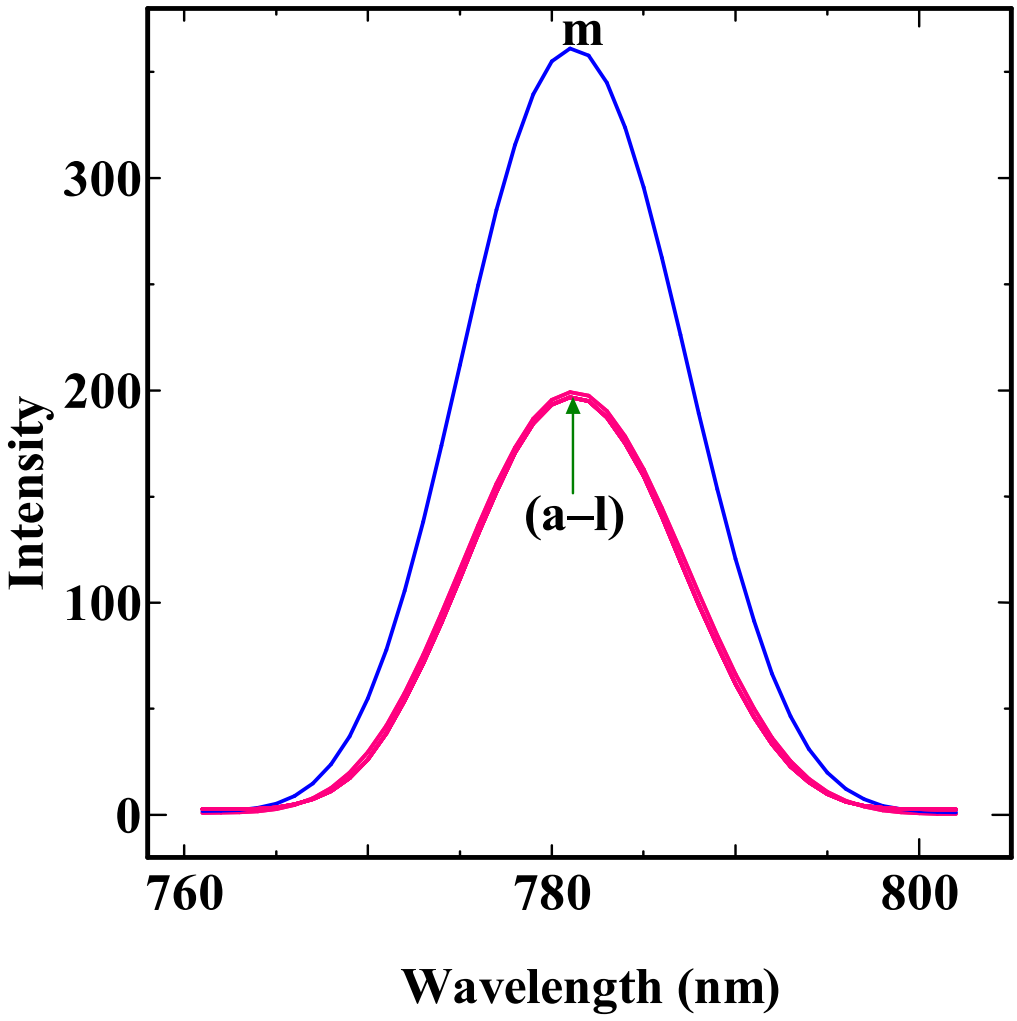

Fig. 5. Emission spectra of (a) AHMP-AuNPs and in the presence of 1 × 10^{-3} M each (b) Ca^{2+}, (c) Zn^{2+}, (d) Cu^{2+}, (e) Pb^{2+}, (f) Hg^{2+}, (g) niacin (h) ascorbic acid, (i) folic acid, (j) riboflavin, (k) pyridoxine, (l) cyanocobalamine and (m) 100 × 10^{-9} M thiamine (λ_{ex} : 520 nm ; λ_{em} : 781 nm).