



Visual detection of melamine in raw milk by label-free silver nanoparticles

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ABSTRACT

A simple, rapid colorimetric method using label-free Ag NPs (silver nanoparticles) as probe for detection of melamine in raw milk was developed in this study. The assay relies on the fact that melamine can induce aggregation of Ag NPs, and thereby results in their yellow-to-red color change. The concentration of melamine in raw milk can be determined by monitoring with the naked eyes or a UV–Vis spectrophotometer. The detection limit of the present method for melamine is 2.32 μM (3σ). The proposed method is a promising mean for on-site screening of melamine adulterant in raw milk without costly instruments.

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1. Introduction

Melamine (2, 4, 6-triamino-1, 3, 5-triazine) is a kind of triazine analog with three amino groups, which is widely used in plastic engineering and agriculture as an important industrial material in the early 1950s. Melamine is forbidden to be used as an additive in food or related ingredients. Because of its high nitrogen content (66%) and low cost, melamine was illegally adulterated in food products in order to increase apparent 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 (Ehling, Tefera, & Ho, 2007). However, melamine could not be metabolized in human body, and could form insoluble complexes with cyanuric acid, depending on urine pH, which could lead to crystallization and subsequent tissue injury, in this way, the excessive intake of melamine will result in the formation of insoluble melamine cyanurate crystal in kidney and finally cause renal failure (Puschner, Poppenga, Lowenstine, Filigenzi, & Pesavento, 2007). In September 2008, melamine was illegally adulterated in infant formulas that led to kidney stone for thousands of infants in China which has been reported (Zhang et al., 2009 & Zhu, Gamez, Chen, Chingin, & Zenobi, 2009).

A safety limit of melamine ingestion has been officially set at 2.5 ppm for adult food and at 1 ppm for infant formula by the US Food and Drug Administration (Zhao et al., 2009; <http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/2008/ucm116960.htm>). The maximum residue level of melamine in infant formula is legally regulated at 1 ppm by Chinese government after the melamine accident (Guo et al., 2010). Currently, several methods have been employed for the determination of melamine in milk and milk-based products, such as gas chromatography (GC) (Yokley, Mayer, Rezaaiyan, Manuli, & Cheung, 2000), high performance liquid chromatography (HPLC) (Ihunegbo, Tesfalidet, & Jiang, 2010; Sun, Wang, Ai, Liang, & Wu, 2010, Venkatasami & Sowa, 2010), high performance liquid chromatography/mass spectrum (HPLC/MS) (Heller & Nochetto, 2008 & Kim et al., 2008), surface enhanced Raman spectroscopy (Lin, He, Awika, Yang, Ledoux, & Mustapha, 2008), capillary zone electrophoresis mass spectrum (CE/MS) (Cook, Klampfl, & Buchberger, 2005), and immunoassay analysis (ELISA) (Garber, 2008). These methods have high sensitivity, but most of them are time-consuming and labor-intensive due to the complicated pretreatment of sample, and require expensive instrumentation and high personnel cost. The above-mentioned disadvantages limit the application of the existing methods. Therefore, developing a rapid, simple, convenient and sensitive method for the determination of melamine has become increasingly attractive and necessary.

In recent years, gold nanoparticles (Au NPs) and silver nanoparticles (Ag NPs) have been widely used as colorimetric probes for chemical sensing and biosensing of various substances (Zhao, Brook, & Li, 2008), such as viruses (Niikura et al., 2009), protein (Wang et al., 2008), DNA (Cho, Han, & Ban, 2008), cancerous cells (Medley et al.,

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2008), metal ions (Fan, Liu, Wang, & Zhang, 2009; Lee, Wang, Liu, & Lu, 2008; Liu & Lu, 2004; Xu, Wang, Jiao, & Yang, 2009) and small molecules (Chen, Parker, Zou, Su, & Zhang, 2010; Li & Li, 2009; Zhang et al., 2008), relying on their unique size-dependent and/or interparticle-distance dependent absorption spectra and solution color. When the nanoparticles approach each other and aggregate, the color of the nanoparticles changes from red to purple (or blue) for Au NPs (Liang et al., 2011; Ai, Liu & Lu, 2009) and from yellow to red (or dark green) for Ag NPs (Han, & Li, 2010), respectively, due to the shift of the surface plasmon band to longer wavelength. Several colorimetric assays based on Au NPs have also been developed for the melamine detection. For example, triple hydrogen-bonding recognition between melamine and a cyanuric acid derivative grafted on the surface of Au NPs has been used for reliable detection of melamine (Ai et al., 2009). Colorimetric sensing methods of melamine have been developed based on the aggregation of crown ether-modified Au NPs through the formation of cavity complexes with amines (Kuang et al., 2011). Melamine in milk products could be detected by visual and light scattering spectrometric methods with polythymine-stabilized Au NPs on the basis of the formation of triple H-bonds between thymine and melamine in aqueous solution (Qi, Wu, Ling, & Huang, 2010). Colorimetric and nonaggregation-based gold nanoparticles probe has been developed for the detection of melamine in which the synthesis of Au NPs was hindered (Cao et al., 2010) or accelerated (Wu et al., 2011) by the presence of melamine. In other reports, simple colorimetric methods base on electrostatic interactions between melamine and citrate-capped Au NPs (Chi, Liu, Guan, Zhang, & Han, 2010; Li, Li, Cheng, & Mao, 2010; Qin, Zhao, Huang, & Wu, 2009; Wei et al., 2010 & Guo et al., 2010) and cysteamine-modified Au NPs (Liang et al., 2011) have been exploited for the detection of melamine.

Compared with Au NPs, Ag NPs have some advantages, for example, lower cost of preparation, higher extinction coefficients relative to Au NPs of the same size (Lee, Lytton-Jean, Hurst, & Mirkin, 2007). Therefore, Ag NPs are also good candidates for colorimetric probes of melamine sensing. Recently, Li group (Han, & Li, 2010) developed p-nitroaniline-modified Ag NPs for visual detection of melamine in infant formula. This method exhibited relatively high sensitivity for melamine, however, the complex modification of nanoparticles limits its potential application. Herein, a simple, rapid, economical and field-portable method has been established based on the color change of label-free Ag NPs for the screening of melamine. An obvious color change from yellow to red could be observed when melamine was added into label-free Ag NPs solution. Raw milk was pretreated with trichloroacetic acid and chloroform to remove the protein and fat, needless of solid phase extraction. The proposed method can be used for the detection of melamine in raw milk by monitoring with the naked eyes or UV–Vis spectroscopy at room temperature. The whole detection process could be completed within 30 min. The detection limit for melamine is $2.32 \mu\text{M}$ ($\approx 0.29 \text{ mg/L}$), which is well below the safety limit (2.5 ppm in USA and EU; 1 ppm for infant formula in China) of melamine ingestion (Zhao et al., 2009; <http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/2008/ucm116960.htm>), and which is comparable with or better than the detection limits of the other methods (Li & Li, 2009; Liang et al., 2011). Besides, the proposed colorimetric method is more convenient than Kjeldahl and Dumas tests, thus, can be used for the rapid detection of melamine in raw milk.

2. Experimental

2.1. Reagents and materials

Melamine was purchased from Aladdin Reagent Company (Shanghai, China). Silver nitrate, sodium citrate, sodium

borohydride, sodium bicarbonate, chloroacetic acid and chloroform were purchased from Beijing Chemical Reagent Company (Beijing, China). All solvents and reagents are of analytical grade and were used without further purification. Doubly-distilled water was used throughout the experiments. A stock solution of 1.0 mM melamine was made by dissolving 126.2 mg melamine in 1 L distilled water. The raw milk was purchased from the local pasture.

2.2. Apparatus

Absorption spectra were recorded on a UV-2550 UV–Vis Spectrophotometer (Shimadzu, Tokyo, Japan) at room temperature. Transmission electron microscopy (TEM) measurements were made on a TECNAI F20 (FEI Co., Holland) operated at an accelerating voltage of 200 kV. The pH measurements were carried out on model pHS-3C pH meter (Shanghai, China). The centrifugation was performed on a CR20B2 Refrigerated Centrifuge (Tokyo, Japan). The ultrasonic treatment was carried out on a KQ-300DE ultrasonicator (Kunshan Ultrasonic Instrument Co., Shanghai, China).

2.3. Preparation of Ag NPs

Ag NPs were prepared by the borohydride reduction method according to the reference with some modifications (Zhao et al., 2009). All glassware used in the following procedure was soaked in a freshly prepared aqua regia for 24 h and rinsed thoroughly in water and oven-dried prior to use. Ag NPs were synthesized as the following procedures. Firstly, 20 mL mixed solution of silver nitrate (0.25 M) and sodium citrate (0.25 M) was placed into a 250 mL round-bottom flask with three necks, then 10 mL of sodium borohydride (10 mM) was added dropwise to the 250 mL round-bottom flask with three necks with high speed stirring for 10 min, then the yellow solution was obtained, the mixture was then cooled to room temperature and stewed for 8 h, finally the yellow solution of Ag NPs was stored at 4°C before use.

2.4. Colorimetric detection of melamine in raw milk

A typical colorimetric analysis of melamine in raw milk was achieved as follows. Firstly, 4.0 mL raw milk was placed into a 15 mL centrifuge tube, and diluted to 10 mL, then 2.0 mL of 10% mixed solution of trichloroacetic acid and chloroform was added and mixed with a vortex for 1 min to deposit protein in the sample matrix. The mixture was sonicated at 20°C for 15 min and centrifuged at 13,000 rpm for 10 min to separate the deposit. Secondly, the supernatant was transferred into another centrifuge tube and adjusted to pH 8.0 with 10% of NaHCO_3 solution. The solution was centrifuged at 10,000 rpm for 10 min to remove the deposit again and the final solution was used for detection. Thirdly, 600 μL of the obtained solution was added to 800 μL Ag NPs solution, and then the mixed solution was allowed to react for 20 min at the room temperature. Finally, the absorption spectrum of the reacted solution was recorded with 1 cm path length cell. The concentration of melamine was quantified based on the absorption ratio (A_{500}/A_{402}) or naked eyes observation. The color change of the mixture solution was also recorded by digital camera.

3. Results and discussion

3.1. Detection principle of melamine using unmodified Ag NPs as colorimetric probe

Ag NPs can be stabilized in aqueous solution by coating it with negatively-charged citrate ions (Pinto, V. V., Ferreira, M. J., & Pereira, C. M. 2010), and electrostatic force counteract the effects of Van der

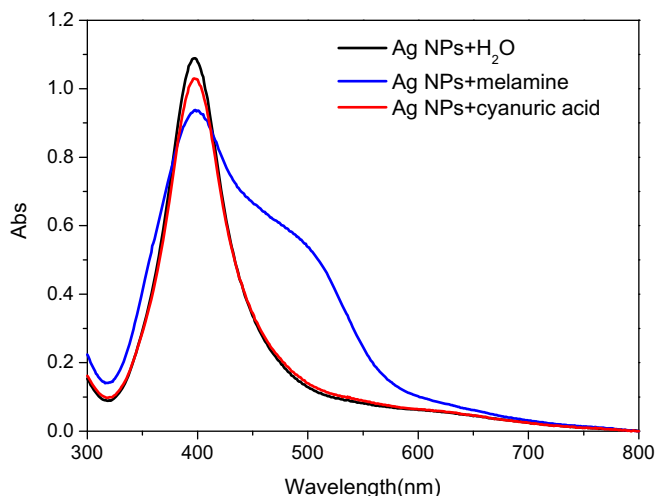


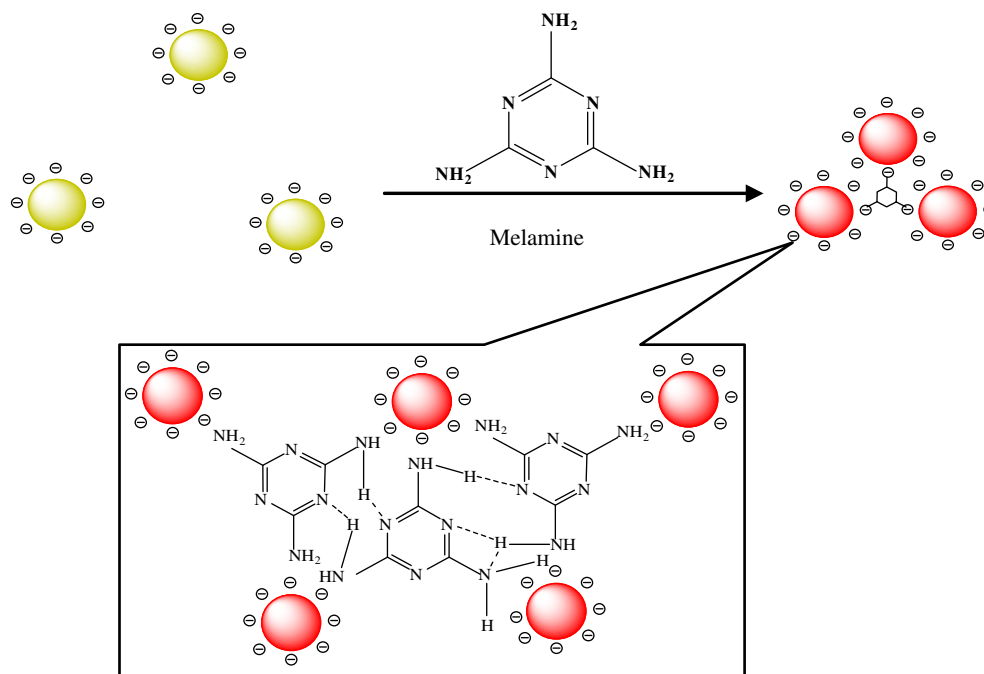
Fig. 1. Absorption spectra of Ag NPs in presence of water (black line), cyanuric acid (red line) and melamine (blue line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Waals' force between molecules, with the result of homodisperse of Ag NPs. Melamine molecule is a small molecule which contains three exocyclic amino groups ($-\text{NH}_2$) and a three-nitrogen hybrid ring. The negatively-charged citrate ions can attach each other with positively charged exocyclic amino groups ($-\text{NH}_2$), with the result that melamine molecules are attached to the surface of Ag NPs. In our experiments, when melamine was added into Ag NPs solution, the Ag NPs aggregated together, simultaneously, the color of Ag NPs solution changed with spectral variations as shown in Fig. 1. The aggregation of Ag NPs might be induced either by the three exocyclic amino groups ($-\text{NH}_2$) or by the three-nitrogen hybrid ring. In order to explore the real reason of Ag NPs aggregation, we carried out the control experiment using cyanuric acid as a substitute of melamine. Melamine is easily hydrolyzed to produce cyanuric acid. In the molecular structure of cyanuric acid, three hydroxy groups

($-\text{OH}$) replace the three amino groups ($-\text{NH}_2$) of melamine, and the three-nitrogen hybrid ring still remains. The experimental results showed that cyanuric acid could not induce the aggregation of Ag NPs, as shown in Fig. 1. It is obvious that only three amino groups of melamine are key groups for the interaction between melamine and Ag NPs. The three amino groups caused a rapid yellow-to-red color change, whereas, the three-nitrogen hybrid ring is irresponsible for the interaction between melamine and Ag NPs. On the other hand, every melamine has six equivalent sites which are to form double $\text{NH}-\text{N}$ hydrogen bonds with a similar site of another melamine molecule (Li et al., 2010; Silly et al., 2008). We reasoned that the neighbor melamine-coated Ag NPs could be cross-linked by $\text{NH}\dots\text{N}$ hydrogen bonds between melamine molecules as shown schematically in Scheme 1. Thereby, the aggregation of Ag NPs could be induced. Scheme 1 depicts the mechanism for colorimetric detection of melamine by label-free Ag NPs.

3.2. UV-visible absorption spectra and TEM images of Ag NPs

The absorption peaks of gold and silver nanoparticles are closely related with space between nanoparticles, when the state of nanoparticles changes from dispersive to aggregated, the absorption peaks of nanoparticles shift obviously with a corresponding change of absorption strength (White, & Rosi, 2008). The absorption spectrum of stable Ag NPs shows a typical surface plasmon resonance absorption peak at 402 nm (Solid line in Fig. 2 A), demonstrating Ag NPs are well dispersed. After addition of melamine into Ag NPs solution, the Ag NPs quickly aggregate together, and the solution color changes from yellow to red as shown with the insert photographs in Fig. 2 A. This aggregation was directly verified by the TEM images of Ag NPs and Ag NPs in the presence of melamine (Fig. 2 B and C). In the presence of melamine, the absorption at 402 nm of the original Ag NPs decreases and shows a slight red shift, meanwhile, a new broad absorption band appears around 500 nm (dashed line in Fig. 2 A). Thus, a simple, rapid and field-portable colorimetric method can be developed for the analysis of melamine by naked eyes or UV-Vis spectroscopy.



Scheme 1. Schematic representation of the Ag NPs colorimetric mechanism for melamine detection.

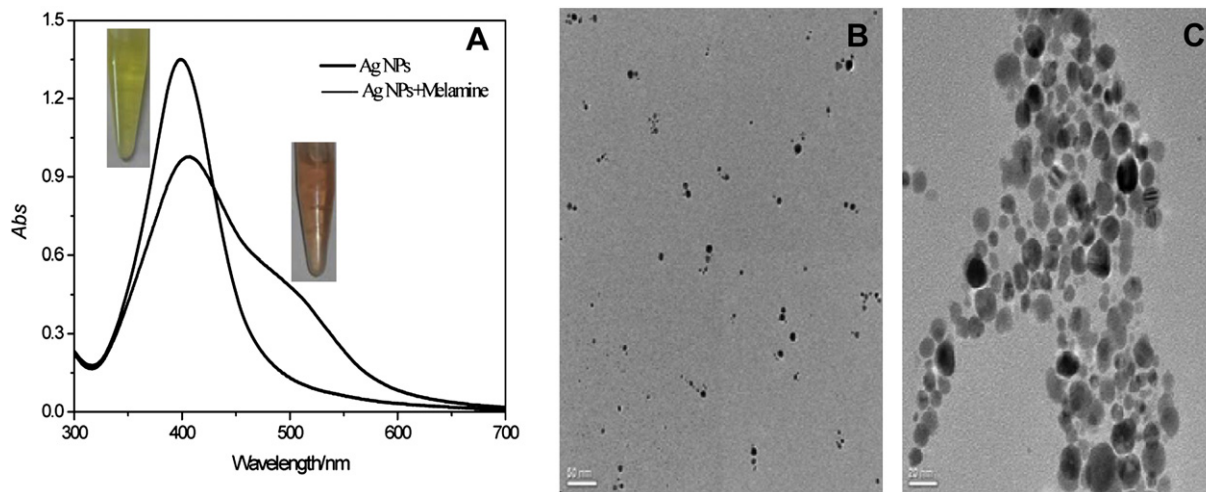


Fig. 2. (A) Absorption spectra of the well-dispersed Ag NPs (solid line) and the aggregated Ag NPs in presence of melamine (dashed line). The inserts are the corresponding photographs: 600 μ L Ag NPs + 600 μ L H₂O (left), 600 μ L Ag NPs + 600 μ L melamine (0.5 mM) (right). (B) The TEM image of Ag NPs. (C) The TEM image of Ag NPs in presence of melamine.

3.3. Optimization of assay condition

The interaction of Ag NPs and small molecules (melamine) can be affected by media pH. Melamine is a weak base with pKa of 5.05. Media pH can also affect the form of melamine in aqueous solution. We investigated the effect of media pH in the range from 4.0 to 12.0. Media pH of Ag NPs was adjusted with hydrochloric acid or sodium hydroxide. To take 0.1 mM melamine for the example, the profile of absorption ratio (A_{500}/A_{402}) versus media pH was obtained, as shown in Fig. 3. It can be seen that in acidic media (pH < 5.0) and basic media (pH > 10.0), the absorption ratio (A_{500}/A_{402}) is lower; at pH 7.0~9.0, the highest absorption ratio (A_{500}/A_{402}) has been obtained. It is probably due to the fact that melamine was hydrolyzed at pH < 5.0 and pH > 10.0, amino groups were gradually replaced by hydroxyl groups, and melamine was finally transformed into cyanuric acid that could not induce the aggregation of Ag NPs (Bozzi et al., 2004). The other reason is that Ag NPs partially aggregate, and could not interact with melamine in the media with relatively low pH (pH < 4.0) or relatively high pH (pH > 12.0) which can be confirmed by control experiments. Thus, pH of media was chosen as 8.0.

The reaction time between Ag NPs and melamine is a key point that affects colorimetric assays, and the relationship between reaction time and absorption ratio (A_{500}/A_{402}) was investigated by

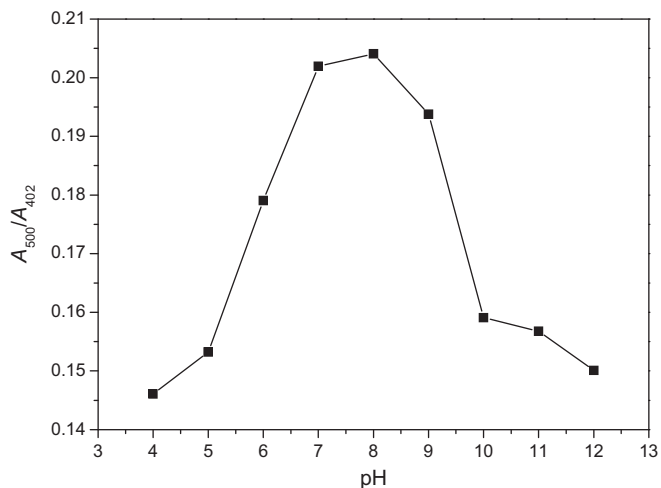


Fig. 3. Effects of media pH on the absorption ratio (A_{500}/A_{402}).

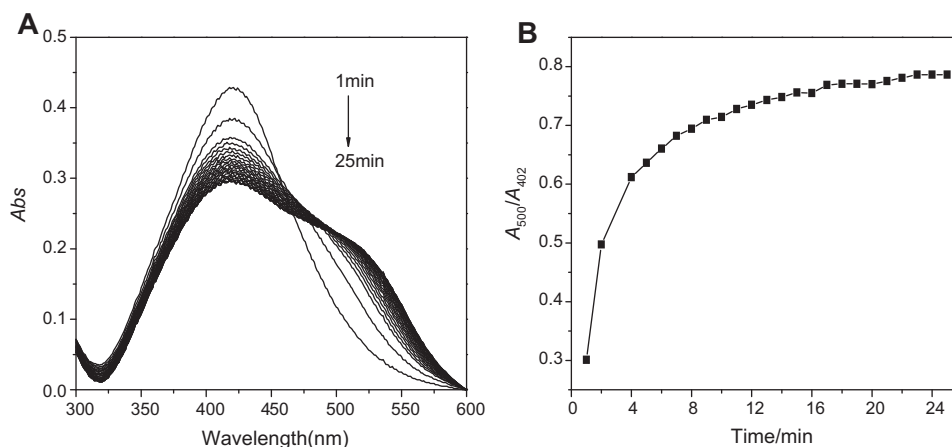


Fig. 4. Time-dependent absorption spectra of Ag NPs-melamine systems (left). Effects of reaction time on the absorption ratio (A_{500}/A_{402}) (right).

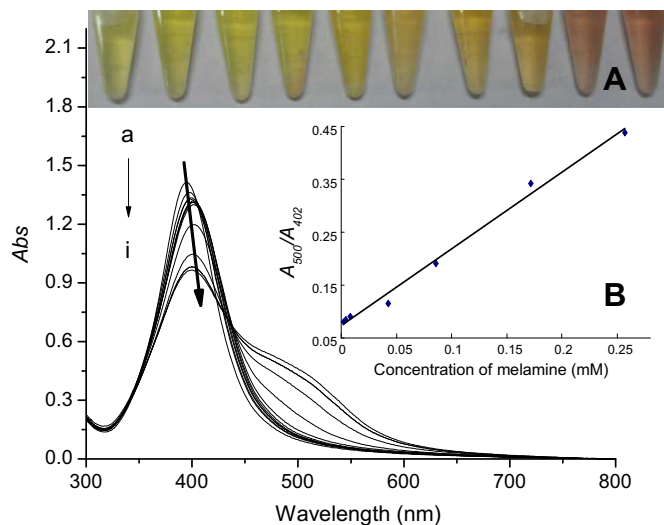


Fig. 5. Absorption spectra of Ag NPs in the presence of different concentrations of melamine. Insert in (A) are photographs of 800 μL Ag NPs solution mixed with 600 μL melamine solution. The concentrations of melamine (from left to right) are 0.002, 0.004, 0.008, 0.042, 0.085, 0.17, 0.25 mM, respectively. Insert in (B) is the calibration curve between A_{500}/A_{402} and concentration of melamine.

adding 600 μL of 1.0 mM melamine solution into 800 μL Ag NPs solution, as shown in Fig. 4. It can be seen that the absorption ratio (A_{500}/A_{402}) increased gradually from 1 min to 18 min, and kept steady from 18 min to 25 min, demonstrating that the aggregation of Ag NPs almost completed within 18 min. Thus, the detection time was chosen as 20 min.

3.4. Analytical performance of Ag NPs-based sensing for melamine in water

In order to quantitatively detect melamine using Ag NPs as colorimetric probes, the aggregation of Ag NPs induced by melamine in water was monitored by UV–Vis spectroscopy. With the addition of melamine from 0.002 to 0.48 mM, the absorbance of Ag

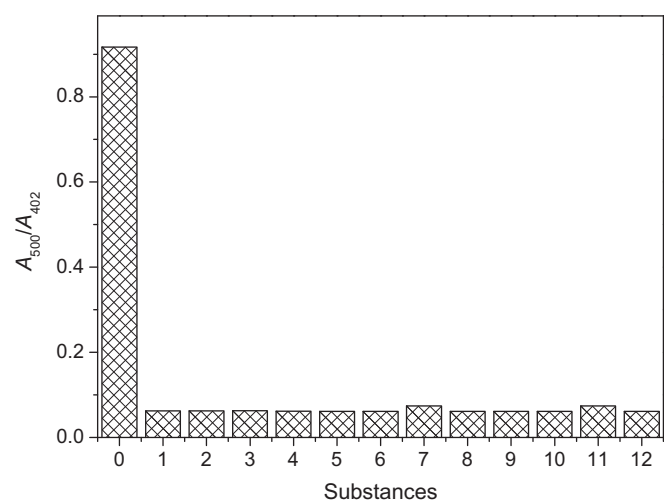


Fig. 6. The absorbance ratio (A_{500}/A_{402}) of Ag NPs solution in the presence of different substances. Substances: 0, melamine; 1, vitamin C; 2, lactose; 3, glucose; 4, polypeptide; 5, NH_4^+ ; 6, Na^+ ; 7, K^+ ; 8, Ca^{2+} ; 9, SO_4^{2-} ; 10, Cl^- ; 11, NO_3^- ; 12, Cl^- . The concentration of melamine is 1.0 mM, and the other substances are all 100.0 mM.

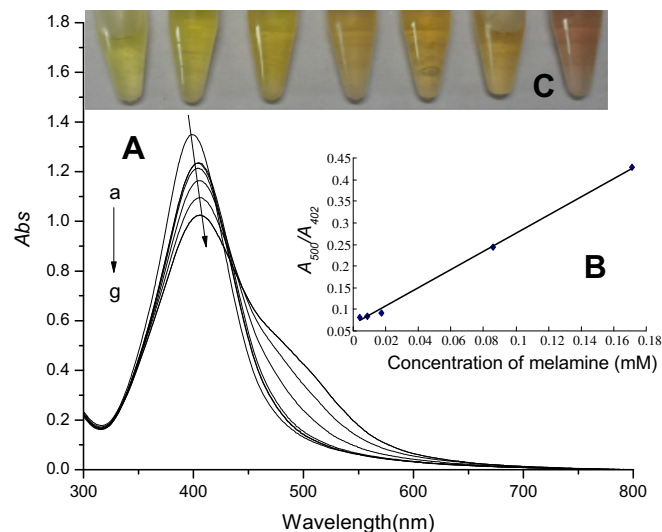


Fig. 7. Concentration-dependent absorption spectra of the label-free Ag NPs (A) and the corresponding plot of A_{500}/A_{402} versus melamine concentration spiked in raw milk (B). Insert in (C) are photographs of 800 μL Ag NPs solution mixed with 600 μL melamine solution. The concentrations of spiked melamine (from left to right) are 0.004, 0.008, 0.017, 0.085, 0.17 mM, respectively.

NPs solution at 402 nm decreased gradually and the absorbance around 500 nm increased obviously. At the same time, the color of the mixture solution changed from yellow to light red progressively. The color change and the absorbance ratio of the mixture solution correlate with the concentration of melamine. Shown as the inset A in Fig. 5, the visible color change could be easily differentiated by naked eyes when the concentration of melamine is over 0.17 mM. The absorbance ratios (A_{500}/A_{402}) were calculated based on the data shown in Fig. 5, and a good linear relationship (the linear equation is $y = 1.4507x + 0.0731$, correlation coefficient $r = 0.9960$) was obtained between A_{500}/A_{402} and concentration of melamine in the range of 2.0–250.0 μM (Fig. 5 B). The detection limit is calculated to be 1.83 μM (3σ).

3.5. Interference of other substances

In order to explore the specific detection of melamine in raw milk using Ag NPs, we investigated the interference of common ions and excipients in raw milk for determination of 1.0 mM melamine. With the tolerable concentration ratios for interference at the $\pm 7\%$ level, the results for Ag NPs solution after adding 100-folds of different substances (100.0 mM) are shown in Fig. 6.

3.6. Analysis of spiked milk samples

In order to quantitatively detect melamine in raw milk using Ag NPs colorimetric probes, the raw milk was pretreated by using the method in Section 2.4. Different concentrations of melamine were

Table 1
Recovery results of using the proposed method to determine melamine spiked in raw milk.

Sample	Added value (mM)	Measured value (mM)	(Recovery \pm RSD) % ($n = 3$)
Raw	0.040	0.046	114.29 \pm 2.83
milk	0.080	0.083	104.70 \pm 3.10
	0.120	0.108	88.83 \pm 2.04

added into the prepared raw milk, and the UV–Vis absorption spectra of Ag NPs in the absence and presence of raw milk preparation with added different concentrations of melamine were recorded. Fig. 7 shows that the absorption ratios (A_{500}/A_{402}) increase with the increment of melamine concentration, and the absorption ratio (A_{500}/A_{402}) of this method exhibited a linear correlation to melamine concentration in the range of 4.0–170.0 μM (the linear equation is $y = 2.1096x + 0.065$, correlation coefficient $r = 0.9989$) (Fig. 7 B). The detection limit is calculated to be 2.32 μM (3σ). The method has been applied to determine melamine spiked in raw milk, and the recovery for the sample was in the range of 88.83%–114.29% with the RSD from 2.04% to 3.10% ($n = 3$), as shown in Table 1.

4. Conclusions

In conclusion, a sensitive, selective, and simple colorimetric assay using Ag NPs to detect melamine in raw milk was reported. The Ag NPs-based assays can transform molecular recognition event between Ag NPs and melamine into the visual color change. Three amine groups of melamine molecule are demonstrated to be the key factor to induce Ag NPs aggregation. The proposed method can be used for the detection of melamine in raw milk, with a detection limit of 2.32 μM . The rapid detection of melamine can be observed by the naked eyes, needless of any advanced instrument and any complex pretreatment. Furthermore, the proposed method is promising for on-site screening melamine adulterant in milk products.

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