In Situ Functionalization of Silver Nanoparticles by Gallic Acid as a Colorimetric Sensor for Simple Sensitive Determination of Melamine in Milk

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ABSTRACT: A simple and green colorimetric sensing assay strategy for highly efficient determination of melamine has been fabricated, which is based on the redox reaction of gallic acid with Ag+. Monodispersed Ag nanoparticles (AgNPs) were obtained using gallic acid as a reducing and stabilizing agent. However, the aggregate behavior of AgNPs was observed, while the melamine was present in the reaction medium. As a result, the color of the solution changed from vivid yellow to brown, and the density of the color was quantitatively correlated with the melamine concentration. The aggregation of AgNPs could be attributable to the formation of hydrogen bonds between melamine and gallic acid. The designed sensor exhibited a good detection limit of 0.099 μM (0.012 ppm), which was much lower than the safety limit in China (1.0 ppm) and EU (2.0 ppm). Additionally, the sensing assay displayed good selectivity toward melamine over other coexisting substances. Consequently, the proposed colorimetric sensor was successfully used for the determination of melamine detection in raw milk samples.

1. INTRODUCTION

Melamine (1,3,5-triazine-2,4,6-triamine), a desirable protein substitute, is predominately emerged in milk, infant formula, and pet food due to its high nitrogen content (66% by mass).1-3 Besides, the addition of melamine could increase the protein content in milk, which can mislead the consumers in the milk product market.4 However, while melamine concentration is higher than the safety limit [2.5 ppm of United States Food and Drug Administration (US FDA) and 1.0 ppm of infant formula in China], an insoluble compound with cyanuric acid that induces kidney stones could be formed, leading to renal failure and even death in humans.6,7 For example, serious renal failure and deaths of infants have been reported due to the addition of melamine into infant formula in 2008.8 The serious detrimental consequences of melamine contamination can pose to public health. Therefore, it is essential to develop reliable methods for the detection of melamine in milk industry.

Until now, a number of analytical methods such as mass spectroscopy (MS), liquid chromatography−MS (LC−MS), gas chromatography−MS, micellar LC, high-performance LC, and nuclear magnetic resonance spectroscopy have been well explored for the detection of melamine in food samples.9-13 These methods have the advantages of high specificity and sensitivity. However, the properties such as high instrumentation costs, complicated preconcentration, and time consumption hindered their utilization in melamine detection.14 Overall, it is of particular importance to establish a simple, low-cost, intrinsic, and sensitive method for the detection of melamine.

Recently, benefiting from the advantages of being simple and cost-effective and having good sensitivity and selectivity for real-time sample analysis,15-17 the metallic nanoparticle-based colorimetric sensing assays have been drawn considerable attention in biological science and analytical chemistry. Among the nanoparticle-based colorimetric assays, gold nanoparticles (AuNPs)18 and silver nanoparticles (AgNPs)19 have been widely used for the design of visual sensors for melamine detection owing to the simple preparation, stability, and biocompatibility. The colorimetric sensing assay technique involves three steps, including synthesis of Au/Ag nanoparticles, functionalization of nanoparticles, and detection of melamine.20 These two nanoparticles aggregate because of the interaction by hydrogen-bonding recognition, electrostatic, or donor−acceptor interaction. The shifting of resonant excitation is due to the introduction of melamine, resulting in the change of the color of the solution from bright yellow to pale red.21 For example, Inamuddin and Kanchi22 first synthesized the AgNPs, then modified the AgNPs with Colocasia esculenta, and finally detected the melamine in biological samples.
Noticeably, the whole protocol was still time-consuming and required complex procedures and toxic reagents, which limited the applicability of the approaches. Therefore, it is necessary to establish a more facile and rapid sensing assay to achieve simple sensing of melamine in the raw milk sample.

Gallic acid possesses hydroxyl groups in the molecular structure and can reduce metal ions to metal nanoparticles. Herein, we employed a colorimetric sensor for highly selective detection of melamine. In this research, gallic acid acted as a reducing and functionalizing agent for AgNPs. More importantly, the synthesis and functionalization of AgNPs and the analysis of melamine could be completed in a simple process. In this study, the as-prepared colorimetric sensing assay provided a simple and convenient method for the detection of melamine.

2. RESULTS AND DISCUSSION

2.1. Mechanism of the Colorimetric Sensor. It is reported that gallic acid can reduce Ag⁺ to AgNPs without the addition of any other reducing materials. In this paper, the role of gallic acid during the formation of AgNPs was investigated by mixing gallic acid and AgNO₃ in alkaline solution (Scheme 1).

The prepared AgNPs reduced by gallic acid exhibited a vivid yellow color (left, inset of Figure 1) and showed a sharp absorption peak characteristic of AgNPs at 410 nm (Figure 1). The results indicated the formation of monodispersed AgNPs. The absorbance decreased dramatically (Figure 1), and the color of the mixture turned brown (right, inset of Figure 1) with the addition of melamine. We speculate that this phenomenon occurred because the AgNPs aggregated in the presence of melamine (Scheme 1). To further verify the speculation, the morphology of AgNPs@gallic acid in the absence and presence of melamine was investigated by transmission electron microscopy (TEM) and high-resolution TEM (HRTEM). As shown in Figure 2a,c, the lattice spacing of 0.26 nm (Figure 2b,d) corresponded to the (004) plane of Ag nanocrystals. Also, the ringlike lattice structure in the selected area electron diffraction (SAED) pattern of the nanoparticles (inset, Figure 2a) suggested its crystal feature. What is more, the AgNPs were monodispersed with the absence of melamine (Figure 2a). The nanoparticles aggregated when melamine was added to the solution (Figure 2c). In addition, the field-emission scanning electron microscopy (FESEM) of the AgNPs is shown in Figure S1. Obviously, the monodispersed AgNPs aggregated in the presence of melamine. All the abovementioned results were in good agreement with our speculation that the color of the solution changed from vivid yellow to brown due to the aggregation of AgNPs because of melamine. Afterward, Fourier transform infrared (FTIR) spectra were used to analyze the characteristic peaks of the AgNPs. As shown in Figure S2, the peaks around 2924 and 2854 cm⁻¹ were attributed to the stretch vibration of aliphatic C−H, whereas the peaks around 3280 and 1629 cm⁻¹ were ascribed to the stretch vibration of catechol−OH and aromatic rings, respectively. These peaks
were observed in both pure gallic acid and AgNPs@gallic acid, suggesting that the AgNPs were modified by gallic acid. These results proved that the AgNPs@gallic acid was successfully synthesized.

Herein, we also investigated the reason for the aggregation of the AgNPs. In the melamine sensing assay of AgNPs@gallic acid, gallic acid exhibited reducing ability due to the presence of −OH groups, which promoted its antioxidiant ability. Therefore, gallic acid functioned as reducing and stabilizing agents for the preparation of AgNPs (Figure S3), and the as-prepared AgNPs displayed an absorption peak at 410 nm in UV−vis absorption curve. However, two different kinds of interactions prevailed when melamine was present in the reaction medium. The first interaction was the hydrogen bonding interaction between melamine and gallic acid28 and the other interaction was the formation of the coordination bond between melamine and Ag+.28 The multiple −OH groups in gallic acid served as binding sites with melamine (Figure S4). Melamine could interact with gallic acid through hydrogen bonding, lowering the availability of reducing agents for reduction of Ag+ to Ag0. Simultaneously, nitrogen atoms of amines and triazine groups in melamine could interact with Ag+ via acceptor−donor interaction.29 Therefore, melamine acted as a barrier according to the above-discussed interactions. Two conditions with melamine existed in solution. The first condition was that the barrier was not strong enough to prevent the close proximity of reducing agents and Ag+ ions when the concentration of melamine was low. In this condition, the aggregated AgNPs were formed. The second condition was that gallic acid and Ag+ ions were sufficiently masked when the concentration of melamine was high,30 and the formation of AgNPs was interrupted.

2.2. Optimization of Experimental Conditions. Several factors, such as pH of the reaction medium, the concentration of AgNO3 and gallic acid and reaction time, might affect the sensitivity of the sensor assay. Therefore, these parameters were well optimized for the sensitive detection of melamine based on the characteristic peak at 410 nm.

2.2.1. Effect of pH. The pH could affect the interaction of gallic acid-functionalized AgNPs with the melamine,31 and therefore, the influence of the pH value on the oxidizing ability of Ag+ toward gallic acid and melamine was investigated. The UV−vis spectra were recorded, and the profile of the change in absorbance against the NaOH concentration is shown in Figure S5. Obviously, the profile absorbance change was achieved when NaOH concentration was 1300 nM, indicating the highest sensitivity for melamine detection. The binding reaction between gallic acid and melamine could be achieved at a fast reaction rate at higher pH, which was due to the formation of cyanuric acid, ammelide, or ammonium through the replacement of −NH2 in melamine with −OH groups. The results demonstrated that the as-prepared sensor assay could be utilized in this NaOH concentration, and 1300 nM was selected for the subsequent studies.

2.2.2. Effect of Concentration of Gallic Acid and AgNO3. In this proposed sensor, gallic acid not only acted as reducing and stabilizing agents but also acted as the binding site for melamine. Therefore, the concentration of gallic acid played a crucial role in the sensitivity and linear range of the sensor. As shown in Figure S6, the value of ΔA410 increased with the concentration of gallic acid from 4 to 8 μM and reached the maximum value at 8 μM, indicating the best sensitivity toward melamine. Therefore, the concentration of gallic acid at 8 μM was selected as the optimum concentration in the subsequent experiments.

Due to the interactions between melamine and Ag+, the effect of AgNO3 concentration on the sensing ability was also optimized. AgNO3 with different concentrations (60, 70, 80, 90, 100, 110, 120, 130, 140, and 150 μM) was tested, while the concentration of gallic acid was fixed at 8 μM. According to Figure S7, a maximum value of ΔA410 was observed at an AgNO3 concentration of 90 μM. As a result, 90 μM was chosen as the optimum concentration of AgNO3.

2.2.3. Effect of the Reaction Time. The reaction time for the oxidation of gallic acid was also studied to investigate the response of the designed sensing assay. Absorbance data for A410 of AgNPs in the presence and absence of melamine were collected every 2 min within 27 min. As shown in Figure S8, the number of nanoparticles was low, which was due to the fact that melamine acts as a barrier when they are present in the solution. In addition, the absorbance data of the system reached a stable value at 10 min, demonstrating that the oxidation reaction was completed or reached equilibrium. Therefore, 10 min was chosen as the incubation time for the sensing assay in the following experiment. Overall, the visual detection process could be achieved quickly in the typical colorimetric sensor.

2.3. Sensitive Detection of Melamine under the Optimized Conditions. To evaluate the sensitivity of the melamine sensing assay, the change of the absorbance intensity at 410 nm under optimized conditions in response to the melamine concentration in the range from 0 to 10 μM was utilized to measure the quantitative determination using UV−vis absorption spectrophotometry. The absorbance intensity decreased (Figure 3) with the increase of melamine concentrations, while the color of the solution changed from yellow to brown (Figure S9). The spectral curves and corresponding calibration (inset of Figure 3) demonstrated that the good linearity was obtained between the melamine concentrations and ΔA410 in the two concentration ranges. The linear equation was \[ y = 0.0754 + 0.09552x - 0.00525x^2 \] with a correlation coefficient \( R^2 = 0.992 \). The limit of detection (LOD) was calculated to be 0.099 μM (0.012 ppm) according to the following equation \[ \text{LOD} = \frac{3.3 \sigma}{m} \]
where $S_d$ represents the standard deviation of the blank solution of the sensor and $s$ is the slope of the calibration curve.

The LOD of the sensing assay was much lower than the safety limit of melamine in EU (2.0 ppm) and China (1.0 ppm) and also compared to those of the colorimetric melamine sensors in the previous reported methods (Table S1). Overall, the proposed calorimetric method was not only fast but also simple when taking all procedures into consideration.

2.4. Selectivity Study of the Optimized Sensor. The interferences of the common analytes presented in the milk were needed to investigate to validate good applicability of the optimized calorimetric sensing assay. Hence, the effect of thymine, cytosine, inorganic biomolecules on the melamine detection was investigated. $\Delta A_{410}$ of the sensor assays was used to evaluate the selectivity of the proposed sensor assays. As shown in Figure 4, melamine led to significant enhancement and color darkening, which could be observed by the naked eye (inset in Figure 4c). Also, copper nitrate, copper chloride, and cytosine have a slight effect on the absorbance ratio. The results indicated that a remarkably high selectivity of the proposed sensor strategy toward melamine over other analytes in the pretreated milk. The excellent antiforeference of the sensor might be because of the fact that gallic acid could bind to $\text{Ag}^+$. Thus, the proposed sensor was appropriate for the selective colorimetric sensing assay of melamine in the pretreated milk.

2.5. Detection of Melamine in Real Samples. We measured the melamine concentration in raw milk with the proposed sensor to test the practical applications of the calorimetric sensor. The milk samples were spiked with three known concentrations of melamine (0.8, 1.0, and 2.0 ppm) to validate the developed strategy and analyzed by the method given in Section 2.3, and all the measurements were repeated three times. The results of the determination were shown in Table 1. The satisfactory recoveries and relative standard deviation (RSD) values were about 93.6–101.1 and 2.65–4.07% on average, respectively. The good synchronity indicated that the sensing assay could be satisfied with the required percentage recoveries applied for the detection of melamine in raw samples.

Table 1. Recovery Test of Melamine in Raw Milk at Different Spiking Concentrations of Melamine

| sample number | added (μM) | found (μM) | recovery (%) | RSD (%) |
|---------------|-----------|------------|--------------|--------|
| 1             | 0.8       | 0.809      | 101.1        | 2.65   |
| 2             | 1.0       | 0.936      | 93.6         | 4.07   |
| 3             | 2.0       | 1.900      | 95.0         | 3.02   |
3. CONCLUSIONS

In summary, a new convenient and economical colorimetric melamine sensor based on gallic acid-stabilized AgNPs was successfully fabricated. Herein, the gallic acid acted as reducing and stabilizing agents for AgNPs. The well-dispersed AgNPs could be obtained after modification by gallic acid. The coordinate bonding between melamine and Ag\(^+\) caused the aggregation behavior of AgNPs. As a consequence, the color of the solution changed from vivid yellow to brown, which made the melamine determination convenient. More importantly, the detection limit of 0.099 \(\mu\)M (0.012 ppm). Simultaneously, the sensor displayed excellent selectivity in the process with a detection limit of 0.099 \(\mu\)M (0.012 ppm).

4. EXPERIMENTAL SECTION

4.1. Chemicals and Reagents. Silver nitrate (AgNO\(_3\)), gallic acid (C\(_{19}\)H\(_{19}\)N\(_7\)O\(_6\)), trichloroacetic acid (C\(_2\)H\(_3\)Cl\(_3\)O\(_2\)), sodium hydroxide (NaOH), and melamine (C\(_6\)H\(_6\)N\(_6\)) pure were purchased from Aladdin Corporation. All of these reagents were of analytical grade and directly used as received. Raw milk was purchased from the local supermarket. Ultrapure water from a Millipore system (18.2 M\(^\Omega\) cm) was employed throughout the whole experiment.

4.2. Instruments. UV–vis absorption spectra were recorded using a Cary 50 UV–vis spectrophotometer (Varian, Inc.) with a quartz cuvette (path length, 1 cm). The FTIR spectrum was measured on the GX FTIR system (Japan) in the range of 400–4000 cm\(^{-1}\). TEM was collected by the Tecnai G2 F20 (FEI) instrument operated at an accelerating voltage of 200 kV.

4.3. Colorimetric Detection of Melamine. First, 30 \(\mu\)L of gallic acid (8 \(\mu\)M) and 30 \(\mu\)L of NaOH (1.3 mM) were added in 3 mL of water in a 5 mL centrifuge tube. Then, 30 \(\mu\)L of melamine solutions with different concentrations from 0 to 10.0 \(\mu\)M and 60 \(\mu\)L of AgNO\(_3\) (0.09 mM) were added into the tube in sequence and kept for 15 min. Finally, the solution was transferred into a quartz cuvette, and UV–vis absorption spectra were recorded to monitor the colorimetric changes. All the experiments were repeated three times.

4.4. Pretreatment of the Raw Milk Sample. Milk samples were pretreated to remove the interfering proteins in raw milk, which might interfere with the colorimetric sensor. Specifically, 10% of 2 \(\mu\)L of trichloroacetic acid was added into 5 \(\mu\)L of raw milk. Then, the solution was sonicated for 20 min and stirred for 10 min to ensure the complete precipitation of proteins in the samples. Subsequently, the mixture was centrifuged at 10,000 rpm for 15 min to separate the precipitate. The obtained supernatant portion was adjusted to pH 7.0 with 1 M NaOH. For recovery experiments, the corresponding amount of melamine was dissolved in the raw milk samples and the spiked-milk samples were performed with the same described procedure as the blank raw milk sample. Finally, the concentration of melamine in the samples was calculated using the linear regression equations, and the recoveries were calculated according to eq 2.

\[
\text{rate of recovery (\%) } = \frac{\text{amount of melamine found} \times 100}{\text{amount of melamine added}}
\]
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