Food Chemistry 174 (2015) 473–479

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Colorimetric detection of melamine based on methanobactin-mediated synthesis of gold nanoparticles

Jia-ying Xin [a,b,*], Lan-xuan Zhang [a], Dan-dan Chen [a], Kai Lin [a], Hong-chen Fan [a], Yan Wang [a], Chun-gu Xia [b]

[a] Key Laboratory for Food Science & Engineering, Harbin University of Commerce, Harbin 150076, People's Republic of China
[b] State Key Laboratory for Oxo Synthesis & Selective Oxidation, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou 730000, People's Republic of China

ARTICLE INFO

Article history:
Received 17 April 2014
Received in revised form 21 October 2014
Accepted 16 November 2014
Available online 23 November 2014

Keywords:
Methanobactin
Gold nanoparticles
Melamine
Colorimetric detection
Liquid milk

ABSTRACT

A simple and rapid field-portable colorimetric method for the detection of melamine in liquid milk was reported. Methanobactin (Mb) could reduce Au (III) to Au (0) and mediate the synthesis of gold nanoparticles (Au-NPs). Upon the addition of melamine, melamine interacted with oxazolone ring of Mb, which interrupted the formation of Au-NPs. Melamine could also stimulate the aggregation of formed Au-NPs. In this paper, these characteristics have been used to detect melamine in liquid milk by naked eyes observation with a detection limit of 5.56×10^{-6} M (0.7 mg/kg). Further, the plasmon absorbance of the formed Au-NPs allowed the quantitative detection of melamine by UV–vis spectrometer. A linear correlation was existed between the absorbance and the melamine concentration ranging from 3.90×10^{-7} M to 3.97×10^{-6} M with a correlation coefficient of 0.9685. The detection limit (3σ) obtained by UV–vis spectrum was as low as 2.38×10^{-7} M (i.e., 0.03 mg/kg).

1. Introduction

Melamine (1,3,5-triazine-2,4,6-triamine, $C_3H_6N_6$) is a triazine heterocyclic organic compound, which is widely used in the production of melamine resins, flame retardants, fertilizer and other products. Because of the high nitrogen content (66% nitrogen by mass), melamine was illegally and unethically adulterated in protein-rich ingredients to increase the apparent protein content measured by Kjeldahl protein analysis (Mauer, Chernyshova, Hiatt, Deering, & Davis, 2009). Melamine can be hydrolyzed to cyanuric acid which in turn associates with melamine to form insoluble complexes, resulting in the formation of insoluble crystals in the kidneys and subsequent tissue injury (Reimschuessel & Puschner, 2010). It is not allowed to use as a food additive in human food or animal feeds. A safety limit of melamine (2.5 mg/kg) in milk and milk-based products was set by Food and Drug Administration (FDA) of USA and European Union (Zhu, Gamez, Chen, Chingin, & Zenobi, 2009). Therefore, there is an increasing need for rapid and reliable methods to detect melamine in milk and other food products. Currently there are a number of analytical techniques for detecting melamine in milk and milk-based products. Amongst them, the most common methods are high performance liquid chromatography (HPLC) (Ehling, Tefera, & HO, 2007), mass spectrum (MS) (Yang et al., 2009), gas chromatography/mass spectrum (GC/MS) (Yokley, Mayer, Rezaaiyan, Manuli, & Cheung, 2000), high performance liquid chromatography/mass spectrum (HPLC/MS) (Kim et al., 2008), capillary zone electrophoresis/mass spectrum (CE/MS) (Vo et al., 2008), surface enhanced Raman spectroscopy (SERS) (Lin et al., 2008) and fluorescence polarisation immunoassay (Wang et al., 2011) etc. Although these methods have high sensitivity, many of them require expensive apparatus (such as MS) and time-consuming sample pretreatment (such as derivatisation or extraction) and are not readily adaptable to on-site detection.

UV-spectrophotometry is also a convenient and widely used method for quantitative analysis. However, the spectrophotometric method requires the analyte presented in the given sample to have different absorption spectrum from other unknown coexistent substances with low spectral overlapping. If the method is used to determine melamine in milk directly, the problem seems more intractable. Numerous nutrition components in milk could interfere the quantitative determination of melamine (Liu, Deng Jian, Liang, Chen, & Wang, 2011).

Recently, gold nanoparticles (Au-NPs) have been widely used as colorimetric probes for chemical sensing and biosensing because of their solution colour, strongly size-dependent and distance-dependent optical property, and extremely high extinction coefficients (10^8–10^{10} M^{-1} cm^{-1}, which is about 3–5 orders of

* Corresponding author at: Key Laboratory for Food Science & Engineering, Harbin University of Commerce, No. 138 Tongda Road, Daoli District, Harbin 150076, Heilongjiang, People's Republic of China. Tel.: +86 451 84838194.
E-mail address: Xinjiayingvip@163.com (J.-y. Xin).

http://dx.doi.org/10.1016/j.foodchem.2014.11.098
0308-8146/

magnitude higher than common organic molecules) (Jin, Wu, Li, Mirkin, & Schatz, 2003; Kuang et al., 2011). There are a few reports on the colorimetric detection of melamine based on the fact that Au-NPs are induced to aggregate by interparticle crosslinking (Ai, Liu, & Lu, 2009; Chi, Liu, Guan, Zhang, & Han, 2010; Kuang et al., 2011). However, the complex modification of nanoparticles limits their potential application. More simple colorimetric methods for the detection of melamine by unmodified nanoparticles were proposed by Li, Li, Cheng, and Mao (2010). But the tedious sample preparation by solid phase extraction is disadvantageous for on-site detection. Colorimetric detection of melamine during the formation of gold nanoparticles has also been reported. However, most of them are based on the interaction between melamine and reducer, which leading to weakening of the Au (III) reducing ability (Cao et al., 2010).

Methanotrophs are a group of gram-negative eubacteria that utilise methane as the sole carbon and energy source (Hanson & Hanson, 1996). It is known that the amount of bioavailable copper regulates the methane monooxygenase (MMO), which is used by methanotrophs to oxidise methane (Hakemian & Rosenzweig, 2007). Methanobactin (Mb) is a copper-binding small peptide that appears to function as an agent for copper sequestration and uptake in methanotrophs (Balasubramanian & Rosenzweig, 2008). The crystal structure of copper-loaded Mb (Cu–Mb) from *Methylosinus trichosporium* OB3b revealed a 1217 Da molecule with a chemical composition of $C_{45}N_{12}O_{14}H_{62}S_5Cu$ (Balasubramanian & Rosenzweig, 2008). Mb can coordinate a single Cu (II) ion by its nitrogens from two oxazolone rings and sulfurs from two enethiol groups and then reduce Cu (II) to Cu (I) (Choi et al., 2006). Mb can also bind and reduce Au (III) to Au (0), which result in the formation of Au-NPs (Xin et al., 2013).

In this paper, we have developed a colorimetric method for the detection of melamine in liquid milk based on the phenomena that melamine can interfere the formation of Au-NPs by Mb. The detection of melamine can be achieved during the formation of Au-NPs, i.e., the synthesis of Au-NPs and the analysis of melamine can occur in one-step just by using Mb as mediator for the generation Au-NPs. This one-step synthesis offers fast, sensitive and convenient colorimetric detection of melamine with the naked eyes or UV–vis spectroscopy within 50 min. The proposed scheme reduces the volume of waste generated during Au-NPs synthesis and purification, and can be an alternative means for onsite detection of melamine without costly instruments.

2. Materials and methods

2.1. Chemicals and materials

Melamine was obtained from Sigma -Aldrich (St. Louis, MO, USA). Chloroauric acid ($HAuCl_4{\cdot}4H_2O$) was obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Other chemicals were of analytical reagent grade and used without further purification. All stock solution were prepared daily with distilled water. The market milk products were bought from local supermarket. The general composition was showed below:

Lipids: 3.3 g/100 g; milk solids-not-fat (MSNF): 8.1 g/100 g; proteins: 2.9 g/100 g (market milk from Inner Mongolia Meng Niu Dairy Co., Ltd. China).

Lipids: 3.4 g/100 g; milk solids-not-fat (MSNF): 8.1 g/100 g; proteins: 2.9 g/100 g (Heilongjiang Province Wondersun Dairy Co., Ltd. China).

Lipids: 3.5 g/100 g; milk solids-not-fat (MSNF): 8.1 g/100 g; proteins: 3.0 g/100 g (Heilongjiang Province Wondersun Dairy Co., Ltd. China) (Inner Mongolia Yili Industrial Group Co. Ltd. China).

2.2. Organism and culture conditions

Methylosinus trichosporium 3011 was obtained from the Institute of Microbiology and Virology (Kiev. Ukraine) and was cultivated with a mineral salt medium according to Xin et al. (2010). Methanol was added to 0.2% (v/v) and supplied on-line to keep the same concentration. Cells were grown at 28–30 °C and an agitation rate of 250–300 rpm. Ambient air was bubbled through the fermentor continuously at 0.5–0.8 L/min. The cultures were grown to stationary phase for Mb production.

2.3. Isolation and quantification of Mb

Mb from the spent medium of *Methylosinus trichosporium* 3011 was isolated as previously described for *Methylococcus capsulatus* Bath by Choi et al. (2005). The cells were removed by centrifugation at 10,000g for 30 min. The supernate was loaded onto a 2.5 × 20 cm Diaion HP-20 column (Mitsubishi Chemical Holdings, Japan). The bound Mb was washed with two column volumes of H_2O and eluted with 40% methanol: 60% H_2O. The eluant was lyophilized for concentration and storage. The freeze-dried samples following chromatography on Diaion HP-20 columns were the source of Mb used in this study. The amount of Mb in the sample was quantified according to Xin et al. (2013).

2.4. Gold coordination

Gold coordination was performed using 0.1 mM aqueous solutions of Mb. Before gold coordination, 50 μL of 10 mM melamine or the same volume of dH_2O were added to 5 mL of Mb solutions and shaken for 1 min. Stock solution of $AuHCl_4$ (10 mM) was added gradually in 0.1 mM aqueous solutions of Mb, mixed and incubated for 10 min before spectral determinations. UV–visible absorption spectroscopy was performed by using a UV-2550 spectrophotometer (Shimadzu, Japan). Fluorescence excitation spectra were recorded on Hitachi F-7000 fluorescence spectrometer. The valence states of elements were analysed by using X-ray photoelectron spectroscopy (ESCALAB210 , VG Scientific). The samples for XPS were prepared by adding 0.2 mL aqueous solution of the Au (III) & Mb mixture onto glass plate and allowing water to completely evaporate. transmission electron microscope (TEM) technique was employed to visualise the size and shape of gold nanoparticles. The samples for TEM were prepared by adding 10 μL of a 1:5 (stock : H_2O) dilute solution of the sample onto carbon-coated copper grids and allowing water to completely evaporate. TEM images were recorded in a Hitachi H-7650 transmission electron microscope.

2.5. Synthesis of AuNPs and colorimetric detection of melamine

Au-NPs were synthesised according to previous method (Xin et al., 2013). Mb was added into the solution of $HAuCl_4$ (0.5 mM) and the mixture was further reacted at room temperature until the wine-red solution of Au-NPs was obtained. The detection of melamine was conducted under the optimal conditions for the synthesis of Au-NPs. First, In 5 mL centrifuge tube, 0.2 mL of Mb (1 mM) and 0.1 mL of different concentrations of melamine solutions were mixed in a 5 mL centrifugal tube, NaOH (0.1 M) was added to adjust pH in the range of 5.0–5.4 and kept for 1 min. Then, 0.1 mL of $HAuCl_4$ (10 mM) and 0.4 mL HQ (1 mM) were added to the tube. The mixture solution was diluted with dH_2O to 2–5 mL and was further incubated at room temperature for 50 min. The absorbance (at 539 nm) of the mixture solution in the presence and in the absence of melamine were recorded, respectively. The spiked-recovery detection of melamine in raw milk was manipulated in the same step.

2.6. Liquid milk sample preparation

For the detection of liquid milk samples, 8 mL market milk bought from local supermarket was placed into a 50 mL centrifuge tube, and 8 mL of 10% trichloroacetic acid was added. After 10 min shaking, the mixture was centrifuged at 10,000 rpm for 10 min to separate the deposit. The supernatant was transferred into another centrifuge tube and adjusted to pH 5.2 with a small amount of 0.1 M NaOH, and then the supernatant was filtered through a 0.22 μm nylon filter to remove lipids. The obtained filtrate was collected for detection.

3. Results and discussion

3.1. Mechanism for the detection of melamine based on Mb-mediated synthesis of Au-NPs

It has been reported that Mb can reduce Au (III) to Au (0), which result in the formation of gold nanoparticles (Au-NPs) (Xin et al., 2013). We found that the formation of Au-NPs was interrupted by melamine. In order to know the mechanism of how Au-NPs synthesis was interrupted by melamine, the spectral properties of Mb binding by Au (III) were examined in the presence and absence of melamine respectively. Mb from *Methylosinus trichosporium* 3011 was light yellow in colour, with weak absorption maxima at 395 nm and strong absorption maxima in the range of 260–280 nm (Fig. 1). Gold coordination experiments were determined by gradual addition of 10 mM solutions of $HAuCl_4$ to 0.1 mM aqueous solutions of Mb. As shown in Fig. 1, at molar ratios of Au (III) to Mb between 0.1 and 1.0, the increases in the absorption maxima at 264 nm and the decreases in the absorption maxima 395 nm in Mb were observed with Au (III) addition. According to previous publication (Choi et al., 2006), the increases in the absorption maxima at 264 nm might represent a charge transfer of phenolic and phenoxide ion forms of tyrosine, and the decreases in the absorption maxima 395 nm suggested the coordination of Au (III) to oxazolone ring. Further, at molar ratios of Au (III) to Mb above 1.0, the surface plasmon resonance (SPR) of Au-NPs was clearly visible as a peak in the range between 530 and 550 nm. Also, an adverse response to Au (III) addition was observed at 395 nm, where an faint increase in absorbance occurred with Au (III) concentration increase. To determine if melamine disturb the binding of Au (III) by Mb, the spectral changes associated with the binding of Au (III) were determined following exposure to melamine. The spectral changes associated with the binding of Au (III) in the absence of melamine was similar to that observed in the presence of melamine suggesting melamine did not disturb the binding of Au(III) by Mb. However, melamine interrupted the formation of Au-NPs. The surface plasmon resonance (SPR) of Au-NPs was not found in the presence of melamine.

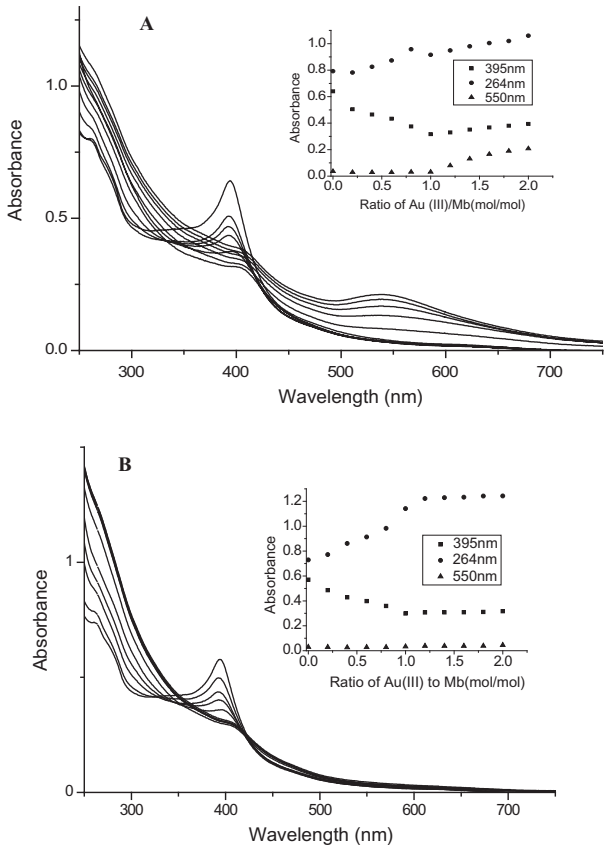

Fig. 1. UV–visible absorption spectra of Mb following addition of Au (III) without (A) or with melamine (B).

The fluorescence spectral properties of Mb binding by Au (III) were also examined. The fluorescence spectra of Mb from *Methylosinus trichosporium* 3011 showed three characteristic emissions at 310, 450 and 610 nm when excited at wavelengths of 254 nm. Based on the UV–visible absorption spectra, the emissions at 450 nm and 610 nm were assumed to be from the oxazolone ring. The emission at 310 nm was the characteristic emission peak of tyrosine. Consistent with the UV–visible absorption titrations, the fluorescence exhibited at 450 nm and 610 nm by oxazolone ring of Mb was rapidly quenched upon addition of Au (III). This suggested that oxazolone ring was involved in Au (III) coordination. The addition of Au (III) also quenched emission from tyrosine which might represent a charge transfer of phenolic and phenoxide ion forms of tyrosine. Further, the interaction between melamine and the Mb was validated by the fluorescence spectra of Mb. The addition of melamine to Mb increased the emission intensity from the oxazolone ring of Mb, This could be attributed to energy transfer between the oxazolone and melamine. However, this interaction between melamine and the Mb did not disturb the coordination of Au (III) by Mb. A similar response to Au (III) concentration was also observed in the presence of melamine. The degree of quenching of the emissions at 310, 450 and 610 nm associated with the addition of Au (III) in the presence of melamine was similar to that observed in the absence of melamine (data not shown).

In order to get a better knowledge on the mechanism, the reaction mixtures of Au (III) and Mb were characterised by X-ray photoelectron spectroscopy (XPS). According to the reports (Huang, Dai, & Fan, 2009), the binding energies of metallic Au 4f7/2 and Au 4f5/2 for Au (0) were 84.0 eV and 87.7 eV, the binding energies of Au 4f7/2 and Au 4f5/2 for the oxidised Au (I) were 85.0 eV and 88.7 eV, respectively. The binding energies of Au 4f7/2 for the oxidised Au (III) were 86.5 eV. In this research, XPS spectroscopy demonstrated the almost whole reduction of Au (III) to Au (0) at Au to Mb ratios approximate 1.0 Au per Mb. XPS of the Au (III) and Mb mixtures have only a gold signals, Au (0). Au (III) and Au (I) were not detected in reaction mixtures. However, XPS showed that the addition of melamine resulted in decrease peak area at 84.0 eV and 87.7 eV and increase peak area at 85.0 eV and 88.7 eV. These suggested that Mb binds and reduces Au (III) catalytically to Au (0) through Au(I) species. The mechanism for the melamine disturbance the formation of Au-NPs mediated by Mb was proposed. Mb appeared to initially coordinate Au (III). This initial coordination was followed by a reduction of Au (III) to Au (I), and then followed by a reduction of Au (I) to Au (0). Melamine might compete with the Au (I) for the oxazolone ring of Mb, which disturbed the reduction of Au (I) to Au (0).

3.2. Optimisation of the synthesis of Au-NPs

To assay the melamine during the formation of Au-NPs, the conditions for the synthesis of Au-NPs by Mb were optimised by UV–visible absorption spectroscopy, which was a convenient way to examine the formation of the metallic nanoparticles in aqueous suspensions by surface plasmon resonance (SPR) (Wiley, Im, McLellan, Seikkinen, & Xia, 2006). It has been found that the ratio of Mb to Au (III) is very important for the formation of Au-NPs. The intensity of the characteristic SPR band for Au-NPs centered around 539 nm was increased when increasing the molar ratio of Mb to Au (III) from 0.05 to 0.20. However, further increase the molar ratio of Mb to Au (III) from 0.25 to 0.35 showed light enhancement in the intensity of SPR feature. This was consistent with previous report (Xin et al., 2013). Taking absorption intensity and sensitivity into account, the chosen mole ratio of Mb to Au (III) for the melamine assay was 0.20.

Au-NPs has been synthesised by Mb-mediated reduction of Au (III) without adding any other reducer. However, the intensity of the characteristic SPR band for Au-NPs centered around 539 nm was weak. The results indicated that Mb have a limited Au (III) reduction capacity and may only reduce limited Au (III) to Au (0). It has been reported that continuous synthesis of Au-NPs by Mb could be achieved when hydroquinone (HQ) was provided as additional reducing power (Xin et al., 2013). To retain synthesis capacity of Au-NPs, HQ was used as an external reductant. It has been found that the absorption intensity of the plasmon band of the Au-NPs was influenced by the concentration of HQ. The absorption at 539 nm was very weak and broad at lower concentrations of HQ and becomes more intense and sharp with the HQ concentration increasing. However, the change of Au-NPs characteristic spectral absorption was small when the ratio of HQ to Au (III) was over 0.4. Therefore, this ratio of HQ to Au (III) was employed for further assay.

It was generally acknowledged that HQ was unable to react with Au (III) on its own in acidic and neural solution (Perrault & Chan, 2009). Therefore, the effect of pH on the formation of Au-NPs was carried out at a pH range from 4.4 to 6.0 (adjusted by 0.1 M NaOH). As shown in Fig. 2, the absorbance at 530–600 nm increased when the pH value increased from 4.4 to 5.0, then followed by a decrease in the range of 5.0–6.0. At the range of pH5.0 to pH 5.4, the maximum absorption of Au-NPs was the largest and the absorbance spectra showed the sharp plasmon band characteristic of the Au-NPs. Therefore, the pH range of 5.2 was selected for further experiments.

To obtain the optimum conditions, the reaction time (10–80 min) was also examined in details. The absorption spectrum of the reaction mixture was recorded at different time intervals to investigate the kinetics of Au-NPs formation. Fig. 3 showed the absorbance of Au-NPs at 539 nm that was recorded at different time intervals. Obviously, the absorbance increased rapidly in the initial 50 min and then kept nearly constant when the time exceeded 50 min. These results indicated that the synthesis of Au-NPs was almost complete within 50 min under this condition. Therefore, 50 min was selected as the reaction time.

3.3. Colorimetric detection of melamine

On the basis of the mechanism described above, we established a new method for the colorimetric determination of melamine. As shown in Fig. 4, In the Mb-mediated Au-NPs synthesis process, exposure of the Au (III) to Mb create small Au (0) metallic clusters. Once these metallic seeds are created, further growth of gold can continue at these particles surface through both the Mb-catalysed and metallic particles surface-catalysed Au (III) reduction processes with HQ as a reductant. HQ is unable to reduce isolated Au (III) ions but is able to reduce those same ions on the surface of metallic clusters (Perrault & Chan, 2009). Once the nucleation center is created, HQ can be used on its own to generate additional Au (0) atoms directly to the growing seeds. Melamine compete with the Au (I) for the binding site of Mb and the formation of Au (0) from Au (I) by Mb usually does not occur once the melamine is bound. Melamine interrupts the formation of Au (0) from Au (I) by Mb, which results in little to no formation of small Au (0) metallic clusters. This decreases the number of Au-NPs. Also, it has been reported that melamine molecule has a strong electrostatic interaction with Au-NPs, which decreases the stability of the Au-NPs (Wei et al., 2010). The aggregation of Au-NPs result in the shift of surface plasmon bands to a longer wavelength and visible colour changes from wine-red to purple or blue.

To investigate above mechanism to develop a simple colorimetric assay for monitoring melamine during the formation of Au-NPs, different amounts of melamine were added to the solutions. The formation of Au-NPs mediated by Mb was monitored by the absorption peak and the colour changes of the system. Fig. 5 showed the spectra and colour changed of the Au (III) & Mb mixture solution with concentrations of melamine. The

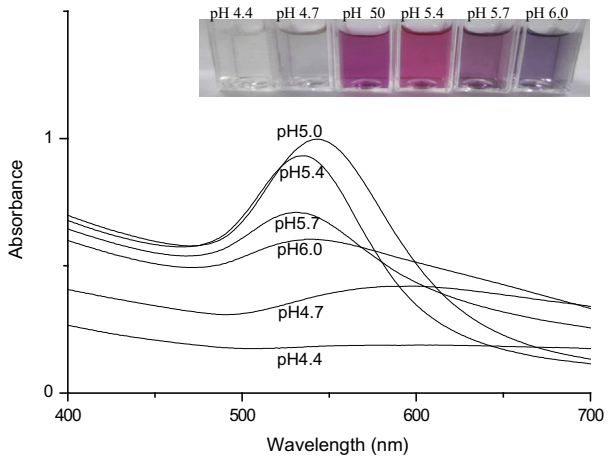

Fig. 2. Effect of pH on the absorbance and colour of Au-NPs solution the molar ratio of Mb to Au (III) was 0.2; the molar ratio of HQ to Au (III) was 0.4; the reaction time was 120 min. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

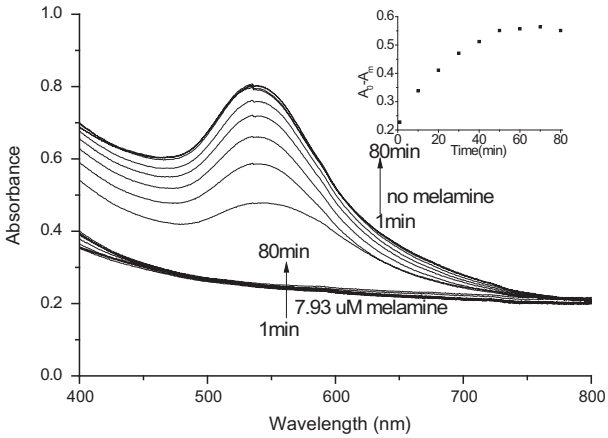

Fig. 3. Effect of reaction time on the absorbance of Au-NPs solution the molar ratio of Mb to Au (III) was 0.2; the molar ratio of HQ to Au (III) was 0.4; pH value was pH 5.0.

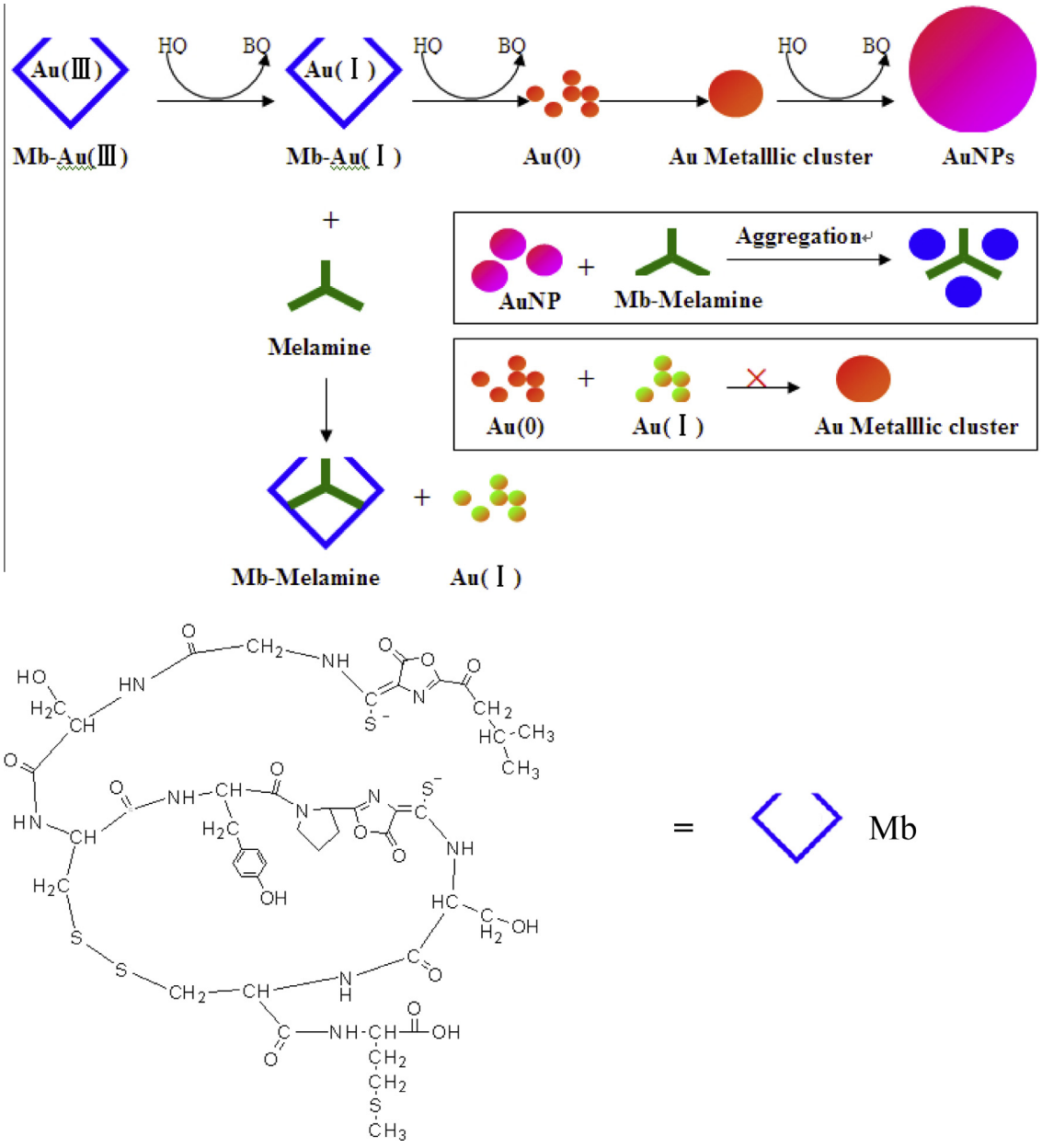

Fig. 4. Schematic representation of the possible mechanism for melamine detection based on Mb-mediated synthesis of Au-NPs.

Au-NP formation was influenced in a melamine concentration-dependent manner. It was clear that, on increasing the concentration of melamine, the absorbance of the formed Au-NPs at 539 nm decreased and red-shifted gradually and disappeared when the concentration of melamine was 5.56×10^{-6} M (0.7 mg/kg). At the same time, the colour of the mixture solution changed from wine-red to purple blue and finally to pale yellow. This implied that the number of the formed Au-NPs was lower as the concentration of melamine increased and the formed Au-NPs were aggregated at high concentrations of melamine. When the concentration of melamine more than 5.56×10^{-6} M (0.7 mg/kg), the formation of Au-NPs was completely disturbed and the colour of the mixture solution kept pale yellow.

The clear colour difference between wine-red and yellow could be easily distinguished by naked eyes when the concentration of melamine was increased to 5.56×10^{-6} M (0.7 mg/kg), indicating that the proposed method could be used to detect as low as 5.56×10^{-6} M (0.7 mg/kg) of melamine by naked eyes observation. The concentration of melamine could also be quantified by the absorption at 539 nm. A good linear correlation (correlation coefficient $R^2 = 0.9685$) existed between absorbance and the melamine concentration in the range from 3.90×10^{-7} M to 3.97×10^{-6} M.

The presence of melamine interrupted the process of synthesis of Au-NPs and stimulated the aggregation of Au-NPs formed. These facts were also evidenced by transmission electron microscopy (TEM) images. The TEM images revealed individual nanoparticles in the absence of melamine. These Au-NPs significantly aggregated in the presence of melamine. No Au-NPs were found when the concentration of melamine was increased to 5.56×10^{-6} M (0.7 mg/kg) (data no show).

In order to determine melamine in liquid milk, the interference of the substances in liquid milk was investigated. The concentrations of melamine was 5.56×10^{-6} M and the concentrations of other interferences were 5.56×10^{-4} M. The concentration of the coexisting substances exceeded the concentration of melamine over 100 times. The values of Absorbance at 539 nm were

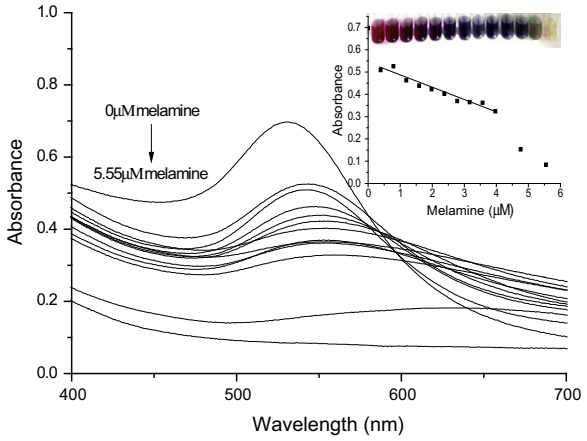

Fig. 5. Absorbance spectra of the Au-NPs generated in the presence of different concentrations of melamine. Inset: Visual colour change and the absorbance at 539 nm of Au-NPs solution at different concentrations of melamine (from left to right: 0, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, 3.2, 3.6, 4.0, 4.8, and 5.6×10^{-6} M). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

displayed in Fig. 6. It was clear that none of phenylalanine, arginine, glutamic acid, alanine, tryptophan, tyrosine, praline, glycine, glucose, sucrose, lactose, Vc, VB_{12}, Na_3PO_4, $MgCl_2$, $CaCl_2$, KI, $FeSO_4$ or glycerol except melamine induced a dramatic decrease in absorption intensity at 539 nm. This revealed good anti-interference ability of the method for detecting melamine in the presence of these coexisting interferents.

To investigate the practical application of this colorimetric method, the detection of melamine in real liquid milk samples was also carried out. Different amounts of melamine were added to the market milk samples (from Heilongjiang Province Wondersun Dairy Co., Ltd) and then the samples were pretreated according to the procedure described in Section 2.6. After the pretreatment of liquid milk sample, proteins were eliminated by precipitating with trichloroacetic acid, lipid micelles were removed by repeated filtrations and lipophilic vitamins were removed together with lipids. The other steps for the detection of the melamine in pretreated liquid milk were the same as those above mentioned.

In the case of liquid milk, the colour different between yellow and wine-red could be clearly distinguished at the samples with or without melamine (5.56×10^{-6} M, 0.7 mg/kg), indicating that as low as 5.56×10^{-6} M (0.7 mg/kg) of melamine in liquid milk could be detected with naked eye observation. Furthermore, melamine in liquid milk could also be analysed quantitatively by the absorption at 539 nm. The average recovery of 1.59×10^{-6} M (0.2 mg/kg), 2.38×10^{-6} M (0.3 mg/kg), and 3.96×10^{-6} M (0.5 mg/kg) of melamine were 97.5%, 102.0% and 103.1%, respectively. The theoretical detection limit (3σ, the concentration necessary to yield a net signal equal to three times the standard deviation of the background) obtained by UV–vis spectrum was calculated to be 2.38×10^{-7} M (0.03 mg/kg), which was below the safety limit (2.5 mg/kg in the USA and UK; 1 mg/kg for infant formula in China). The relative standard deviation (RSD) for measurement of 2.38×10^{-6} M (0.3 mg/kg) melamine for 3 independent determinations was 0.8%, indicating that the proposed method has good reproducibility and could be used for the direct visual measurement of melamine.

Milk samples are typically complex matrices that are difficult to analyse because of the abundance of proteins, lipids, and other various components. There are many market milk products in the world with the different composition of protein and lipid. Detection of melamine may be different due to the influence of proteins and lipids in milk. In order to determine whether this colorimetric assay could be applied to melamine detection in various market milk products, the detection of melamine in real liquid milk samples was also carried out by using three kinds of market milk samples (from Inner Mongolia Meng Niu Dairy Co., Ltd, Heilongjiang Province Wondersun Dairy Co., Ltd and Inner Mongolia Yili Industrial Group Co. Ltd.), and the steps were the same as those described in Section 2.6. Analytical results showed that the melamine concentrations in all of the spiked samples could be well quantified after the sample pretreatment. The detection limits of the melamine detection for three kinds of market milk samples were also similar. These results indicated that the influence of proteins and lipids in different market milk products was eliminated by precipitating with trichloroacetic acid and the pretreatment method worked well for different kinds of market milk products.

4. Conclusions

In this work, a sensitive and simple colorimetric assay to detect melamine during the formation of Au-NPs was reported based on the Mb-mediated gold nanoparticles (Au-NPs) production. Detailed mechanistic investigations indicate that melamine interrupts the formation of Au-NPs by Mb and accelerates the aggregation of formed Au-NPs. Concentrations of melamine could be directly

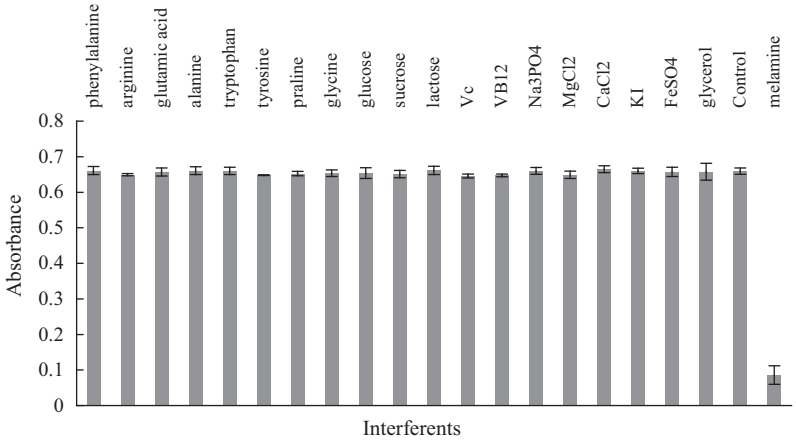

Fig. 6. Absorbance of the solution in the presence of melamine or other interferences Spectra were recorded after a fixed time interval of 50 min. Error bars show the standard deviations of measurements taken from three independent experiments.

monitored according to the colour change resulted from the formation and aggregation of Au-NPs, which made the melamine determination convenient and easy. Milk is a complex system and its various components serve as potentially interactive species alongside melamine regardless of the detection methodology used. In our study, market milk samples were pretreated by a simple precipitation and filtration procedure. The results indicated that the potential interference from the sample can be eliminated by this simple pretreatment. Also, the analysing method itself showed excellent anti-disturbed ability to the common interferents in the sensing of melamine. In contrast to traditional Au-NPs colorimetric detection, this novel method was based on the fact that melamine could not only interrupt the formation of Au-NPs but also stimulate the aggregation of formed Au-NPs. The method realised the determination during the formation of Au-NPs, which allowed the simple and efficient recognition and quantification of melamine. Furthermore, this method has high sensitivity and enables accurate quantification of melamine as low as 3.90×10^{-7} M.

Acknowledgements

The authors thank the National Natural Science Foundation of China (20873034, 21073050) and Heilongjiang Provincial Funds for Distinguished Young Scientists (JC201106) for support.

References

Ai, K. L., Liu, Y. L., & Lu, L. H. (2009). Hydrogen-bonding recognition-induced color change of gold nanoparticles for visual detection of melamine in raw milk and infant formula. *Journal of the American Chemical Society, 131*, 9496–9497.

Balasubramanian, R., & Rosenzweig, A. C. (2008). Copper methanobactin: a molecule whose time has come. *Current Opinion in Chemical Biology, 12*, 245–249.

Cao, Q., Zhao, H., He, Y. J., Li, X. J., Zeng, L. X., Ding, N., et al. (2010). Hydrogen-bonding-induced colorimetric detection of melamine by nonaggregation-based Au-NPs as a probe. *Biosensors and Bioelectronics, 25*, 2680–2685.

Chi, H., Liu, B. H., Guan, G. J., Zhang, Z. P., & Han, M. Y. (2010). A simple, reliable and sensitive colorimetric visualization of melamine in milk by unmodified gold nanoparticles. *Analyst, 135*, 1070–1075.

Choi, D. W., Antholine, W. E., Do, Y. S., Semrau, J. D., Kisting, C. J., Kunz, R. C., et al. (2005). Effect of methanobactin on the activity and electron paramagnetic resonance spectra of the membrane-associated methane monooxygenase in *Methylococcus capsulatus* Bath. *Microbiology, 151*, 3417–3426.

Choi, D. W., Zea, C. J., Do, Y. S., Semrau, J. D., Antholine, W. E., Hargrove, M. S., et al. (2006). Spectral, kinetic, and thermodynamic properties of Cu(I) and Cu(II) binding by methanobactin from Methylosinus trichosporium OB3b. *Biochemistry, 45*, 1442–1453.

Ehling, S., Tefera, S., & HO, I. P. (2007). High-performance liquid chromatographic method for the simultaneous detection of the adulteration of cereal flours with melamine and related triazine by-products ammeline, ammelide, and cyanuric acid. *Food Additives & Contaminants, 24*(12), 1319–1325.

Hakemian, A. S., & Rosenzweig, A. C. (2007). The biochemistry of methane oxidation. *Annual Review of Biochemistry, 76*, 223–241.

Hanson, R. S., & Hanson, T. E. (1996). Methanotrophic bacteria. *Microbiological Reviews, 60*, 439–471.

Huang, J., Dai, W. L., & Fan, K. N. (2009). Remarkable support crystal phase effect in Au/FeOx catalyzed oxidation of 1,4-butanediol to γ-butyrolactone. *Journal of Catalysis, 266*, 228–235.

Jin, R., Wu, G., Li, Z., Mirkin, C. A., & Schatz, G. C. (2003). What controls the melting properties of DNA-linked gold nanoparticle assemblies. *Journal of the American Chemical Society, 125*, 1643–1654.

Kim, B., Perkins, L. B., Bushway, R. J., Nesbit, S., Fan, T., Sheridain, R., et al. (2008). Determination of melamine in pet food by enzyme immunoassay, high-performance liquid chromatography with diode array detection and ultra-performance liquid chromatography with tandem mass spectrometry. *Journal of AOAC International, 91*, 408–413.

Kuang, H., Chen, W., Yan, W. J., Xu, L. G., Zhu, Y. Y., Liu, L. Q., et al. (2011). Crown ether assembly of gold nanoparticles: melamine sensor. *Biosensors & Bioelectronics, 26*, 2032–2037.

Li, L., Li, B. X., Cheng, D., & Mao, L. H. (2010). Visual detection of melamine in raw milk using gold nanoparticles as colorimetric probe. *Food Chemistry, 122*(3), 895–900.

Lin, M., He, L., Awika, J., Yang, L., Ledoux, D. R., Li, H., et al. (2008). Detection of melamine in gluten, chicken feed, and processed foods using surface enhanced Raman spectroscopy and HPLC. *Journal of Food Science, 73*(8), 129–134. http://dx.doi.org/10.1111/j.1750-3841.2008.00901.x.

Liu, Y. T., Deng Jian, A. L., Liang, J., Chen, F., & Wang, H. (2011). Spectrophotometric determination of melamine in milk by rank annihilation factor analysis based on pH gradual change-UV spectral data. *Food Chemistry, 126*, 745–750.

Mauer, L. J., Chernyshova, A. A., Hiatt, A., Deering, A., & Davis, R. (2009). Melamine detection in infant formula powder using near- and mid-infrared spectroscopy. *Journal of Agriculture and Food Chemistry, 57*(10), 3974–3980. http://dx.doi.org/10.1021/jf900587m.

Perrault, S. D., & Chan, W. C. W. (2009). Synthesis and surface modification of highly monodispersed, spherical gold nanoparticles of 50–200 nm. *Journal of the American Chemical Society, 131*, 17042–17043.

Reimschuessel, R., & Puschner, B. (2010). Melamine toxicity-stones vs. crystals. *Journal of Medical Toxicology, 6*, 468–469. http://dx.doi.org/10.1007/s13181-010-0107-5.

Vo, T. D. T., Himmelsbach, M., Haunschmidt, M., Buchberger, W., Schwarzinger, C., & Klampfl, C. W. (2008). Improved analysis of melamine-formaldehyde resins by capillary zone electrophoresis-mass spectrometry using ion-trap and quadrupole-time-of-flight mass spectrometers. *Journal of Chromatography A, 1213*, 83–87.

Wang, Q., Haughey, S. A., Sun, Y. M., Eremin, S. A., Li, Z. F., Liu, H., et al. (2011). Development of a fluorescence polarization immunoassay for the detection of melamine in milk and milk powder. *Analytical and Bioanalytical Chemistry, 399*(6), 2275–2284. http://dx.doi.org/10.1007/s00216-010-4599-2.

Wei, F., Lam, R., Cheng, S., Lu, S., Ho, D., & Li, N. (2010). Rapid detection of melamine in whole milk mediated by unmodified gold nanoparticles. *Applied Physics Letters, 96*, 133702-1–133702-3.

Wiley, B. J., Im, S. H., McLellan, J., Seikkinen, A., & Xia, Y. (2006). Maneuvering the surface plasmon resonance of silver nanostructures through shape-controlled synthesis. *Journal of Physical Chemistry B, 110*, 15666–15675.

Xin, J. Y., Cheng, D. D., Zhang, L. X., Lin, K., Fan, H. C., Wang, Y., & Xia, C. G. (2013). Methanobactin-mediated one-step synthesis of gold nanoparticles. *International Journal of Molecular Sciences, 14*(11), 21676–21688. http://dx.doi.org/10.3390/ijms141121676.

Xin, J. Y., Zhang, Y. X., Dong, J., Zhou, Q. Q., Wang, Y., Zhang, X. D., et al. (2010). Epoxypropane biosynthesis by whole cell suspension of methanol-growth Methylosinus trichosporium IMV 3011. *World Journal of Microbiology & Biotechnology, 26*, 701–708.

Yang, S. P., Ding, J. H., Zheng, J., Hu, B., Li, J. Q., Chen, H. W., et al. (2009). Detection of melamine in milk products by surface desorption atmospheric pressure chemical ionization mass spectrometry. *Analytical Chemistry, 81*(7), 2426–2436. http://dx.doi.org/10.1021/ac900063u.

Yokley, R. A., Mayer, L. C., Rezaaiyan, R., Manuli, M. E., & Cheung, M. W. (2000). Analytical method for the determination of cyromazine and melamine residues in soil using LC–UV and GC–MSD. *Journal of Agriculture and Food Chemistry, 48*(8), 3352–3358.

Zhu, L., Gamez, G., Chen, H. W., Chingin, K., & Zenobi, R. (2009). Rapid detection of melamine in untreated milk and wheat gluten by ultrasound-assisted extractive electrospray ionization mass spectrometry (EESI-MS). *Chemical Communications*, 559–561. http://dx.doi.org/10.1039/b818541g.