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Visual detection of melamine in milk samples based on label-free and labeled gold nanoparticles

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ABSTRACT

Melamine that can cause serious damage to the organs of animal or human beings was found to bind to polythymine via hydrogen bonding. With this novel discovery, colorimetric detection of melamine based on label-free and labeled gold nanoparticles was developed, respectively. Both of the methods revealed good selectivity for melamine over other components that may exist in milk and good anti-influence ability. The raw milk samples were pretreated according to the National standard method combined with a solid phase extraction monolithic column. The accurate quantification of melamine as low as 41.7 nM and 46.5 nM was obtained, respectively. It also guarantees fast and reliable readout with naked eyes, making visual detection possible. Further comparison between label-free and labeled based methods was discussed in this paper.

1. Introduction

Contamination of the milk products with melamine has been an important worldwide concern for several years. Because of the high nitrogen content (66% by mass) of melamine, it is added to the milk products illegally to produce an incorrect reading high in the measurement of protein content. Melamine in food will cause serious damage to the organs of animals [1] or human beings. It can be hydrolyzed to cyanuric acid which in turn associates with melamine to form reticulations, resulting in the formation of highly toxic concretions [2,3]. Hence, the great importance of detecting melamine in milk products has been well recognized. Most methods measure melamine through HPLC [4,5], MS [6–8] and LC–MS [9,10], which require expensive instrumentation and considerable time for identification and quantification. In view of the huge amount of samples to be screened, visual detection methods would be extremely attractive because of the possibility of readily reading out with the naked eyes, in some cases at the point of use [11]. Recently, melamine detection utilizing modified gold nanoparticles (AuNPs) by color changes of the solution has been invented [12], in which a thiol-functionalized cyanuric acid derivative is labeled on the AuNPs. It has advantages over the other reported methods, as it requires no expensive and complicated instruments, making on-site and real-time melamine sensing possible. However, the functional legend in this research work was laboratory synthesized, which making the application of this method limited.

The ability of pyrimidine to form hydrogen bonds with purines has been well documented, and the hydrogen bonding between thymine and 2,6-diamino-5-methylpyrimidin-4-one (W), which has a similar molecular structure as melamine, was reported (Scheme 1a) [13]. Melamine can be fixed by electrostatic or hydrogen bonding as reported [14–16]. Besides, the uncoiled single-stranded oligonucleotide has been found to stabilize AuNPs against aggregation because of the association of their bases with AuNPs, whereas double-stranded oligonucleotides do not have such function [17]. These findings gave us a hint that the single-stranded oligonucleotide (containing thymine) may also bind to melamine via hydrogen bonding (Scheme 1b). Accordingly, the stabilization effect of this oligonucleotide on AuNPs would disappear (or be weakened), resulting in the aggregation of AuNPs in solutions, which causes distinct color changes of the solution (Scheme 1c). A newly report which was published as we submitted our manuscript gave a certain proof to our assumption [18]. Based on this thought, we herein describe easy-to-handle colorimetric

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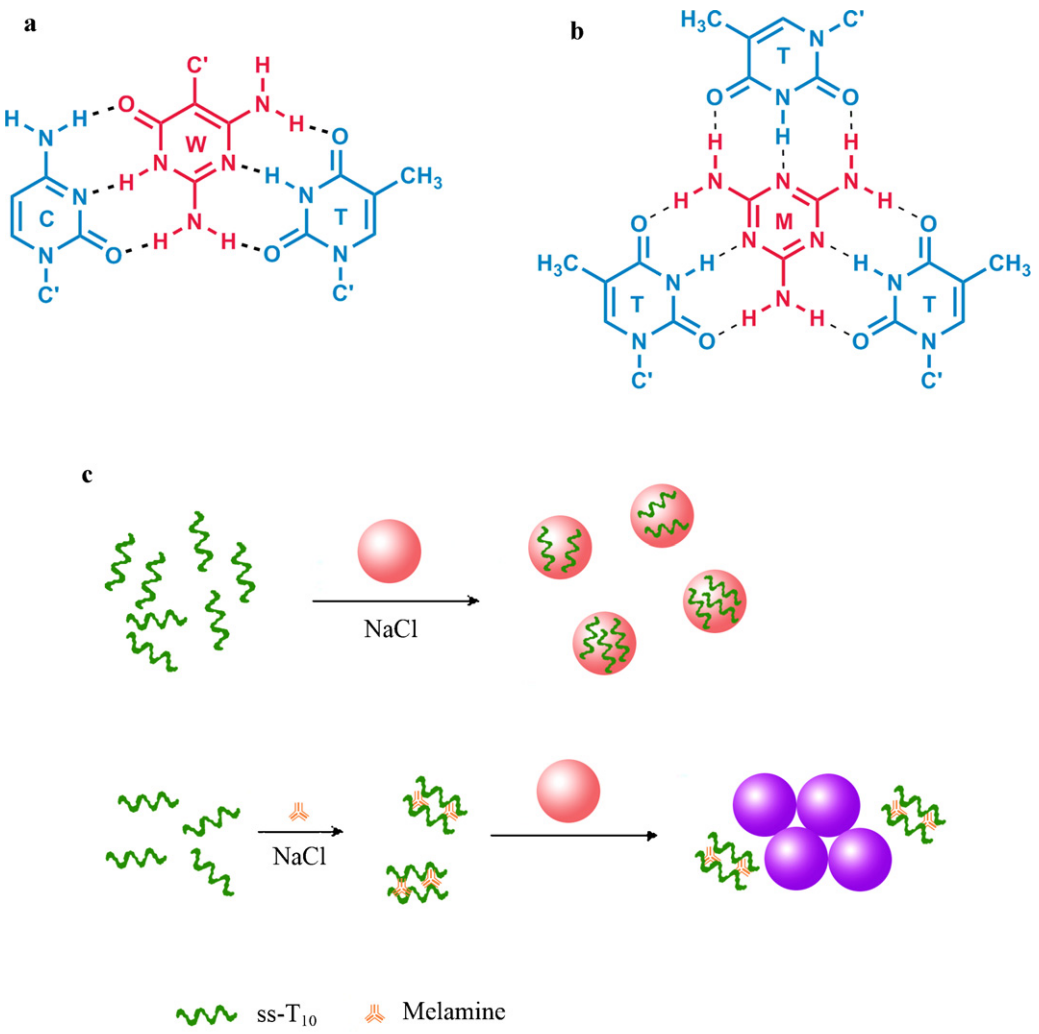


Scheme 1. (a) A Janus–Wedge base triplet: the third-strand residue W binds to the W–C faces of both target residues (J. Am. Chem. Soc. 2004, 126, 70–71). (b) The structure of the triple hydrogen-bonding recognition between melamine and the thymine. (c) The working principle of the melamine detection. Label-free AuNPs sensor for the optical analysis of melamine.

methods for the determination of melamine using single-stranded oligonucleotide, polythymine (poly-T_n), an easily available commercial reagent. More attractively, both label-free and label-based protocols can be followed, providing satisfying results. The proposed methods enable accurate quantification of melamine as low as 41.7 nM (label-free) and 46.5 nM (label-based) in raw milk product, respectively, just using a spectrophotometer. It also guarantees fast and reliable readout with naked eyes, making visual detection possible. The simplicity of the approach eases the popularization of the method. A comparison of the performances between the two methods was carried out.

2. Materials and methods

2.1. Reagents and chemicals

All chemicals were purchased from Sigma–Aldrich, except that the oligonucleotides with the following sequences (poly-T_2, poly-T_5, poly-T_{10}, poly-T_{25}, poly-T_{50}, poly-T_{100}, HS-poly-T_{10} and HS-ssDNA) were synthesized by Shanghai Sangon Biological Engineering Technology & Service Co. Ltd. Milli-Q water (18.0 MΩ cm) was used in all of the experimental processes.

2.2. Instrumentation

Extinction measurements were performed on a UV-2550 Spectrophotometer (SHIMADZU, Japan). The pH of all of the buffer solution was examined with a Model PB-10 pH meter (Sartorius, Germany). TEM pictures were obtained on a FEI Tecnai G2 20 TWIN 200 kV transmission electron microscope.

2.3. Synthesis of AuNPs

AuNPs in a size of 13 nm used in our experiment was synthesized following the literature procedure [19]. All glassware was cleaned with aqua regia, rinsed copiously with deionized water and dried prior to use. Briefly, the mixture of 1.88 mL of $HAuCl_4$ (w/v = 2%) and 100 mL deionized water was brought to boiling. Then 10.0 mL of sodium citrate tribasic dihydrate (0.1141 g) solution was added quickly with vigorous stirring. (The ratio of concentration between $HAuCl_4$ and sodium citrate tribasic dihydrate was 1:3.88.) Boiling was continued for 10 min and the solution turned from yellow to clear, to black, to purple and to deep red. Then the stirring was maintained for another 15 min without heating and the solution was cooled to room temperature (23–25 °C). According to the literature, AuNPs with different diameters (10–100 nm) can be prepared by the sodium citrate reduction method through controlling the

amounts of sodium citrate [20]. Hence, AuNPs with the diameter of 40 nm was prepared following above procedure with a different amount of sodium citrate tribasic dihydrate. Prepared AuNPs were stored at 4 °C protected from light before use.

2.4. Preparation of HS-ssDNA labeled AuNPs

HS-ssDNA modification of AuNPs was performed with similar procedures to those previously reported [21]. Briefly, a solution of HS-ssDNA in water was added to an aqueous solution of AuNPs. The mixture was incubated for 16 h at room temperature, and then a buffer solution (0.01 M phosphate buffer (PB), 0.1 M NaCl, pH 7.4, containing 10^{-4} M L-cysteine) was added dropwisely to the vial with gentle hand shaking. It was further incubated for 40 h at room temperature and the resulting ssDNA–AuNPs conjugates were purified by repeated centrifugation and dispersion in PBS (0.1 M PB, 0.1 M NaCl, pH 7.4) for at least three times. DNA functionalized AuNPs were stored at 4 °C to prevent the degradation of DNA [22].

2.5. Label-free and label-based procedure for melamine detection

For the label-free procedure, different concentrations of melamine (30 μL) were incubated with poly-T_n (10^{-5} M, 30 μL) for 5 min in reaction buffer (0.1 M PB, 0.3 M NaCl, pH 7.4). Then, 300 μL of AuNPs (mixture of 200 μL of AuNPs solution which were synthesized by ourselves and 100 μL deionized water) was added. A UV-2550 Spectrophotometer was used and the calibration curve was made based on the absorbance value at 520 nm.

Labeled AuNPs typical procedure for the detection of melamine was that 20 μL of melamine (dissolved in reaction buffer) at different concentrations were added into 10 μL of AuNPs of which concentration was about 60 nM. The mixture was incubated for 5 min at 95 °C, cooled to room temperature, mixed with 400 μL of deionized water thoroughly and then detected by UV–vis absorbance. The calibration curve for label-based method was made based on the difference between A520 and A700.

2.6. Treatment of raw milk sample

The raw milk samples were pretreated according to the National standard method (GB/T 22388-2008, Determination of melamine in raw milk and dairy products. National standards of PR China) combined with a solid phase extraction monolithic column [23]. Briefly, milk sample (1.0 mL) and 5.0 mL of trichloroacetic acid (v/v = 1%, pH 3.0) was mixed thoroughly and centrifuged at 16.2× 1000 × g for 10 min to get proteins precipitated. The precipitate was discarded and the supernatant was collected after being filtered through a 0.22 μm membrane. Driven by a double-injection pump (TS2-60), the sample solutions flew through the solid phase extraction monolithic column at a speed of 100 μL/min. Then the column was washed with deionized water and methanol. After eluting with 5% ammonium hydroxide in methanol, the eluent was dried with a stream of argon at 50 °C and the residue was dissolved in PBS and filtered through a 0.22 μm membrane.

3. Result and discussion

3.1. Principle for the label-free and label-based detection of melamine

The principle of the straightforward label-free method was illustrated in Scheme 1c. Basically, the difference of the electrostatic properties between poly-T_n and the poly-T_n-melamine-poly-T_n complex was employed to develop this colorimetric method. In the absence of melamine, AuNPs were dispersed homogeneously in phosphate buffer with certain salinity. The bases of the ssDNA are uncoiled partially and can be exposed to the AuNPs, because the ssDNA is sufficiently flexible. Under these conditions, the negative charge on the backbone is sufficiently distant so that attractive van der Waals forces between the bases and the gold nanoparticle are sufficient to cause ssDNA to stick to the gold, which prevented the aggregation of particles [17]. Upon hydrogen bonding between the bases and melamine, a complex with switch-back or triple-stranded structure formed (Scheme 1 b), which caused exposure of the phosphate backbone of the oligonucleotide. Therefore, the repulsion between the negatively charged phosphate backbone and the citrate ions dominated the electrostatic interaction between the poly-T_n-melamine-poly-T_n complex and AuNPs, and thus the complex no longer bound to AuNPs. In this situation the Au particles readily aggregated in the presence of salt and provoked a red-to-blue color change owing to the interparticle coupled plasmon excitons in the aggregated states [24].

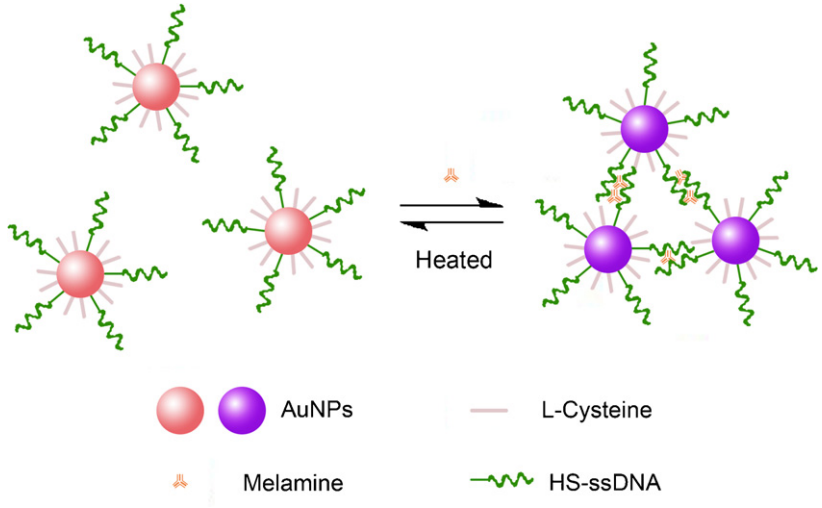
For the label-based procedure, the single-stranded oligonucleotide labeled AuNPs was used for melamine determination, which was also based on the combination of thymine with melamine (Scheme 2). The DNA functionalized AuNPs can assemble when the oligonucleotides hybridize, which causes the changes of colors [25] and can be used for quantitative detection [26,27]. Substances inducing hybridization include small molecules [28–33], metal ions [11,34–39], proteins [40,41] and oligonucleotides [17,42–44]. When two complementary DNA–AuNPs combine, they form DNA-linked aggregates that can dissociate reversibly with a concomitant color change [45]. In our assay procedure, the HS-ssDNA (HS-(CH2)6-TAGCTATGGAATTCCTCGTAGGCATTTTTT) was first attached to the surface of 13-nm gold particles. The functionalized AuNPs assembled upon binding of the oligonucleotide to melamine, which caused the red-to-blue color change. This assembly process was reversible, and dissociation occurred upon thermal denaturation accompanying a blue-to-red color change, which partly proved the existence of hydrogen bond (Fig. 1).

3.2. Determination of melamine

Some influence factors in label-free or label-based method were studied as described in detail in the Supplementary Data. Under an optimized condition, the aggregation occurred in a melamine concentration-dependent manner for both the label-free and label-based methods.

It should be noted that PBS was selected as the buffer solution because it not only accelerated the hydrogen bonding between melamine and the poly-T_{10} but also supplied the salt needed for the aggregation of AuNPs in the label-free method. With the concentration of melamine increasing, the red-shifted band corresponding to the aggregated AuNPs was intensified, and this was accompanied by a decrease of absorbance at 520 nm (Fig. 2a). With the melamine concentration ranging from 41.7 to 417 nM, the color varied as a function of melamine concentration. Concentrations that lower than 41.7 nM did not cause an obvious color change. When the concentration was higher than 417 nM, the absorbance of the system kept constant since almost all poly-T_{10} were exhausted by melamine, and blue-color AuNPs precipitate was generated. All the color changes described above presented in 5 min and could be observed with naked eyes (Fig. 2b). The transmission electron microscopy (TEM) images revealed individual nanoparticles in the absence of melamine (Fig. 3a) and aggregation of AuNPs in the presence of melamine (Fig. 3b and c).

For the HS-ssDNA labeled AuNPs based method, in order to improve the sensitivity and the stability of the AuNPs labeled system, L-cysteine was introduced to take up free positions on the surface of AuNPs, which would otherwise combine with HS-ssDNA. Similar to the label-free system, the absorbance at 520 nm declined and enhanced at 700 nm simultaneously according to the increase



Scheme 2. The dissociation and aggregation of the labeled AuNPs used in the colorimetric screening of melamine.

of melamine concentration (Fig. 4) under the optimized condition. A linear response range of concentration from 46.5 nM to 46.5 μM, which was larger than that of label-free method, was obtained. The melamine-induced aggregation of labeled AuNPs was also evidenced by TEM images (Fig. 5).

Fig. 6 depicts the calibration curves of label-free and label-based methods. Both of calibration curves in buffer and milk samples were obtained. It should be noted that the best figure of merit of label-free method corresponds to the absorbance at 520 nm with different concentrations of melamine while that of label-based method corresponds to the difference in absorbance between 520 nm and 700 nm. It was found that calibration curves in buffer and milk samples of label-based method match better than those of label-free method, because labeled AuNPs are more robust to salt and temperature variations than label-free AuNPs. Easy performance and a better sensitivity were found to be the advantages of label-free method. For the label-based method, despite the need of long preparation time of DNA–AuNPs, longer assay time and the difficulty in controlling the assembly process, the better accuracy and larger response range of concentration makes it abstractive.

3.3. Selectivity for the melamine detection

The selectivity of our methods was evaluated in the presence of other species that may exist in milk products. Our results

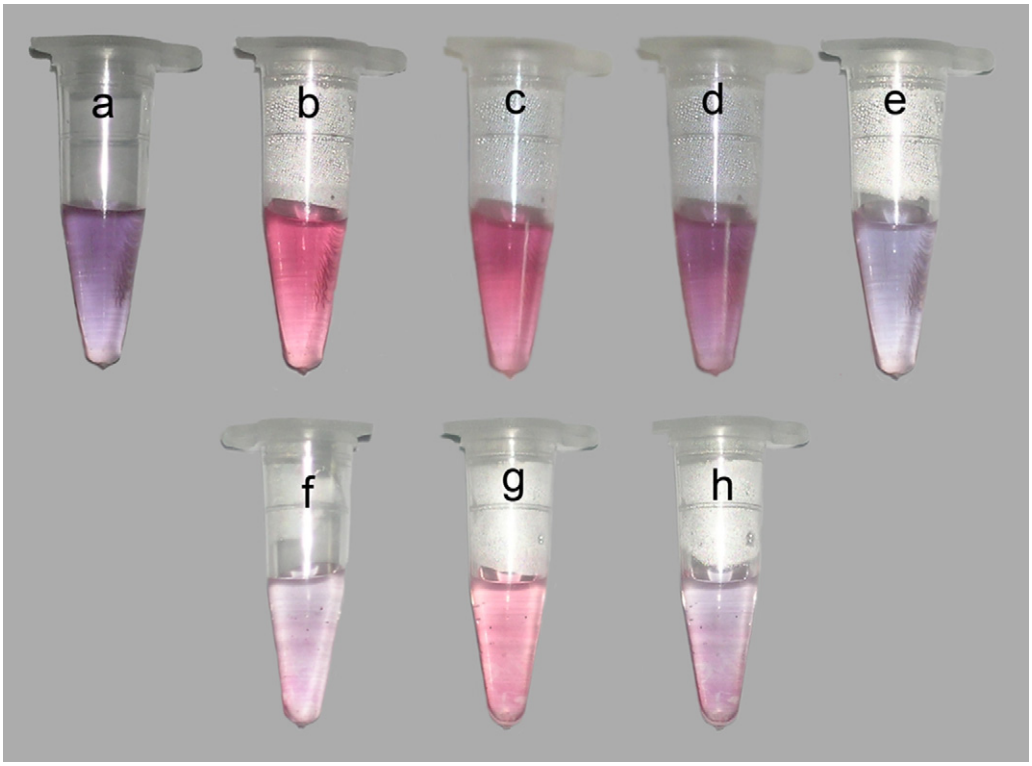


Fig. 1. Color changes of ssDNA labeled AuNPs mixed with melamine upon heating from: (a) the room temperature to (b) 95 °C for 5 min, then (c) cooling for 30 s, (d) cooling for 1 min, and (e) cooling for 3 min. Compared with poly-A_n labeled AuNPs mixed with melamine: (f) at room temperature, (g) heated at 95 °C for 5 min, and (h) cooling for 10 min.

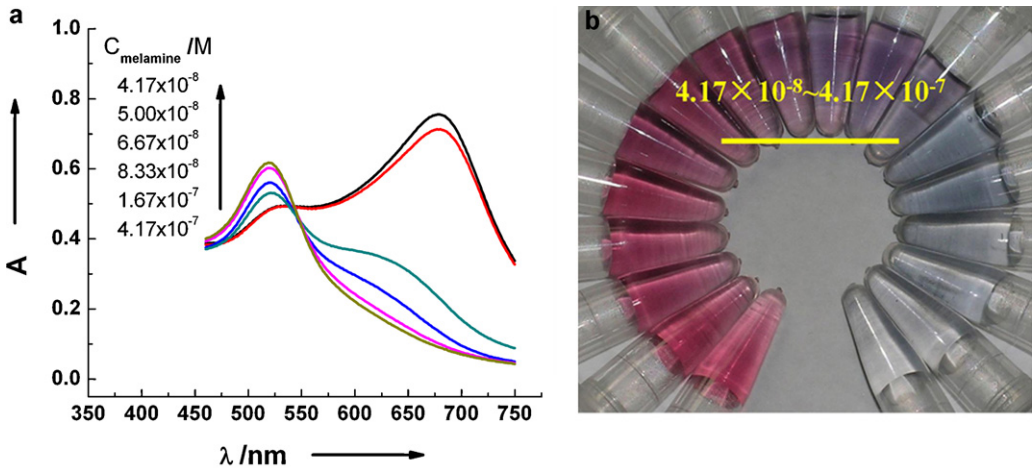


Fig. 2. (a) UV–vis spectra corresponding to the analysis of different concentrations of melamine using the label-free method. (b) Visual color changes of label-free AuNPs upon the addition of varying concentrations of melamine in the presence of NaCl and the final concentrations of poly-T_{10} were 8.33×10^{-7} M. When the final concentration of melamine was 41.7 nM, the solution began to turn pink. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

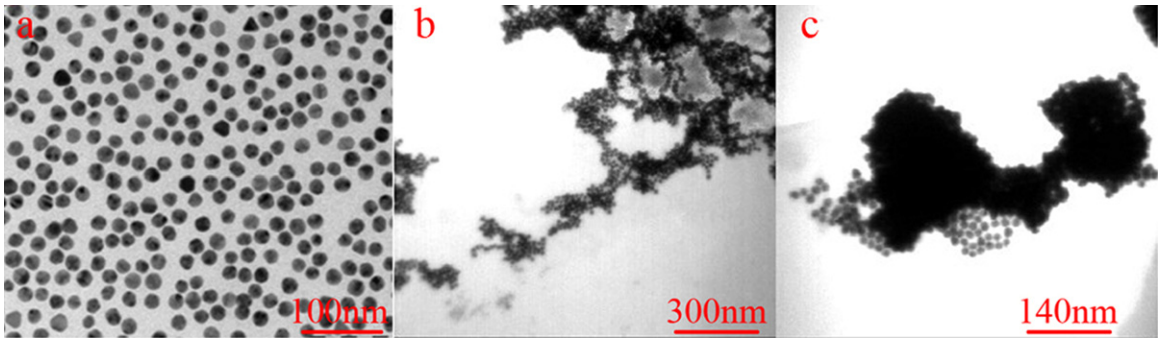


Fig. 3. TEM images of (a) AuNPs in the presence of 8.33×10^{-7} M poly-T_{10}, (b) AuNPs in the presence of 8.33×10^{-7} M poly-T_{10} and 4.17×10^{-8} M melamine (c) AuNPs in the presence of 8.33×10^{-7} M poly-T_{10} and 8.33×10^{-6} M melamine.

indicated that both of the methods showed excellent selectivity over K^+, lactose, Fe^{2+}, Mg^{2+}, Cu^{2+}, Ca^{2+}, Zn^{2+} and vitamin C (Vc) even at low concentration of melamine (Fig. 7). The allowable concentration of the coexisting substances exceeded the concentration of melamine over 1000 times. This revealed good anti-interference ability and the possibility of these methods for detecting melamine in the presence of these coexisting components. This high selectivity was mainly attributed to the high specificity of triple hydrogen bonding recognition between thymine and melamine.

3.4. Applicability to real milk samples

The applicability of our colorimetric methods to real milk samples was examined. After the pretreatment of milk sample, the influence of proteins in milk sample was eliminated by precipitating with trichloroacetic acid. Combining with a treatment with solid phase extraction monolithic column, an enrichment of melamine could be achieved.

No melamine was detected in the pretreated milk sample, and then the sample was analysed with a standard-addition method

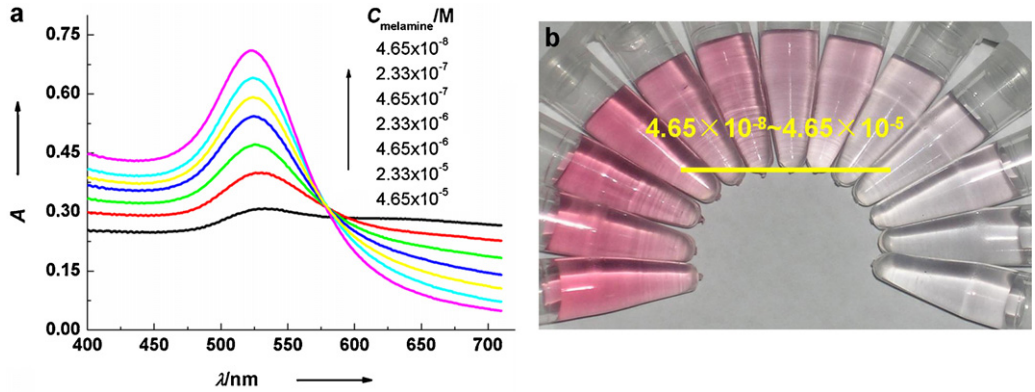


Fig. 4. (a) UV–vis spectra corresponding to the analysis of different concentrations of melamine using the label based method. (b) Visual color changes upon treatment of the labeled AuNPs with different concentrations of melamine in the presence of NaCl.

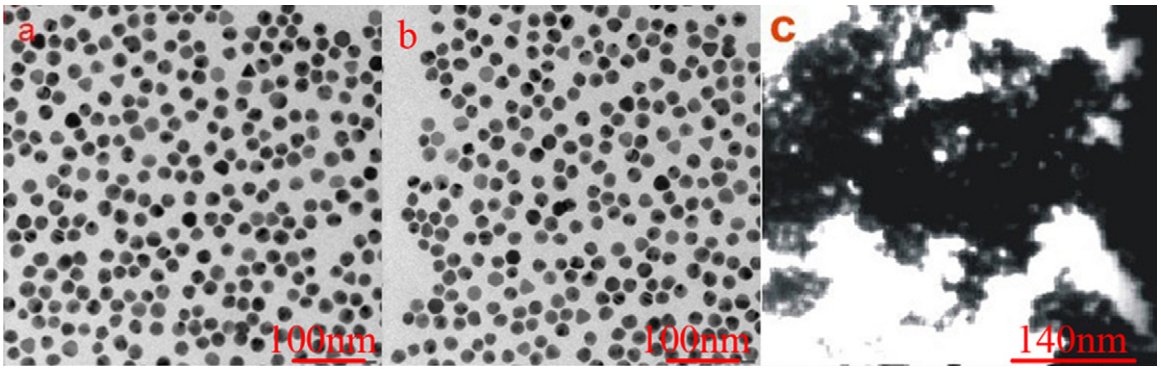


Fig. 5. TEM images: (a) the label-free AuNPs, (b) labeled AuNPs, and (c) labeled AuNPs mixed with melamine.

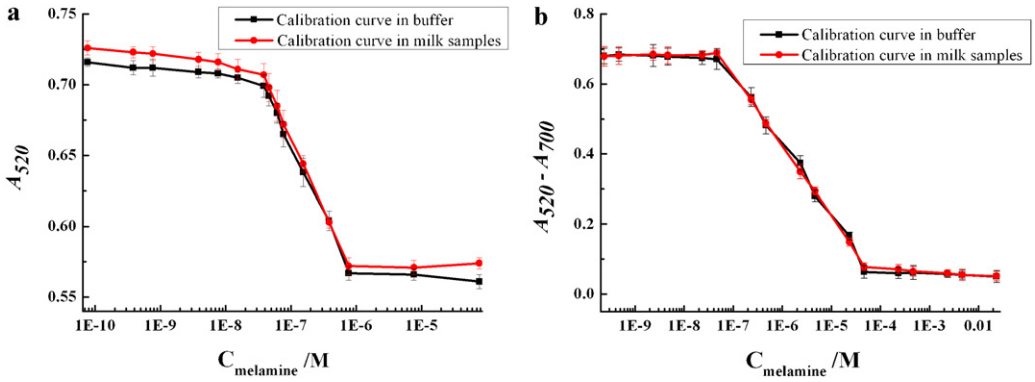


Fig. 6. The calibration curves of (a) the optimized label-free sensor and (b) the optimized label-based sensor in buffer and in milk samples.

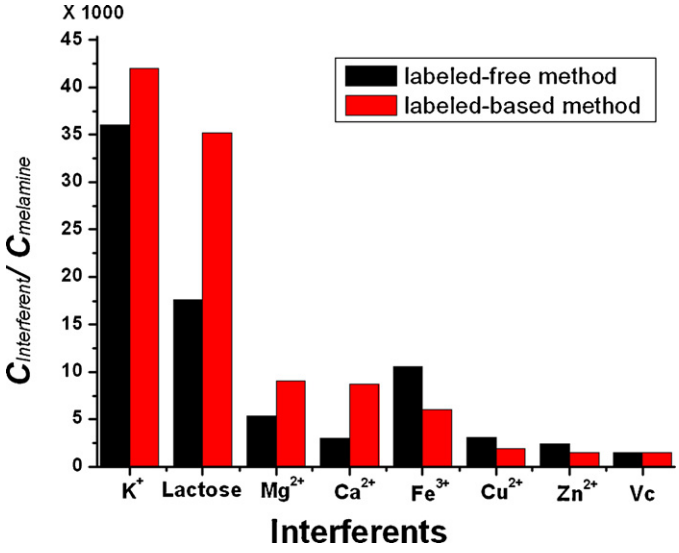


Fig. 7. Selectivity study corresponding to the analysis of melamine by label-free AuNPs and ssDNA-labeled AuNPs in comparison to a series of other components. The final concentration of the melamine was 4.17×10^{-8} M and 2.33×10^{-6} M, respectively. The relative standard deviation was ±5.00%.

Table 1
Determination of melamine in milk samples by label-free method.

Milk samples	Added (10^{-8} M)	Found (10^{-8} M)			Average (10^{-8} M)	Average recovery (%)	RSD (%)
1	3.85	3.16	3.20	3.22	3.19	82.9	0.96
2	4.62	3.96	3.92	3.90	3.93	85.1	0.78
3	6.15	5.34	5.27	5.29	5.30	86.2	0.68
4	7.69	7.22	7.12	7.17	7.17	93.2	0.70
5	15.4	13.8	13.8	13.5	13.7	89.0	1.26
6	38.5	35.7	35.6	35.1	35.5	92.2	0.91
7	76.9	73.0	72.9	72.2	72.7	94.5	0.60

Table 2
Determination of melamine in milk samples by label-based method.

Milk samples	Added (10^{-8} M)	Found (10^{-8} M)			Average (10^{-8} M)	Average recovery (%)	RSD (%)
1	4.65	4.72	4.78	4.79	4.76	102.3	0.80
2	23.3	21.9	22.0	21.4	21.8	93.6	1.48
3	46.5	45.9	46.8	46.0	46.2	99.4	1.07
4	233	227	221	218	222	95.3	2.06
5	465	415	422	423	420	90.3	1.04
6	2330	2107	2155	2148	2138	91.8	1.21
7	4650	4714	4809	4796	4773	102.6	1.08

with adding a certain amounts of melamine to the sample solution. The experimental results are shown in Tables 1 and 2. The recovery of label-free method was between 82.9% and 94.5%, as those of label-based method was between 90.3% and 102.6%, and the relative standard deviation (RSD) was less than 1.26% and 2.06%, respectively. This demonstrated the potential practical application of our methods to measure the melamine in raw milk samples, and the label-based method affords a better accuracy compared with label-free method.

4. Conclusion

To conclude, we have compared two new AuNPs-thymine-based assays for the optical analysis of melamine. The assays are based on the color changes derived from the AuNPs aggregation at a given salt concentration and reaction temperature, involving only one or two-steps operation and can be achieved either with the naked eye or with a common spectrophotometer for reliable detection of melamine. These methods show several analytical advantages. Firstly, they have high sensitivity and enable accurate quantification of melamine as low as 41.7 nM and 46.5 nM, respectively. Secondly, they exhibit excellent selectivity for melamine over other components that may exist in milk. Thirdly, they take only approximately 10 min to determine the concentration of melamine in milk sample after being pretreated. As compared with the reported method [12], they do not involve synthesis of special chemical, which ensures the popularization of the method. Since the label-based method affords a better stability, a better accuracy and a larger response range, compared with label-free method or the reported method [18], we propose the label-based method is more applicable.

The proposed method can be extended to detect other molecules or ions by substituting the thymine in our study with synthetic artificial bases that selectively bind other molecules or ions.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2011.05.006.

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