ORIGINAL PAPER

Rapid detection of melamine with 4-mercaptopyridine-modified gold nanoparticles by surface-enhanced Raman scattering

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Abstract A surface-enhanced Raman scattering (SERS) strategy based on 4-mercaptopyridine (MPY)-modified gold nanoparticles (AuNPs) was developed for the rapid and sensitive detection of melamine in milk powder. The SERS measurement of melamine strongly relied on the "hotspot" effect, in which AuNPs immediately aggregated upon the addition of melamine, leading to significantly enhanced Raman intensity of the reporter molecule MPY and a color change for the solution from red to blue-gray. The limit of detection based on a signal to noise of 3 (S/N=3) was found to be as low as 0.1 ppb of melamine, with an excellent linearity of 0.5–100 ppb, demonstrating a higher sensitivity and a wider quantitation range than direct SERS sensing methods based on enhanced substrate. An impressive specificity for melamine detection over various common metal ions and excipients in dairy products, even at concentrations of 100-fold higher than melamine, was achieved. Good recoveries of 88.5% and 111.7% were obtained from milk samples spiked to 20 and 100 ppb levels, respectively. The proposed method is potentially applicable for the rapid in situ determination of melamine in complex matrices.

Keywords Melamine · Surface-enhanced Raman scattering · Gold nanoparticle · 4-Mercaptopyridine

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Introduction

Melamine (1,3,5-triazine-2,4,6-triamine) is an important organic chemical material for producing melamine resins [1], which are usually used in glues, adhesives, and plastics. However, it was recently added to milk and various dairy products illegally due to its low cost and high nitrogen content (66% by mass), which can lead to incorrectly high readings for the apparent protein content when conventional standard tests are performed [2, 3]. It has been reported that these high levels of melamine in milk killed thousands of pets [4] and caused renal function failure and even death in infants [5].

This emergency highlighted an urgent need for rapid and reliable methods of detecting melamine in food. Currently, various methods—including thin-layer chromatography [6], gas chromatography (GC) [7, 8], high-performance liquid chromatography [9–12], enzyme-linked immunosorbent assay technology [13], matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) [14], and surface desorption atmospheric pressure chemical ionization mass spectrometry [15]—have been used to detect melamine in food. However, these methods usually suffer from drawbacks, such as the need for a complicated sample preparation process, clean-up steps, and a time-consuming determination process, so they cannot fulfill the requirement for fast in situ

detection. Therefore, the development of simple, quick, cost-effective and sensitive methods for the detection of melamine is of particular importance.

Surface-enhanced Raman scattering (SERS) has attracted lots of attention due to its high sensitivity and speed of detection. Since the discovery of the SERS effect in the 1970s [16–18], SERS has been applied to a wide variety of biomedical and environmental analytical applications at the level of molecules, pathogens, cells, and even whole living animals [19–22]. The enhancement associated with SERS— up to 10^{14} times [23]—is widely attributed to two primary mechanisms: the short-range chemical effect and the long-range classical electromagnetic effect. Recently, several groups have reported the determination of melamine using SERS by detecting the signal from melamine directly with roughened gold substrates [24], silver colloid substrate [25] or other SERS substrates [26–28]. However, the signal from melamine itself is relatively weak, and the detection limits of these direct sensing methods are unsatisfactory (most are more than 10 ppb). Here, a novel indirect method of melamine detection using 4-mercaptopyridine (MPY) as the Raman reporter is described. The detection is based on the enhanced SERS signal from MPY due to the melamine-induced aggregation of MPY-labeled gold nanoparticles.

Experimental

Materials and apparatus

Melamine, 4-mercaptopyridine (MPY) and fluorescein isothiocyanate (FITC) were obtained from Sigma-Aldrich. Chloroauric acid tetrahydrate ($HAuCl_4 \cdot 4H_2O$), trisodium citrate, rhodamine B and acetonitrile were purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). All aqueous solutions were prepared with deionized water (18.2 MΩ).

SERS spectra were recorded using a Thermo Scientific RFS 100 Raman system equipped with a microscope and a 632.8 nm diode-pumped He:Ne laser source. UV-Vis spectra were measured with a UV-Vis spectrophotometer (Beckman Coulter Inc., USA).

MPY-modified gold nanoaggregates

SERS probes for melamine detection were synthesized by attaching appropriate reporter molecules to AuNPs. The AuNPs were prepared by reducing the $HAuCl_4$ with trisodium citrate [29]. Typically, 10 mL of trisodium citrate (38.8 mM) were rapidly injected into a boiling solution of $HAuCl_4$ (100 mL, 1 mM) while stirring, and the solution was further refluxed for another 30 min into a wine-red suspension. The suspension was gradually cooled to room temperature under stirring. The size of the AuNPs was about 13 nm and the concentration was about 13 nM, as determined by UV-Vis spectrometry. Different amounts of three tested Raman reporters (MPY, FITC and rhodamine B) were spiked with the AuNP suspension as prepared. After gentle shaking for several minutes, the reporter molecules were linked onto the surfaces of the AuNPs via Au–S or Au–N bonds, thereby generating SERS probes.

Milk powder sample treatment

Melamine-spiked milk powder was used as an experimental sample; this was created by mixing the milk powder bought from a local supermarket and melamine. Briefly, 1 g of milk powder was spiked with 0.1 g of melamine, and then 10 mL of 50% acetonitrile aqueous solution was added. After 15 min of sonication and 10 min of shaking, the mixture was centrifuged at 10,000 rpm for 10 min. The supernatant was filtered with a 0.22 μm filter film and diluted to give a total volume of 10 mL with 50% acetonitrile aqueous solution. The control sample was the same milk powder but with no added melamine.

SERS measurement

The mixed solution of melamine and 50% acetonitrile was spiked into the MPY-modified AuNP suspension to a volume ratio of 1:1. After gentle shaking, the mixed solution was placed in a capillary tube for detection. During the SERS measurements, a diode laser was directed and focused onto the sample with a microscope stage through a 10× objective. Raman scattering signals were detected by a CCD array detector. The measurement was conducted with an exposure time of 4 s and a laser power of 8 mW.
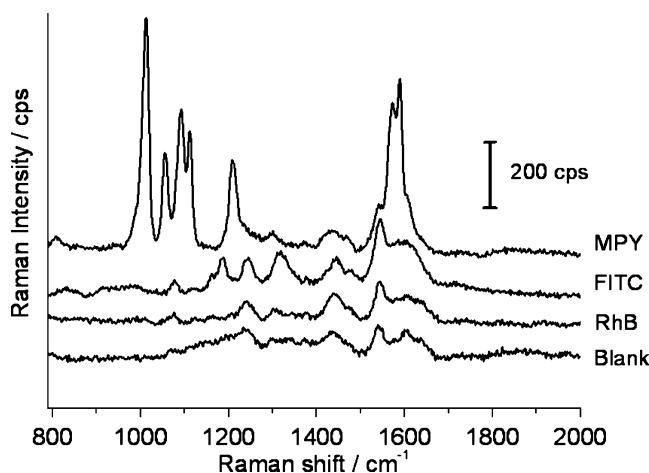

Fig. 1 Raman spectra of blank AuNPs as well as MPY-, RhB-, and FITC-modified AuNP colloids mixed with melamine. Concentration of melamine: 0.5 ppm; concentration of MPY/RhB/FITC: 5×10^{-6} mol/L
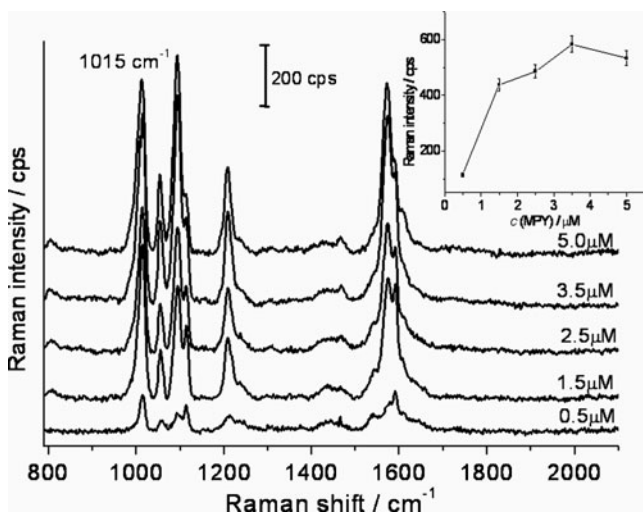
Fig. 2 Raman spectra of SERS probes modified with different concentrations of MPY (from 5×10^{-7} to 5×10^{-6} mol/L) in a solution of melamine (concentration of melamine: 1 ppm)

Results and discussion

Selection of the Raman reporter

The Raman reporter plays an important role in SERS-based molecule detection. Different reporters exhibit different SERS spectral profiles and signal-enhancing abilities. In order to acquire satisfactory detection sensitivity for melamine, three Raman reporters (MPY, FITC and rhodamine B) were used to produce SERS probes, while blank AuNPs were also tested for the control experiment. Figure 1 shows the Raman spectra of the SERS probe labeled with different Raman reporters in 0.5 ppm melamine solution. It can be seen that MPY is an ideal candidate because the spectrum is easily discernable and exhibits strong Raman-enhancing signals. By contrast, the signals of the other two reporters are much weaker. Thus, MPY was chosen as the Raman reporter in this study. Interestingly, the indirect SERS sensing method using MPY as the Raman reporter is actually potentially more sensitive than the direct sensing method; although melamine could also induce the aggregation

Fig. 3 a Schematic diagram of the indirect SERS method of measuring melamine using MPY-modified AuNPs. **b** UV-Vis spectra of MPY-modified AuNPs with different concentrations of melamine in 50% acetonitrile
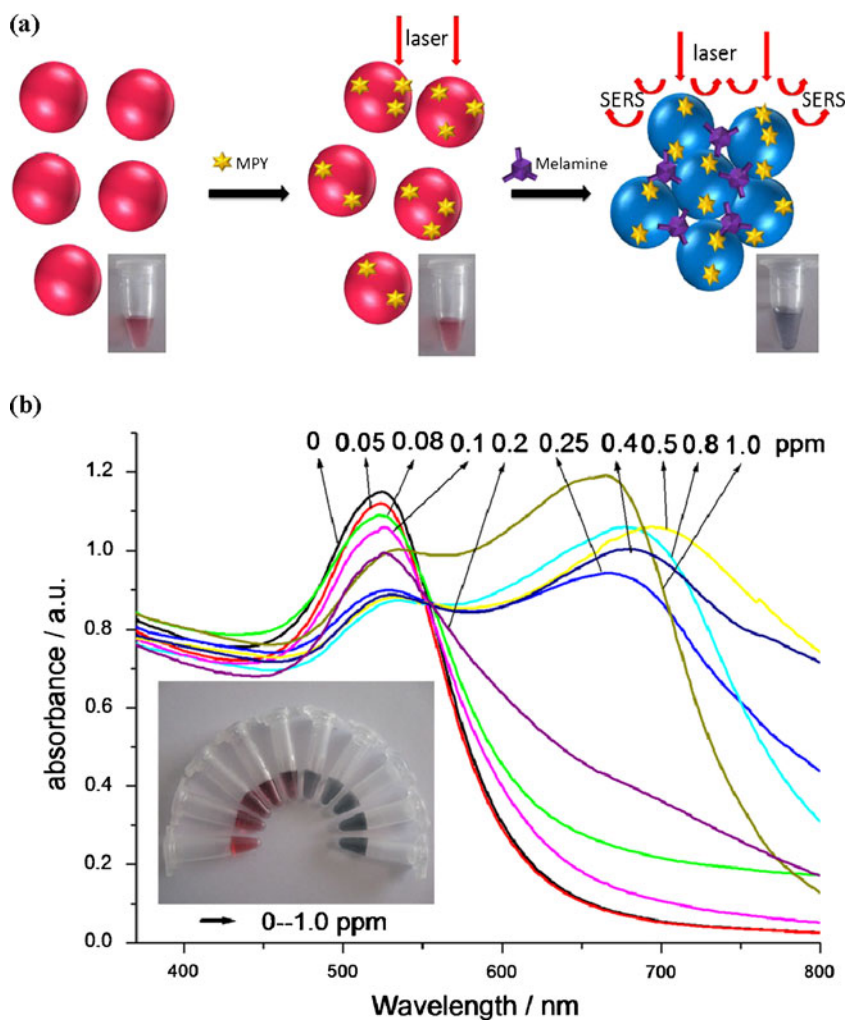
of bare AuNPs, an apparent SERS signal from melamine was not observed.

Concentration of the Raman reporter

The effect of the concentration of the reporter on SERS signal intensity was investigated. Five different concentrations of MPY ranging from 5.0×10^{-7} to 5.0×10^{-6} mol/L were tested, and the corresponding Raman spectra are shown in Fig. 2. It was found that a concentration of 3.5×10^{-6} mol/L induced the largest signal. When the concentration of MPY was increased further, there was no obvious enhancement of the signal. Moreover, a high concentration of MPY would also induce AuNP aggregation. Thus, a concentration of 3.5×10^{-6} mol/L of MPY was used throughout the rest of the study.

Mechanism of determination

Figure 3a shows a schematic diagram the mechanism of melamine determination. During the process of SERS probe synthesis, the tested Raman reporters were able to link to the AuNPs through S–Au or N–Au bonds and replace the citrate stabilizer. Upon adjusting the amount of reporters added, the color of the colloid remained wine red, indicating a monodispersion of the reporter-labeled AuNPs. After the addition of melamine, primary amines with electron-rich nitrogen atoms were more likely to be bound to the surfaces of the AuNPs through coordinating interactions that neutralized the citrate-stabilized AuNPs (which are rich in negative charge). This decreased the stability of the citrate-stabilized AuNPs, thus leading to a dramatic aggregation of AuNPs and visible color changes [30]. Figure 3b shows the UV-Vis spectra of different concentrations of melamine in 50% acetonitrile mixed with an AuNP colloid solution in the volume ratio of 1:1. The results revealed that the size of the gold–melamine particle aggregation depended on melamine concentration. The color of the colloid shifted from red to blue when the melamine concentration was increased from 0.12 to 0.49 ppm, which agrees with the results reported by Wei et al. [31]. Meanwhile, the aggregated AuNPs produced a number of hotspots, and therefore the Raman scattering signal from the MPY reporters adsorbed on the AuNPs was greatly increased. This approach therefore has good potential for use as a rapid and sensitive assay of melamine in milk products.

Detection of melamine in milk powder

The prepared MPY-based SERS probes were then applied to the detection of melamine in milk powder. Different concentrations of melamine were spiked into solutions of
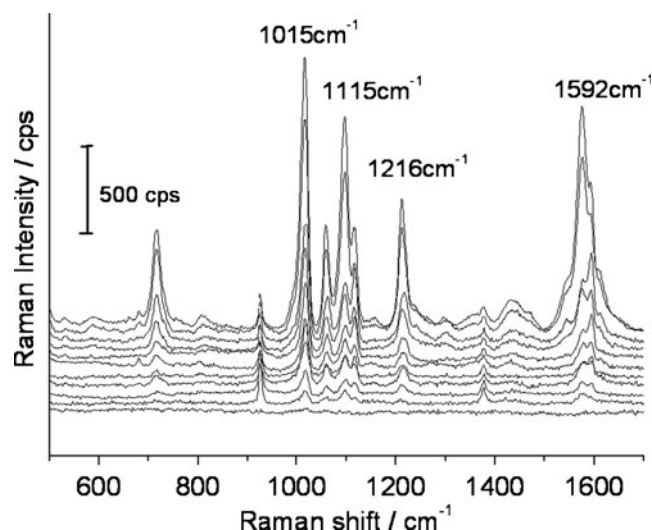

Fig. 4 Raman spectra of MPY-labeled SERS probes with different concentrations of melamine in 50% acetonitrile solution (from 0 to 1 ppm melamine)

milk powder that were filtered by a 0.22 μm film and extracted by 50% acetonitrile aqueous solution. The extract solutions were then analyzed by Raman spectroscopy. As shown in Fig. 4, there are many spectral features that are characteristic of MPY that can be used for the quantitative determination of melamine, such as those at 1592 cm^{-1}, 1216 cm^{-1}, 1115 cm^{-1}, 1060 cm^{-1}, 1015 cm^{-1}, and 714 cm^{-1}. The strongly enhanced band at 1115 cm^{-1} corresponding to the ring-breathing/C–S stretching mode indicates that MPY is adsorbed onto the surfaces of the AuNPs through the sulfur atom [32–34]. This is also supported by the C–S stretching mode at 714 cm^{-1}, which displays an increase in intensity. The band at 1592 cm^{-1} corresponds to the C–C stretching mode, and C–H mode bands at 1060 cm^{-1} and 1216 cm^{-1} are also observed. The
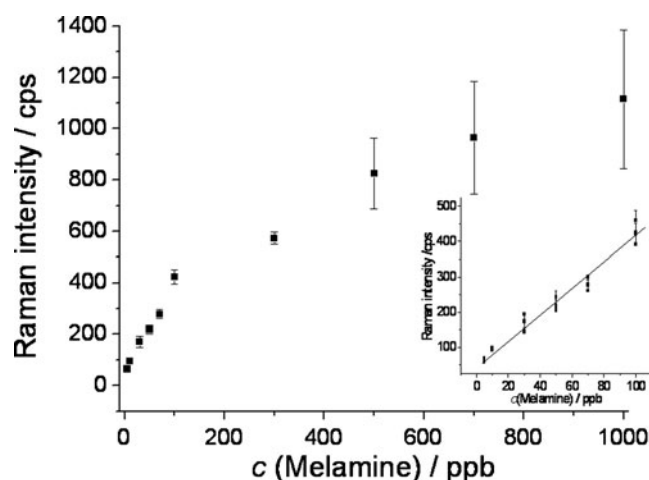

Fig. 5 Calibration curve for different concentrations of melamine in milk powder solution. *Inset*: calibration curve at very low melamine concentrations

Table 1 Comparison of methods based on the SERS detection of melamine

Methods	LOD	Linear range	Reference
SERS on silver colloid substrate	10 ppb	0.5–20 ppb	[24]
SERS using gold substrate	33 ppb	–	[23]
SERS using a roughened silver electrode	12.6 ppb	–	[26]
SERS on gold nanosubstrate	1.1 ppm	2.5–100 ppm	[35]
SERS on roughened gold substrate	200 ppb	–	[36]
SERS on silver nanorod (AgNR) array substrate	0.1 ppm	0.1–10 ppm	[37]
Indirect method based on aggregation of AuNPs	0.1 ppb	0.5 ppb to 0.1 ppm	This work

band at 1015 cm^{-1} is related to ring-breathing vibrations. According to the spectra, the peak around 1015 cm^{-1} was the most prominent one, and its intensity was very sensitive to the concentration of melamine. Therefore, it was selected as an instructive peak for the quantitative analysis of melamine.

A calibration curve based on the concentration of melamine and the Raman intensity of the peak at 1015 cm^{-1} is shown in Fig. 5. At low concentrations (from 0.5 ppb to 0.1 ppm), the curve shows excellent linearity, with a correlation coefficient (R) of 0.9926 (inset of Fig. 5). With the further addition of melamine, the rate of increase in Raman intensity slowed down, and the curve began to level off at around 0.5 ppm. This suggested that, at these high concentrations of melamine, most of the AuNPs have already aggregated, so the number of "hotspots" cannot increase linearly like it does at low concentrations of melamine. The SERS assay was capable of rapidly detecting melamine concentrations as low as 0.5 ppb in the milk powder solution extracted by 50% acetonitrile solution, and a limit of detection (LOD) of 0.1 ppb was obtained based on a signal to noise of 3. The indirect SERS method based on the aggregation of AuNPs displayed a higher sensitivity and a wider quantitation range for
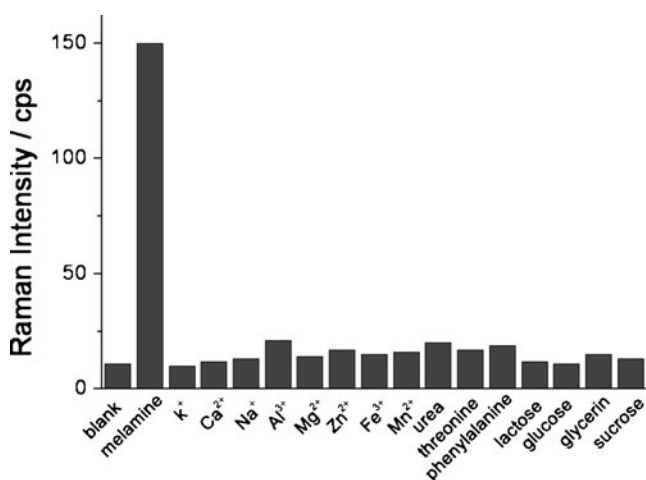
melamine measurement than previous SERS methods using gold/silver substrates, although all of the other methods were direct detection methods, as shown in Table 1. The method's precision and accuracy were determined by analyzing milk powder samples spiked with 50 ppb melamine. The relative standard deviation (RSD)—which was found to be 4.7% ($n=6$)—was relatively small, indicating that the method is quite reproducible.

To study the selectivity of this SERS method for melamine, some commonly found metal ions and excipients in dairy products were chosen for use in an interference investigation, including K^+, Ca^{2+}, Na^+, Al^{3+}, Mg^{2+}, Zn^{2+}, Fe^{3+} and Mn^{2+}, as well as urea, threonine, phenylalanine, lactose, glucose, glycerin and sucrose. Figure 6 shows the SERS response to 0.5 μM (63 ppb) melamine and 50 μM of the individual metal ions or excipients mentioned above. Only melamine was found to greatly increase the Raman intensity; the others had negligible effects (even at a 100-fold higher concentrations than melamine). This indicated that only melamine could induce the aggregation of AuNPs. Therefore, a highly selective SERS strategy was attained with an impressive specificity for melamine.

To demonstrate the feasibility of the method for real sample analysis, different concentrations of melamine were added to the milk sample using the standard addition method. The recoveries of melamine were 88.5%, 119.2% and 111.7% for the 20, 50 and 100 ppb spiked sample, respectively (Table 2). Various factors can lead to the low or high recoveries, such as the matrix effect, method errors and operational errors, but the recoveries were still acceptable. These results hold great promise for the rapid in situ monitoring of melamine in dairy products.

Fig. 6 Selectivity of the MPY-modified AuNP probe toward 0.5 μM (63 ppb) melamine versus other tested metal ions and additives (0.5 mM each)

Table 2 Recoveries of the developed SERS method based MPY-modified AuNPs for the determination of melamine in milk samples

Samples	Added (ppb)	Found (ppb)	Recovery (%)
1	20.0	17.7±0.36	88.5
2	50.0	59.6±2.89	119.2
3	100.0	111.7±5.81	111.7

Conclusions

In summary, a novel fast SERS strategy for measuring melamine contamination in milk powder was successfully developed. This strategy uses the enhanced optical properties of AuNPs modified with the Raman reporter MPY, and the specific inductive effects of melamine. The assay proved simple, cost-effective, sensitive and selective for the in situ, rapid detection of melamine in food. With the appropriate choice of Raman labels and SERS substrate, melamine-detection SERS platforms could be developed for the routine monitoring of on-site food quality control and market surveillance in order to assure food supply safety.

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