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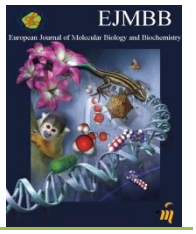

VISUAL DETECTION OF MELAMINE USING SILVER NANOPARTICLES

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ABSTRACT

Here we are reporting a new homogeneous assay for detection of melamine using silver nanoparticle synthesized and functionalized by Gallic acid (2, 4, 6-trihydroxy benzoic acid). Various concentrations of melamine were added to test the linearity and sensitivity of the method. The detection of melamine was based on changes in absorbance resulting from aggregation of silver nanoparticles induced by melamine and thereby results in their yellow to greenish color change. A new peak at around 625 nm, in addition to the peak of the AgNP at 415 nm was observed. With the increase of concentration of melamine, the absorbance at 415 nm peak decreased and that of 625 nm increased gradually. The calibration curve obtained from the ratio of the absorption coefficients of these two peaks ($Ex_{625\ /\ 415}$ versus concentration of melamine enables one to estimate quantitatively the amount of melamine present in water at ppm levels. The assay relies on the fact that melamine can induce aggregation of silver nanoparticles. The presence of melamine in raw milk can be determined by monitoring with the naked eye or a UV-Visible spectrophotometer. The proposed method is a promising mean for on-site screening of melamine adulterant in raw milk without costly instruments.

INTRODUCTION

Melamine (2, 4, 6-triamino-1, 3, 5-triazine) is a small polar compound which is very rich in nitrogen (67% by mass). It has been found to be adulterated in milk products and animal food to give false impression of high protein content. Standard tests such as the Kjeldahl and Dumas tests estimate protein levels of food products by measuring the nitrogen content, thus melamine was adulterated in protein rich diets by unethical manufacturers [1]. Melamine contamination has been reported in products such as milk, infant formulas, frozen yogurt, pet food biscuits, candy and coffee drinks [2]. However, human body cannot metabolize melamine and it may form insoluble complex with cyanuric acid at a certain urine pH. This insoluble melamine - cyanurate complex is being stored in kidney which causes renal failure [3]. In early 2007, it became a topic of discussion when hundreds of pet death occurred due to pet food contamination [4]. In September 2008, melamine was illegally contaminated in infant formulas which caused the death of thousands of infants in China [5]. Numerous cases of renal complications in children have been attributed to consumption of tainted product [6, 7] which contained melamine.

Recently, thorough proactive steps have been adopted by several countries and organizations to ensure that the food supply is not affected by melamine adulterated products. In December 2008, WHO (World Health Organization) suggested the limit of melamine content in infant formula should be 1.0 mg/kg [8]. A safety limit of melamine ingestion has been officially set at 2.5 ppm for adult food and 1ppm for infant formula by Food and Drug Administration (FDA) in the USA [9]. The maximum residue level of melamine in infant formula is

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legally regulated at 1 ppm by Chinese government after the melamine accident [10]

Currently, several methods have been employed for the determination of melamine in milk based products, like high performance liquid chromatography (HPLC) [11], high performance liquid chromatography/mass spectrum (HPLC/MS) [12], gas chromatography [13], immunoassay analysis (ELISA) [14], capillary zone electrophoresis mass spectrum (CE/MS) [15] and surface enhanced Raman spectroscopy [16]. Though these methods are very much useful, a rapid, simple, convenient and sensitive method for the determination of melamine is still found challenging and attractive.

In recent years, gold and silver nanoparticles have been widely used as colorimetric probes for chemical sensing and biosensing of various substances [17] such as viruses [18], cancerous cells [19], toxins [20], heavy metals [21- 23], pesticides [24-26], many inorganic and organic pollutants in water [27, 28]. The methods of determination based on the solution color and the corresponding absorption spectra. The absorption spectra depend on the size and inter particle distance between them. When the nanoparticles approach each other and aggregate, the color of the sol changes due to the shift of the surface plasmon band to a longer wavelength. Several colorimetric methods based on gold and silver nanoparticles have been developed for the melamine detection [29-32] so far. For example, triple hydrogen bonding recognition between melamine and cyanuric acid derivative grafted on the surface of AuNPs has been used for reliable detection of melamine [33]. Melamine in milk products was detected by visual and light scattering spectrometric methods with polythymine – stabilized AuNPs on the basis of the formation of triple H-bonds between thymine and melamine in aqueous solution [34]. Simple colorimetric methods based on electrostatic interactions between melamine and citrate-capped AuNPs and AgNPs [35, 36] have been exploited for the detection of melamine.

In our present study, we have also used the functionalized silver nanoparticles for detection of melamine in milk and milk products. Gallic acid has been employed for reduction of silver and functionalization of silver nanoparticles. The phenolic OH groups present in Gallic acid are expected to act as the stabilizer for AgNP [37].

MATERIALS AND METHODS

Chemicals

Silver nitrate and trichloroacetic acid were purchased from Sigma-Aldrich Chemical Ltd. Sodium hydroxide and Gallic acid (2, 4, 6-trihydroxy benzoic acid) was purchased from Merck. Melamine (2, 4, 6-triamino-1, 3, 5-triazine) and Chloroform ($CHCl_3$) were purchased from Hi-media. All solvents and reagents were of analytical grade and were used without further purification. Double distilled de-ionized water was used in all experiments.

Apparatus

The absorbance spectra of the AgNP were analyzed by using a 'SHIMADZU' UV 1800 spectrophotometer and TEM images were taken using JEOL-JEM 2100 high resolution transmission electron microscope (HR-TEM). 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.

Preparation of AgNP

AgNP was produced by the reduction of silver nitrate solution by Gallic acid in alkaline medium (pH 8). 10 ml of $3x10^{-3}$ (M) Gallic acid solution was taken in a beaker. The pH was maintained by dropwise addition of sodium hydroxide solution (0.15 N). The beaker was cooled in ice cold water, and then 6 ml of $3X10^{-3}$(M) aqueous silver nitrate solution was added to the mixture drop-wise under stirring. The colourless solution gradually became yellowish. The yellow colour indicated the formation of silver nanoparticle.

Colorimetric detection of melamine

The colorimetric detection of melamine was studied using this functionalized AgNP solution at room temperature. To observe the effect of melamine on the functionalized AgNP, 600 µl portions of melamine solution having different concentrations (5-200 ppm) was added one at a time in 2 ml AgNP solution and the resulting mixture was then allowed to stand for 2 minutes when the color changed from yellow to greenish. The intensity of the color of the solution was found to increase with the increase of the melamine concentration.

Colorimetric detection of melamine in raw milk and milk products

In order to detect any presence of melamine, we carried out experiment by taking three milk samples from market including health drink and infant formulas. 0.300 mg of each of the sample was added to 50 ml of double distilled water. Then the solutions of the milk samples were treated as described below. In the first step, 4.0 ml of milk sample was diluted to make it 10 ml and 2.0 ml of 10% mixed solution of trichloroacetic acid and chloroform was added in it. The solution was then mixed with a vortex for 1 min to deposit protein in the sample matrix. Then the mixture was centrifuged at 10000 rpm for 10 min to separate the deposit. The supernatant was transferred into another centrifuge tube and adjusted to pH 8 with sodium hydroxide solution. The solution was centrifuged again and the final supernatant solution was used for melamine detection. To the 600 µl portion of the obtained solution, 2 ml of functionalized AgNP was added one at a time and

the UV-Visible absorption spectra of the solution was taken after two minutes.

RESULTS AND DISCUSSIONS

Detection principle of melamine using AgNP as colorimetric probe

UV-Visible absorption spectroscopy was used to investigate the optical properties of the functionalized AgNP. The absorption spectrum of stable AgNP prepared by this method showed a sharp surface plasmon band at 415nm (Fig. 1A) suggesting the mono-dispersed nature of the sol.

After addition of melamine solution into the sol, the color changes from yellow to greenish (Fig 2C) and a change was observed in the UV-Visible spectrum. A new peak at 625 nm emerged in addition to the peak due to the AgNP at 415 nm. With the gradual increase of the concentration of melamine in the sol, the absorbance at 415 nm decreased and consequently an increase in the 625 nm occurred (Fig 2A). Based on this fact a visual detection of melamine by naked eye is possible. The absorption coefficient ratio of 625 nm and 415 nm peak ($Ex_{625/415}$) could be employed to measure the changes in absorption peak explicitly by the addition of melamine. The change in coefficient confirmed that the ratio increased with the increase of concentration of the melamine. A calibration curve between the absorption co-efficient ratio and the concentration of melamine in the range 5- 200 ppm shows a linear relationship. Thus this method would be useful for the estimation of melamine present in a sample quantitatively (Fig 2B). During the addition of melamine solution into the AgNP sol, the pH of the solution was strictly maintained at 8 because melamine hydrolyses to form cyanuric acid at below pH 5 (pH<5) and above pH 10 (pH>10) [36]. The hydrolysed product, cyanuric acid is unable to induce aggregation of AgNP [38].

HRTEM studies

The TEM picture of functionalized AgNP reveals that the particles are mostly spherical and mono-dispersed (Fig.3A). In this work, Gallic acid was used for reduction which also acts as the stabilizer for the AgNP produced by forming a coating of it on the AgNP surface with the help of the negatively charged hydroxyl ions.The electrostatic force counteract the effects of van der Waals force between molecules, which results the mono dispersed nature of AgNP. Fig.3B shows the TEM picture of the functionalized AgNP in presence of melamine. The picture confirms the formation of aggregation of AgNP. The negatively charged AgNP can attach Melamine molecule with positively charged exocyclic amino groups ($-NH_2$). On the other hand every melamine has six equivalent sites which could produce double NH….N hydrogen bonds with a similar site of another melamine molecule. Hence the aggregation might be induced. The aggregation is schematically depicted in scheme 1.

Quantitative analysis of melamine in samples from market

The milk samples purchased from market were treated as section 2.5. After treatment, 600 µl portion of the obtained solution was added to 2 ml of produced AgNP at pH 8 and after 2 minutes the UV-Visible absorption spectra of the solutions were taken. The colour changing from yellow to greenish yellow and the appearance of a peak near 625 nm (Fig. 4) confirmed the presence of melamine in the samples. The absorption co-efficient ratio of these two peaks ($Ex_{625/415}$) for each milk sample was calculated for quantification and the ratio found was 0.188, 0.177 and 0.200 for the samples 1, 2 and 3 respectively. Now comparing these values with the values obtained from the calibration curve between the absorption co-efficient ratio ($Ex_{625/415}$) and the known concentration of melamine added (Fig. 2B), it is found that the samples 1, 2 and 3 contain respectively 200, 180 and above 200 ppm melamine. The result shows that the milk samples available in market contain much greater amount than the safety limit assigned by various countries.

Figure 1 A-B. (A) UV-Visible spectrum of functionalized AgNP and (B) the digital photographic image of the sol.

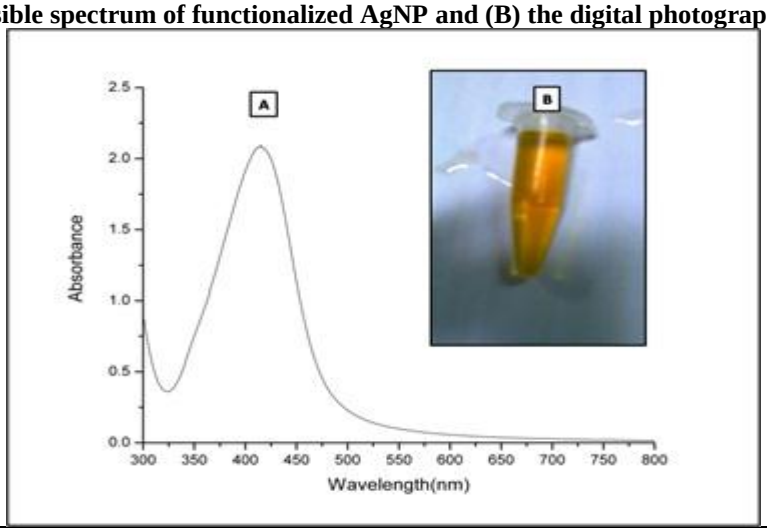


Figure 2. (A) UV-Visible spectra of AgNP (a), varying concentration of melamine in spiked raw milk from 5 – 200 ppm (b to f); (B) Calibration curve between Ex $_{625/415}$ versus concentration of melamine;(C) the photographic images of pure AgNP [1] and AgNP in presence of melamine [2].

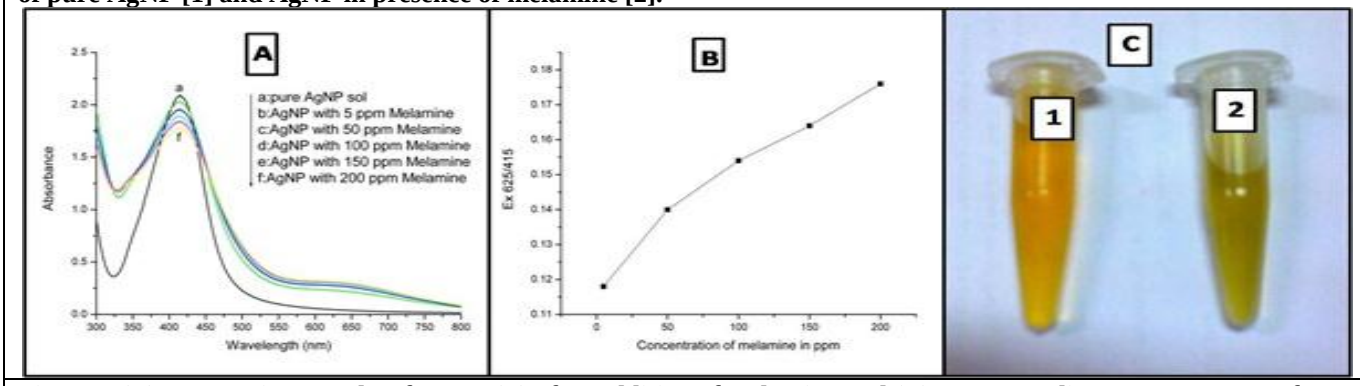


Figure 3 (A). TEM micrographs of AgNP (B) after addition of melamine and (C) corresponding SAED pattern of B.

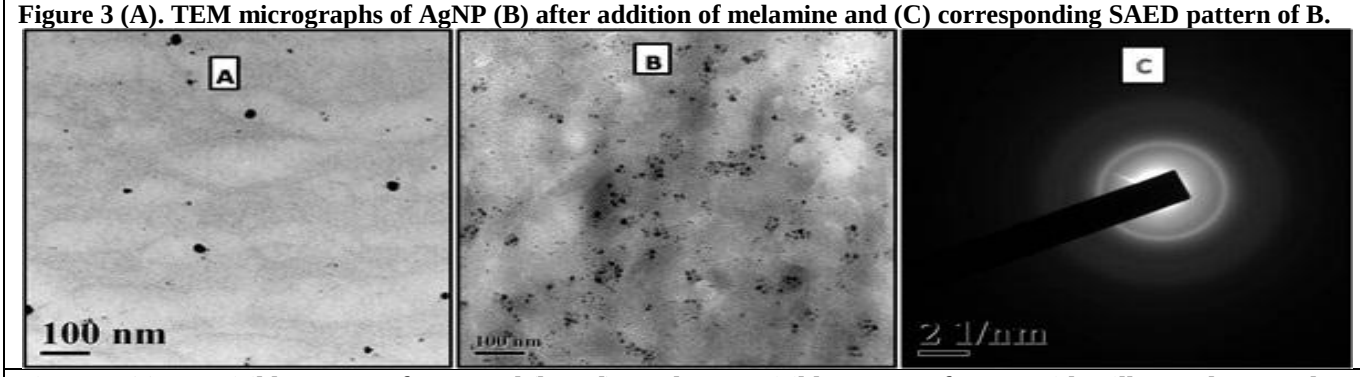
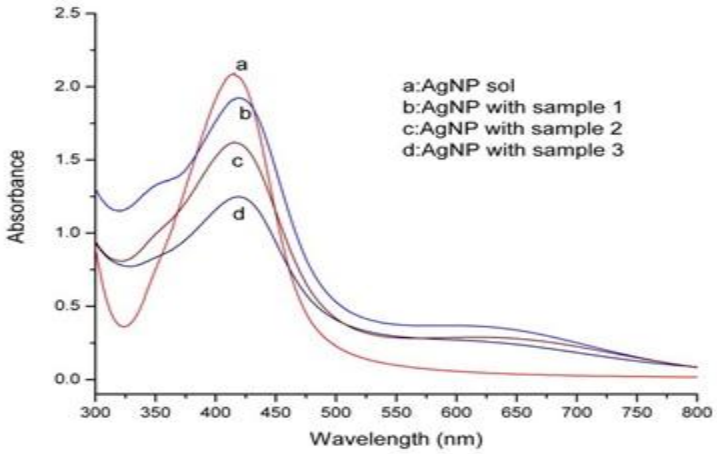
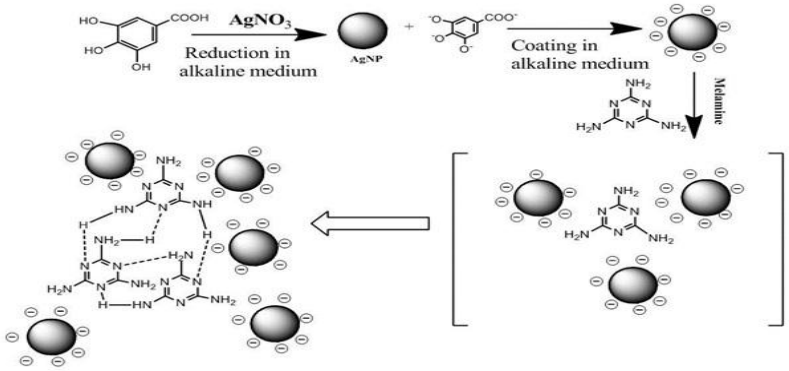


Figure 4. (a) UV-Visible spectra of AgNP sol (b, c, d) are the UV-Visible spectra of AgNP with milk sample1, 2 and 3.



Scheme 1. Schematic representation of aggregation of AgNP



CONCLUSIONS

Here we have reported a new homogeneous assay for detection of melamine using silver nanoparticle synthesized and functionalized by Gallic acid (2, 4, 6-trihydroxy benzoic acid). Various concentrations of melamine were added to test the linearity and sensitivity of the method. The detection of melamine was based on changes in absorbance resulting from aggregation of silver nanoparticles induced by melamine and thereby results in their yellow to greenish color change. A new peak at around 625 nm, in addition to the peak of the AgNP at 415 nm was observed. With the increase of concentration of melamine, the absorbance at 415 nm peak decreased and that of 625 nm increased gradually. The calibration curve obtained from the ratio of the absorption coefficients of these two peaks ($Ex_{625/415}$ versus concentration of melamine enables one to estimate quantitatively the amount of melamine present in water at ppm levels. The assay relies on the fact that melamine can induce aggregation of silver nanoparticles. The presence of melamine in raw milk can be determined by monitoring with the naked eye or a UV-Visible spectrophotometer. The proposed method is a promising mean for on-site screening of melamine adulterant in raw milk without costly instruments.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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