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Selective extraction of melamine using 11-mercaptoundecanoic acid–capped gold nanoparticles followed by capillary electrophoresis

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ABSTRACT

This study describes the use of 11-mercaptoundecanoic acid–capped gold nanoparticles (MUA–AuNPs) for selective extraction of melamine prior to analysis by capillary electrophoresis with UV detection. The highest degree of melamine-induced aggregation of MUA–AuNPs was found to occur at pH 5.0, indicating that the NP aggregation is mainly because of hydrogen bonding between the carboxylate groups of MUA and the amine groups of melamine. Moreover, the degree of melamine-induced NP aggregation gradually increased when the chain length of the mercaptoalkanoic acid was increased from two to 12 carbon atoms. At pH 5.0, the extraction efficiency of melamine was highly dependent on the concentration of MUA–AuNPs, the concentration of dithiothreitol (DTT), the extraction time between MUA–AuNPs and melamine, and the incubation time between melamine-adsorbed AuNPs and DTT. The separation of the extracted melamine and DTT (releasing agent) was accomplished using a solution of 10 mM phosphate (pH 6.0) containing 1.6% (v/v) poly(diallyldimethylammonium chloride). Under the optimum extraction and separation conditions, the limit of detection at a signal-to-noise ratio of 3 was estimated to be 77 pM for melamine, with linear range of 1–1000 nM. The proposed method was successfully applied to the determination of melamine in tap water and in milk.

1. Introduction

Melamine is a nitrogen-rich (66% by mass) compound, used as a flame retardant, and a monomer for the production of plastic resins and fertilizer [1]. Owing to its high nitrogen content, melamine has been added to milk products to boost its apparent protein content. Melamine can bind to it analogues, such as cyanuric acid, and thereby form insoluble melamine cyanurate crystals in kidneys [2,3]. Thus, ingestion of melamine at levels above the safety limit (2.5 ppm in the USA and EU; 1.0 ppm for infant formula milk powder in China) may result in renal failure and even death in infants [4,5]. Low concentrations of melamine can migrate into food and beverages from plastic food packaging materials and dairy product containers [6]. Considering its potential toxicity, a rapid, convenient, and sensitive method is required for routine analysis of melamine in food and infant formula.

Current methods for melamine assay include gas chromatography with mass spectrometry and high performance liquid chromatography (HPLC) with ultraviolet absorption/mass spectrometry [7–9]. Without sample pretreatment, direct analysis of melamine has been successfully achieved using various methods, such as ultrasound-assisted extractive electrospray ionization MS [10], low-temperature plasma probe combined with tandem MS [11], matrix-assisted laser desorption/ionization MS [12], and surface enhanced Raman scattering [13]. Although these methods all provide high sensitivity and selectivity toward melamine, they require expensive and sophisticated instrumentation. Capillary electrophoresis (CE) coupled with UV absorption is a promising alternative for the analysis of melamine, because it offers low sample consumption, excellent separation efficiency, low cost, near universal detection, and simple set-up. However, this method suffers from poor sensitivity, due to short optical length and small sample volume. To overcome these problems, some researchers utilized liquid–liquid extraction and solid-phase extraction techniques for purifying and concentrating melamine from food samples prior to CE–UV analysis [14–16]. Additionally, the combination of an on-line concentration approach and CE–UV was employed to determine melamine in food products [17–19].

Recently, gold nanoparticles (AuNPs) have been shown to be capable of extracting a variety of biomolecules from a complex matrix because they exhibit high absorption capacity (high surface area-to-volume ratios) and provide a surface for facile functionalization. Citrate–capped AuNPs were extensively used for selective enrichment of proteins [20], indoleamines [21], and polycyclic aromatic hydrocarbons [22]. Researchers successfully concentrated

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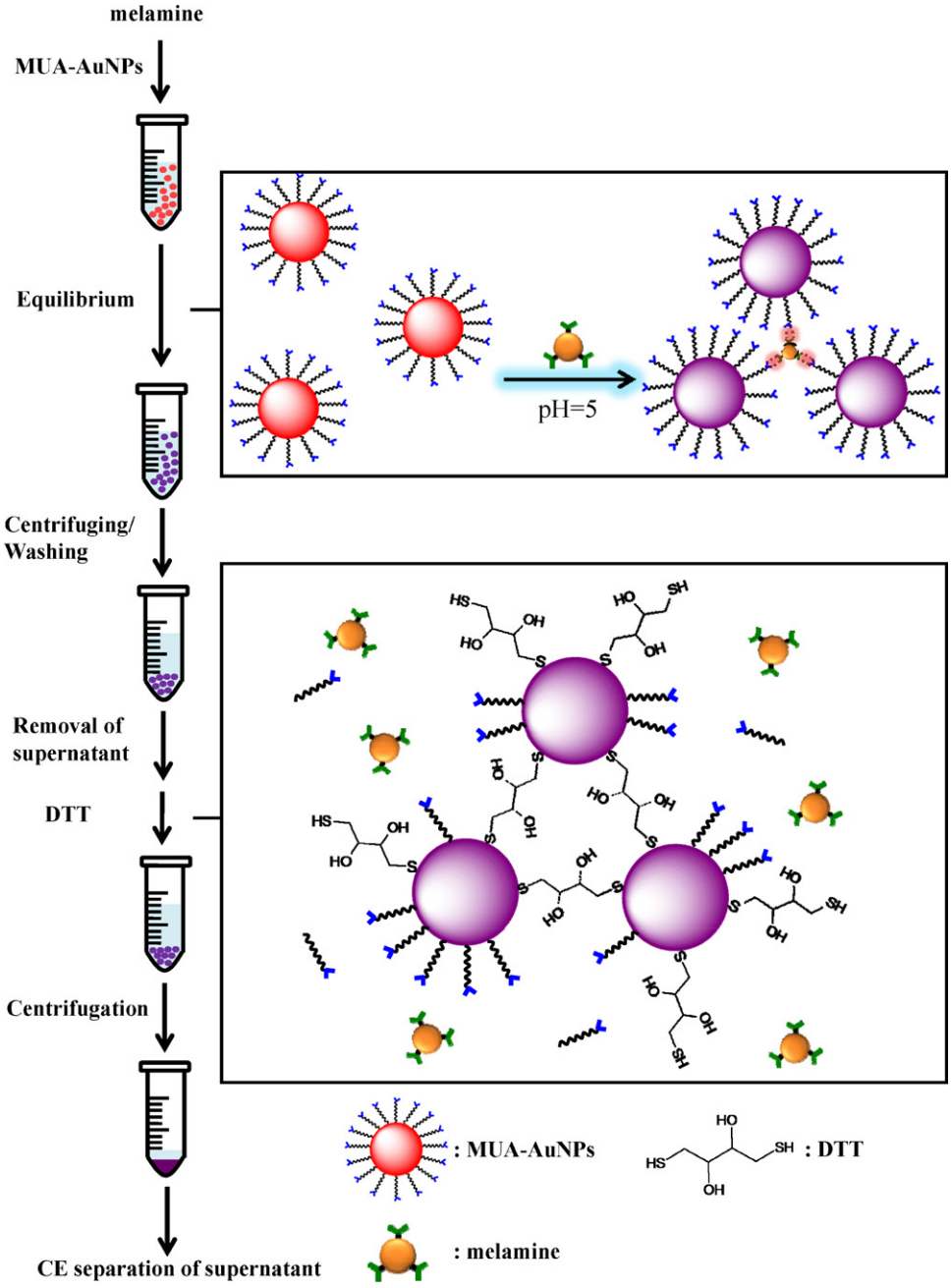
aminothiols from urine and serum using Tween 20-modified AuNPs [23,24]. It is well-known that a stable complex of melamine and cyanuric acid is formed through hydrogen-bonding interaction [25]. Thus, when ligands coordinated to AuNPs form hydrogen-bonding interactions with melamine [26], we deduce that this interaction makes AuNPs suitable for selective enrichment of melamine.

This study found that melamine forms a stable complex with 11-mercaptoundecanoic acid–modified AuNPs (MUA–AuNPs) through interaction between the carboxylate group of MUA and the amine groups of melamine. Thus, MUA–AuNPs act as adsorbents for selective enrichment of melamine (Scheme 1). The extraction of melamine is divided into four steps: (a) capture of melamine with MUA–AuNP; (b) centrifuging and washing of melamine-adsorbed AuNPs; (c) liberation of melamine from the Au surface through dithiothreitol (DTT); and (d) separation of liberated melamine by CE–UV. To demonstrate its practicality, MUA–AuNP was further applied to the extraction of melamine from tap water and from milk powder.

2. Experimental

2.1. Chemicals and preparation

Melamine, ammeline, ammelide, cyanuric acid, thioglycollic acid, 3-mercaptopropionic acid, 6-mercaptohexanoic acid, 8-mercaptooctanoic acid, MUA, 12-mercaptododecanoic acid, trisodium citrate, DTT, poly(diallyldimethylammonium chloride) (PDDAC; 20 wt% in water, MW 400,000–500,000), H_3PO_4, NaH_2PO_4, Na_2HPO_4 and Na_3PO_4 were purchased from Sigma–Aldrich (St. Louis, MO, USA). Hydrogen tetrachloroaurate(III) dehydrate was purchased from Alfa Asear (Ward Hill, MA).



Scheme 1. Step using MUA–AuNPs as the affinity probes to trap melamine from an aqueous solution, followed by CE–UV analysis.

2.2. Synthesis of the AuNPs

Citrate–capped AuNPs were prepared by the chemical reduction of metal salt precursor (hydrogen tetrachloroaurate, $HAuCl_4$) in a liquid phase. A solution of $HAuCl_4$ (200 mL, 1 mM) was brought to a vigorous boil with stirring in a round bottom flask fitted with a reflux condenser. We rapidly added trisodium citrate (20 mL, 38.8 mM) to the heated solution and heated the resulting solution under reflux for another 15 min. The size of the AuNPs estimated by TEM images was 13 ± 1 nm. The surface plasmon resonance (SPR) peak of the AuNPs was 520 nm. Using Beer's law, the concentration of the AuNPs was estimated to be 14 nM; the extinction coefficient of 13 nm AuNPs at the optical wavelength of 520 nm is approximately $2.78 \times 10^8\ M^{-1}\ cm^{-1}$. MUA–AuNPs were synthesized by adding MUA (10 mM, 200 μL) to a solution of citrate–capped AuNPs (200 mL, 14 nM). The resulting solution was equilibrated at ambient temperature overnight. To investigate the effect of surface ligands on the melamine-induced NP aggregation, we replaced MUA with thioglycollic acid, 3-mercaptopropionic acid, 6-mercaptohexanoic acid, 8-mercaptooctanoic acid, or 12-mercaptododecanoic acid, once at a time.

2.3. Characterization of the AuNPs

The extinction spectra of the AuNPs were recorded using a double-beam UV–vis spectrophotometer (JASCO V-530, Tokyo, Japan). A H7100 transmission electron microscopy (TEM) (Hitachi High-Technologies Corp., Tokyo, Japan) operating at 75 keV was used to collect TEM images of the AuNPs. The photo images of dispersed and aggregated AuNPs were recorded using a Coolpix 5400 digital color camera (Nikon, Tokyo, Japan).

2.4. Capillary electrophoresis

The fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) were 65 cm long and had an inner diameter of 75 μm (effective length: 45 cm). Electrophoresis was driven by a high-voltage power supply (Bertan, Hicksville, NY, USA). A commercial UV absorbance detector (Spectra System UV3000 HR, Thermo Separation Products, USA) was used to detect melamine as the detection wavelength was set at 200 nm. Data acquisition (10 Hz) and control were performed by DataApex Software (DataApex, Prague, Czech Republic). New capillaries were washed with 0.5 M NaOH by syringe pumping (KD scientific, New Hope, PA) at a flow rate of 1.0 μL/min. After 30 min, the capillary treated with a solution of 10 mM phosphate (pH 6.0–8.0) containing 1.6% (v/v) PDDAC overnight, resulting reversed EOF. Prior to conducting separations, the capillary was filled with 1.6% (v/v) PDDAC by syringe pumping at a flow rate of 1.0 μL/min. After 30 min, 1–1000 nM melamine was injected by hydrodynamic injection at 20-cm height for 15–240 s. All separations were performed at −140 V/cm.

2.5. Extraction procedure

MUA–AuNPs were prepared in 20 mM phosphate solutions at pH 2.0–12.0. Different concentrations of melamine (500 μL, 0.1–2 μM) were added to a solution of MUA–AuNPs (500 μL, 280–2800 pM). We incubated the resulting solutions for 0–15 min before measuring their extinction spectra. To investigate the selectivity of MUA–AuNPs toward melamine, we replaced melamine with ammeline, ammelide, or cyanuric acid, once at a time.

Prior to extraction, melamine was prepared in a solution of 20 mM phosphate at pH 5.0. After that, we added MUA–AuNPs (175 μL, 14–420 nM) to a solution of melamine (1.0 mL, 1–1000 nM). The resulting solutions were incubated at ambient temperature for 40 min. The precipitates were collected by centrifuging AuNP solutions at 17,000 rpm for 15 min and then washing with deionized water. After two centrifugation/washing cycles, the supernatant was removed, up to a residual volume of 3 μL. The collected particles were resuspended in 2 μL of a freshly prepared solution of 1 M DTT to liberate melamine. After 20 min, the released melamine was isolated from the MUA–AuNPs by centrifugation at 17,000 rpm for 15 min. The supernatant was placed in another 200 μL tube and analyzed by PDDA-filled CE.

2.6. Analysis of melamine in tap water and milk

Tap water was collected from the National Sun Yat-sen University campus. We prepared a series of samples by spiking them with standard melamine in the range of 5–5000 nM. These spiked samples (500 μL) were added to a solution of MUA–AuNPs (175 μL, 140 nM). We equilibrated the resulting solution at ambient temperature for 20 min. These spiked samples (1 mL) were extracted with 175 μL of 140 nM MUA–AuNPs. Note that MUA–AuNPs were prepared in a solution of 20 mM phosphate at pH 5.0. The following steps, including incubation, centrifuging, washing, and liberation were the same as used in extraction procedure.

Milk samples were bought in a local supermarket. 250 mg of milk powder was dissolved in 250 mL of deionized water. Milk samples (500 μL, 1 mg/mL) were spiked with different concentrations of standard melamine (5 μL, 0.06–6 μg/mL). To remove milk proteins, the resulting mixture was filtered with the 3 kDa Nanosep centrifugal device (Pall Co., East Hills, NY) at 7000 rpm with 25 min. The obtained solution (200 μL) was diluted with 800 μL of 20 mM phosphate solution (pH 5.0). After that, we added 175 μL of 140 nM MUA–AuNPs to these solutions and equilibrated for 40 min. The following steps, including centrifuging, washing, and liberation were the same as used in extraction procedure.

3. Results and discussion

3.1. Melamine-induced aggregation of the AuNPs

MUA–AuNP was prepared through ligand exchange between citrate and MUA. We assumed that MUA is a strong interaction between the carboxylate groups of MUA and the amine groups of melamine (Scheme 1). As shown in Fig. 1A, adding 1.0 μM melamine to a solution of MUA–AuNPs resulted in a decreased SPR peak at 520 nm and an increased SPR peak at 660 nm. Moreover, the coordination of melamine changed MUA–AuNP to a violet color (inset in Fig. 1A). These changes are characteristic of AuNP aggregation. The aggregation of MUA–AuNP was confirmed by TEM images, revealing individual AuNP particles in the absence of melamine and aggregated AuNPs in the presence of melamine (Fig. 1B).

To further demonstrate this aggregation process, we investigated the effect of pH on the response of MUA–AuNPs to melamine. The SPR extinction values of a MUA–AuNP solution at 520 nm and 660 nm equate to the quantities of dispersed and aggregated AuNPs, respectively. Thus, the ratio of aggregated to dispersed AuNPs corresponds to the ratio of the extinction value *Ex* at 660 nm to that at 520 nm ($Ex_{660\,nm}/Ex_{520\,nm}$). Above pH 4.0, a small value of $Ex_{660\,nm}/Ex_{520\,nm}$ reflects that MUA–AuNPs were dispersed in the absence of melamine, mainly because of strong electrostatic repulsion between negatively charged carboxylate groups of MUA (Fig. 2A). However, below pH 4.0, repulsive forces between MUA–AuNPs reduced with decreasing the pH of the solution. As a result, the aggregation of MUA–AuNPs occurred through interparticle hydrogen bonding between carboxylic acid groups of MUA [27]. The degree of melamine-induced aggregation of MUA–AuNP aggregation was observed to be highest at pH 5.0. Note that the first protonation of melamine ($pK_a = 5.1$) occurs on one of the three equivalent nitrogen's on the melamine triazine ring (N1, N3, or N5),

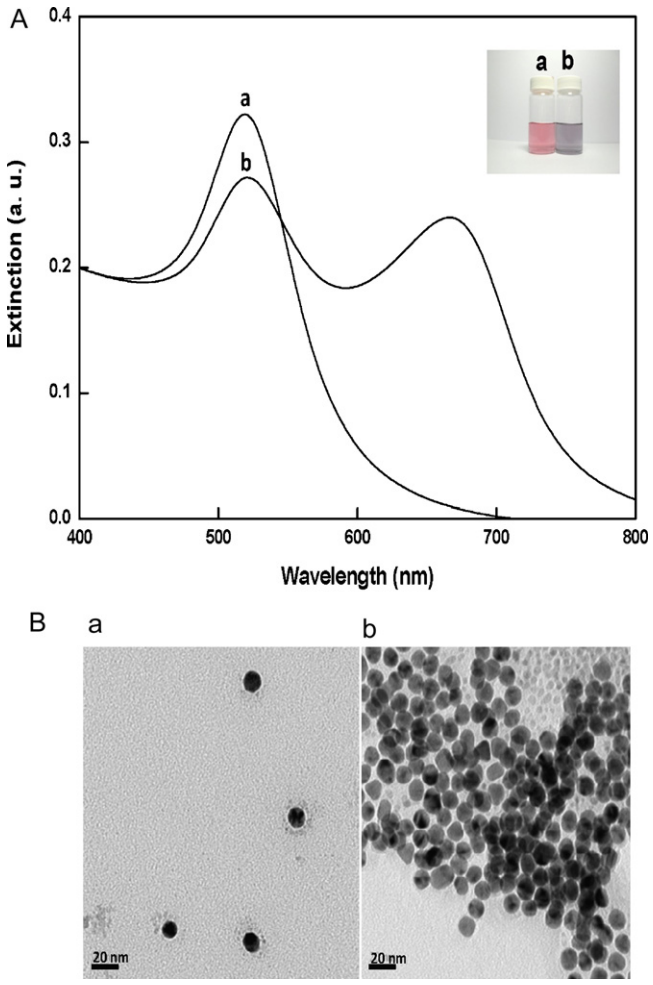


Fig. 1. (A) Extinction spectra and photo images and (B) TEM images of solutions of 1.4 nM MUA–AuNPs (a) before and (b) after the addition of 1 μM melamine. MUA–AuNPs were incubated with melamine for 5 min when they were prepared in 20 mM phosphate solution at pH 5.0.

rather than on one of the three equivalent amine groups (N2, N4, or N6) [28]. Thus, the amine groups of melamine could interact with the negatively charged carboxylate group of MUA ($pK_a = 4.8$) through hydrogen bonding, thereby resulting in the aggregation of the AuNPs. Moreover, the melamine-induced aggregation of MUA–AuNPs gradually increased as the solution pH decreased from 12.0 to 5.0. This phenomenon is probably due to the relatively weak electrostatic repulsion between each AuNP at low pH. Another reason may be that alkaline hydrolysis of melamine produces ammeline, ammelide, and cyanuric acid [29–31]. We explored the impact of chain length of the mercaptoalkanoic acid on the degree of melamine-induced aggregation of MUA–AuNP. Upon increasing alkyl chain length from two to twelve carbon atoms, the degree of melamine-induced NP aggregation gradually increased and reached a plateau at eleven carbon atoms (Fig. 2B). This finding discloses that surface ligands with longer alkyl chain lengths can reduce steric hindrance between each AuNP, thereby providing a greater opportunity to access melamine. Note that interparticle distances increase with increasing alkyl chain length.

To determine MUA–AuNP selectivity toward melamine, triazine analogs—including ammeline, ammelide, and cyanuric acid—were examined under identical conditions. Fig. 2C shows that triazine analogs exhibited the following trend in the degree of the aggregation of MUA–AuNPs: melamine > ammeline > ammelide > cyanuric acid. Because the keto form of cyanuric is more stable than its enol form [28], the weak interaction between cyanuric acid and melamine resulted in a low degree of cyanuric acid-induced aggregation of MUA–AuNPs. Similar reasoning explains the case of ammeline (two amines and one hydroxyl group) and ammelide (one amine and two hydroxyl groups). This result supports the notion that the aggregation of MUA–AuNP induced by melamine is due to the formation of hydrogen bonding.

3.2. Extraction of melamine with MUA–AuNPs

Because the degree of melamine-induced aggregation of MUA–AuNPs was the highest at pH 5.0, the extraction of melamine with MUA–AuNPs was performed at the same pH. The use of high concentration of cationic PDDAC, as a buffer additive in the CE, is an effective way to avoid the adsorption of cationic analyte on the capillary surface [23,24,32]. Thus, we analyzed positively charged melamine using CE–UV when the background electrolyte consisted of 1.6% PDDAC and 10 mM phosphate (pH 6.0). Fig. 3A displays the effect of the concentration of (14–420 nM; 14 nM = 1.6×10^{12} particles/mL) of 175 μL MUA–AuNPs on the extraction of melamine (1 mL, 1 μM). Excess amount of DTT (1 M) was added to the precipitates to liberate melamine from the Au surface. Note that the precipitates were obtained from the centrifugation of a solution containing melamine and MUA–AuNPs. The peak areas of melamine gradually increased with an increase in the concentration of MUA–AuNPs and reached a saturation level above 140 nM. We suggest that 175 μL of 140 nM MUA–AuNPs (1.6×10^{13} particles/mL) can completely extract 1 mL of 1 μM melamine. Thus, the loading of melamine on a single MUA–AuNP was estimated to be 40 molecules. The optimum extraction time between melamine and MUA–AuNPs was found to be 40 min when the concentration of MUA–AuNPs was fixed at 140 nM (Fig. 3B). On the other hand, we investigated the effect of DTT concentration on the release of melamine from the Au surface. Fig. 3C shows that the peaks area of melamine gradually increased with raising the concentration of DTT and reached a plateau at 800 mM DTT. This result clearly reflects that melamine release relies strongly on the DTT concentration. To ensure that melamine was completely released from Au surface, we set the concentration of DTT at 1 M. Upon the addition of 1 M DTT, the extracted melamine was completely liberated after 20 min (Fig. 3D).

Compared to the peak area of 100 μM of standard melamine (Fig. 4A), the extraction of melamine (1 μM, 1 mL) with MUA–AuNPs (175 μL, 140 nM) resulted in a 300-fold improvement in the sensitivity (Fig. 4B). The initial (before extraction) and final sample (after extraction) volumes were 1 mL and 5 μL, respectively. This result provides clear evidence that MUA–AuNPs are capable of extracting melamine from an aqueous solution. Under extraction and separation condition, Fig. 4C shows that the detection of 1 nM melamine was successfully achieved by the combination of NP-based extraction and CE–UV. The limit of detection (LOD) at a signal-to-noise (S/N) ratio of 3 for melamine is 77 pM; this value was estimated from Fig. 4C. The limit of quantification calculated by multiplying the LOD by a factor of 3 is 0.2 nM. The relative standard deviation of the migration time and peak area of the extracted melamine were 1.2% and 6.1%, respectively. A linear calibration curve was obtained by plotting the peak areas versus the concentrations of melamine over the range of 1–1000 nM (Fig. 4D). In contrast to other reported methods for determining melamine (Table 1), the combination of NP-based extraction and CE can provide better sensitivity toward melamine.

3.3. Analysis of melamine in tap water and milk powder

To demonstrate a real-world application of this method for a complex mixture, MUA–AuNPs were applied to the extraction of

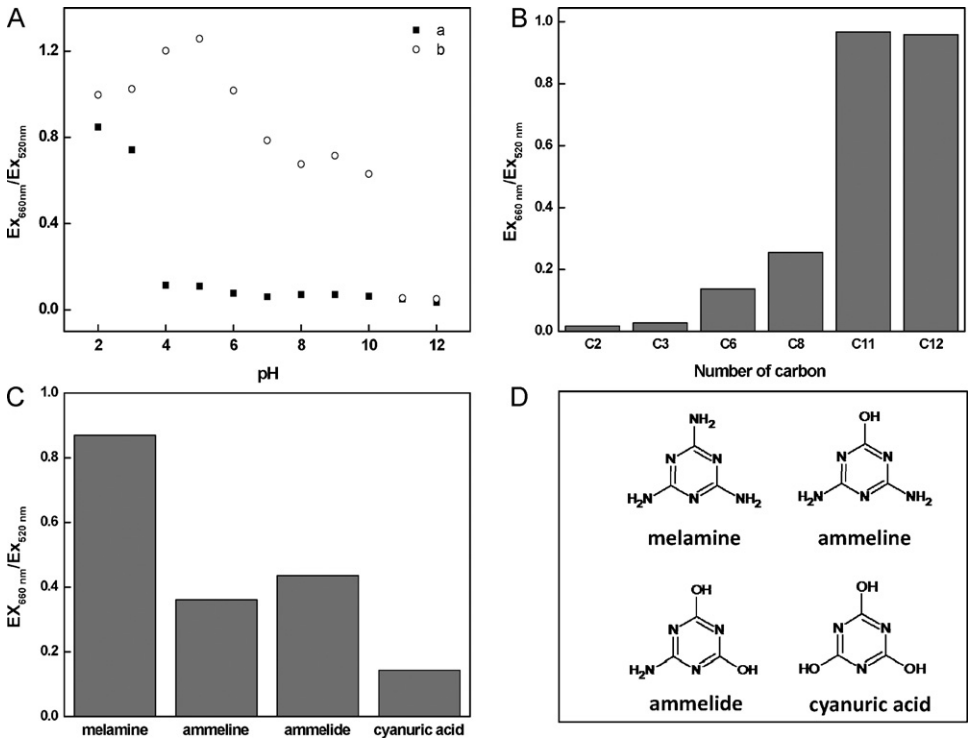


Fig. 2. (A) Effect of solution pH on the value of $Ex_{660\,nm}/Ex_{520\,nm}$ (a) before and (b) after the addition of 1 μM melamine to MUA–AuNPs. (B) Effect of surface ligands on the value of $Ex_{660\,nm}/Ex_{520\,nm}$ after the addition of 1 μM melamine to MUA–AuNPs. (C) Selectivity of MUA–AuNPs. The concentration of each analyte is 1 μM. (D) Chemical structures of melamine, ammeline, ammelide, and cyanuric acid. (A–C) MUA–AuNPs (1.4 nM) were incubated with melamine for 5 min when they were prepared in 20 mM phosphate solution at pH (A) 2.0–12.0 and (B and C) 5.0.

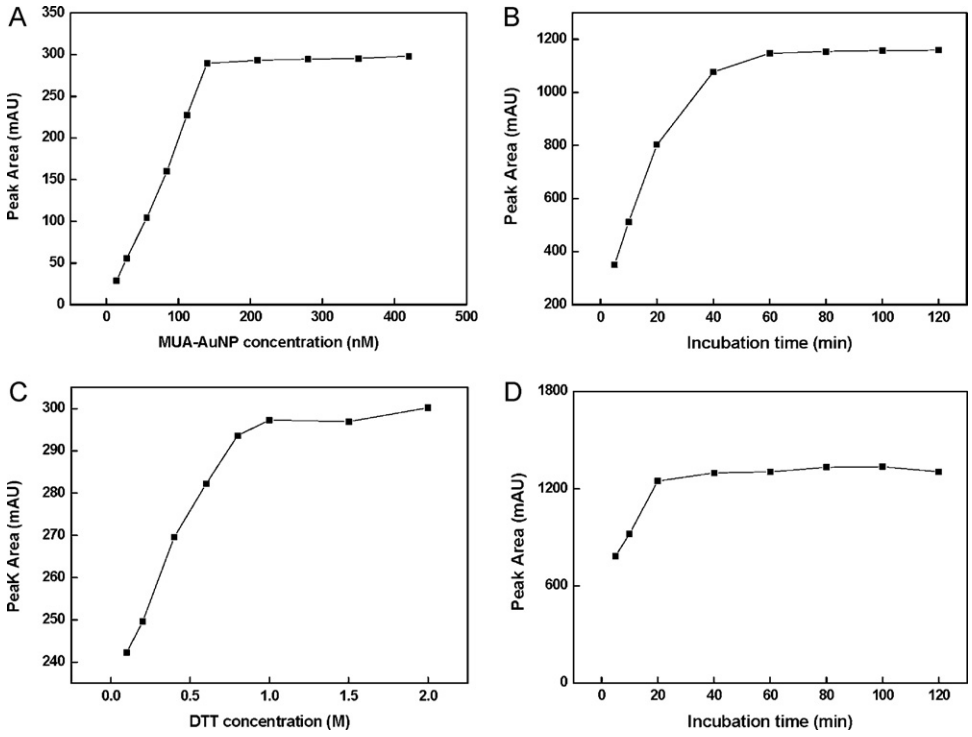


Fig. 3. Effects of (A) the concentration of MUA–AuNPs, (B) the extraction time between MUA–AuNPs and melamine, (C) the concentration of DTT, and (D) the incubation time between DTT and melamine-adsorbed AuNPs on the extraction efficiency. A solution of 20 mM phosphate (pH 5.0) containing 1 μM melamine was extracted using (A) 14–420 nM and (B–D) 140 nM of 175 μL Tween 20-AuNPs. The extraction time between melamine and MUA–AuNPs was (A, C and D) 40 and (B) 5–120 min. Melamine adsorbed onto the surface of AuNPs was liberated upon the addition of 2 μL of (A, B and D) 1 M and (C) 0.1–2 M DTT. The incubation time between 1 M DTT and melamine-adsorbed AuNPs was (A, B and C) 20 and (D) 5–120 min. A 5 μL of samples are hydrodynamically injected by raising the capillary inlet 20-cm height for 210 s. A capillary with effective length of 45 cm (20 cm to detector) is filled with 1.6% (v/v) PDDAC solution, which is prepared in 10 mM phosphate solution at pH 6.0. The applied voltage is −9 kV while the electric current is 40 μA. The detection wavelength is set at 200 nm.

Table 1
Determination of melamine by the combination of CE–UV with concentration method.

Concentration method	Linear range	LOD (S/N = 3)	Real sample	Reference
No	0.7–50 mg/L	0.2 mg/L	Feed, egg, and milk	[14]
No	0.05–100 mg/L	47 μg/L	Milk	[15]
Solid-phase extraction	0.5–100 mg/L	0.1 mg/L	Milk, egg, hog tissue, fish tissue, chicken tissue, wheat protein	[16]
Sweeping	0.1–10 mg/L	9.2 μg/L	Infant formula	[17]
Field amplified sample stacking	Not given	0.5 μg/L	No	[17]
Transient isotachophoretic stacking	12.6–1260 μg/L	6.3 μg/L	Milk and pet feed	[18]
Reversed electrode polarity stacking	0.05–5 mg/L	2.8 μg/L	Tableware and flour products	[19]
AuNP-based extraction	0.126–126 μg/L	9.7 ng/L	Tap water and milk	This study

melamine from tap water and from milk prior to CE. If the 70-kg adult consumes 2 L of drinking water daily, the permissible maximum level of melamine in drinking water is 0.9 mg/L [33]. Fig. 5A shows that tap water samples spiked with standard melamine (0, 1, and 10 nM) were analyzed by the combination of AuNP extraction and CE separation. The peak area of melamine increased upon increasing the spiked concentration of melamine in the tap water. A good linear relationship (R^2 = 0.9981) between peak area and melamine concentration was observed in the range of 0–100 nM. The recoveries of these measurements were valued at 97–101%. Fig. 5B shows analysis results for milk samples (1 mg/mL) spiked with standard melamine (0, 0.6, and 6 ng/mL). Prior to extraction, protein was removed from milk samples by centrifugal ultrafiltration (molecular weight cutoff of 3 kDa). Comparison of peak areas in the electropherograms of the milk samples with (electropherograms b and d in Fig. 5B) and without (electropherogram a in Fig. 5B) standard melamine identified the peak at 17.1 min corresponding to melamine. By applying a standard addition method, the concentration of melamine in milk (1 mg/mL) was estimated to be

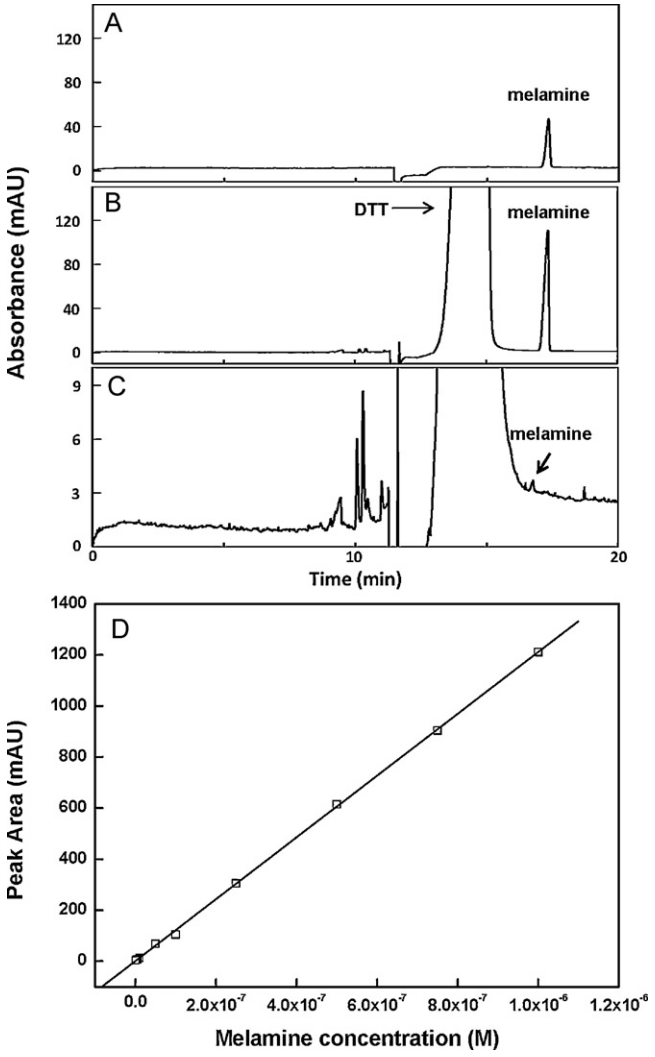


Fig. 4. Analysis of (A and B) 1 μM and (C) 1 nM melamine by CE-UV (A) before and (B and C) after extraction with MUA–AuNPs. (D) A calibration curve for quantitative analysis of melamine by the combination of NP-based extraction and CE–UV. A solution of 20 mM phosphate (pH 5.0) containing melamine (1–1000 nM) was extracted with 175 μL of 140 nM MUA–AuNPs. The extraction time between melamine and MUA–AuNPs was fixed at 40 min. The incubation time between 1 M DTT and melamine-adsorbed AuNPs was 20 min. The CE conditions are the same as those in Fig. 3.

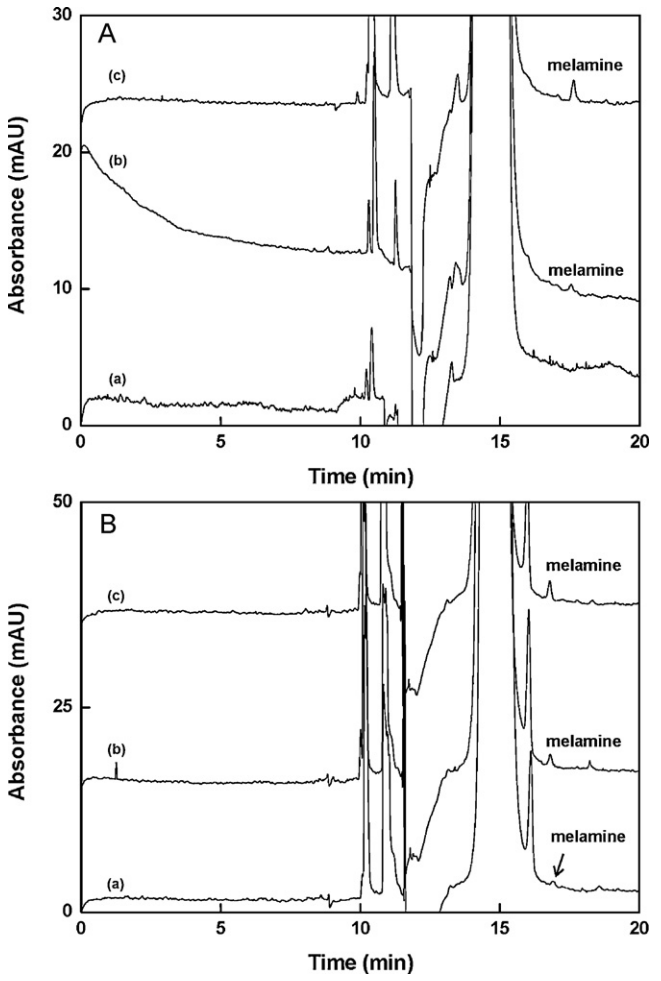


Fig. 5. Detection of melamine in tap water and milk by the combination of NP-based extraction and CE–UV. (A) Tap water samples were spiked with (a) 0, (b) 1, and (c) 10 nM melamine. (B) Milk samples were spiked with (a) 0, (b) 0.6, and (c) 6 ng/mL melamine. These melamine-spiked milk samples were filtered with the 3 kDa Nanosep centrifugal device. The obtained solution (200 μL) was diluted with 800 μL of 20 mM phosphate solution (pH 5.0). The extraction and CE conditions are the same as those in Fig. 4.

0.27 ± 0.03 ng/mL (2.1 ± 0.2 nM). The recoveries of these measurements ranged from 95% to 99%.

4. Conclusion

This study demonstrated that MUA–AuNP can be utilized for the extraction of melamine from a complex matrix based on the interaction between the carboxylate group of MUA and the amine groups of melamine. The highest degree of melamine-induced aggregation of MUA–AuNPs was observed while MUA–AuNPs were prepared at pH 5.0. Under optimum extraction and CE conditions, the linear range and LOD for melamine were 1–1000 nM and 77 pM, respectively. The distinct advantages of our proposed method for the analysis of melamine are its simplicity, high selectivity, and high sensitivity. This was evident when this method was applied to the analysis of tap water and milk containing low concentrations of melamine. In our opinion, one possible extension of this method would be to extract melamine from human urine and animal feeds prior to CE analysis.

Acknowledgment

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