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Silver deposited polystyrene (PS) microspheres for surface-enhanced Raman spectroscopic-encoding and rapid label-free detection of melamine in milk powder

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ABSTRACT

Silver nanoparticles coated amino modified polystyrene microspheres (PS-NH_2/Ag NPs) with extremely high surface enhanced Raman scattering (SERS) activity and uniform surface morphology were created by precise controlling of deposition time. Nanojets that were formed underneath the individual microspheres could be used for nondestructive analysis of species adsorbed on the smooth gold or glass surface. A 10 fold enhancement of SERS was observed between PS-NH_2/Ag NPs and gold surface compared to glass surface due to more effective coupling of surface plasmonic resonance. These microsphere SERS substrates could detect 2-Mercaptopyridine down to 10^{-9}M. Four different thiol compounds have been successfully utilized as tags to prepare SERS encoded PS-NH_2/Ag NPs microspheres. Furthermore, the potential application of these SERS substrate for rapid detection of melamine in milk powder was explored. A linear relationship was observed between SERS intensity and logarithm of melamine concentrations with the limit of detection (LOD) of 1.9×10^{-8} mol/L. The promising advantages of easy sample pretreatment, low protein background interference, short detection time and low cost makes the PS-NH_2/Ag NPs substrate a potential detection tool in the field of food safety.

1. Introduction

Noble metal surface with ideal roughness can dramatically enhance the Raman scattering of analytes adsorbed on the surface [1–3]. Some novel substrate with enhancement factor of more than 10^{10} at so called "hot spot" allows the detection of analytes even at single-molecule level [4–7]. Due to its high sensitivity and finger-printing capability, Surface-enhanced Raman scattering (SERS) spectroscopy opens some new opportunities for biological sensing and trace analysis.

Over the past decade, many attempts have been made to produce favorable nanostructure substrates with high SERS activity, among which metal nanoparticle coated microspheres are popular [8–10]. These nanoparticle coated microspheres have uniform micrometer size while containing well defined nano structures on the surface. Polymeric microspheres provide excellent supports for producing the nanostructural SERS active surfaces. Nanosphere lithography is one of the most powerful techniques that use spin-coated microspheres layers as template for silver or gold deposition to produce substrate with high SERS activity, however the thermal evaporation needs special equipment [11,12]. Another widely used method to create microspheres are the in-situ (nanoparticles grown inside polymer microsphere) [13,14] or ex-situ (nanoparticles assembled or formed on the microsphere surface) [15,16] methods in order to prepare metal nanoparticles coated mono-dispersed microspheres. These metal coated microspheres are more favorable to homogenous SERS analysis in aqueous solution and the synthetic process does not require expensive instruments unlike the lithography. Thus, the synthesis and application of metal nanoparticles coated microsphere has drawn much attention in recent years.

Some researchers have reported the possibility of in-situ reducing gold or silver nanoparticles inside different types of microspheres, such as PEG grafted polystyrene(PS) [17], thermosensitive Poly(NIPAM-co-MAA) microsphere [18], poly(styrene-co-acrylic acid) microsphere [19] and silica nanosphere [20]. Nevertheless, the synthetic steps are usually time-consuming and low metal coverage on the microspheres frequently occurs. For ex-situ techniques, however, the distribution of size, and uniformity of the

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metal nanoparticle coated microspheres are easier to control than that of the in-situ techniques. Piao reported SERS-active PS microspheres with finely tuned Au network structure using stepwise electroless growth of Au NPs layers [21]. Silver coated microspheres also attracted much interest due to the stronger enhancement of Raman scattering comparing to gold. Tollen's test was the first attempt to create silver islands on dielectric beads [22]. Silver-coated silica microparticles with less than 25% metal coverage are transparent which can [23–26] be used as optically trappable Raman probes for SERS spectroscopy and bioimaging. Silver nanoparticle-embedded suffocated PS beads with a silica shell are more stable and biocompatible [27]. The Ag-Coated Fe_3O_4@-SiO_2 three-ply composite microspheres prepared through the well-known Ag-mirror reaction posses both ferromagnetic and SERS properties [28]. Yet the inhomogeneous enhancements and distributions observed on these Ag-coated microspheres also could not be ignored. Another study using self-assembly of dye-functionalized Ag nanoparticle tag onto SiO_2 microbeads via biotin-avidin binding shows highly uniformed SERS responses but the synthesis of the tags before assembly is a bit complicated [29]. Therefore, simple protocols that can easily control the morphology and density of silver nanoparticles on the microspheres with highly SERS activity in a reproducible manner needs to be explored.

Functionalized microspheres such as those containing amino groups allow favorable deposition of silver on the surface and can be easily obtained commercially. [21]. In this work, a PS-NH_2/Ag NPs SERS substrate is prepared using the sodium citrate reducing method for the deposition of silver nanostructure coating directly on the commercially bought amino modified PS microspheres. The self-assembled monolayers of thiol compounds on the PS-NH_2/Ag NPs could be easily detected. The relationship between deposition time, silver surface morphology, and SERS enhancement are studied in detail. The potential use of PS-NH_2/Ag NPs in preparing SERS encoded PS microspheres and ultrasensitive detection in real complex sample as melamine in a complex sample such as milk powder was demonstrated.

2. Experimental section

2.1. Materials and reagents

The major chemicals used in this work including silver nitrate and trisodium citrate dehydrate were obtained from Sinopharm Chemical Reagents (Shanghai, China) and have a purity of 99.5%. Amino polystyrene microsphere (PS-NH_2; 5% w/v; 5.0–5.9 μm) was purchased from Baseline ChromTech Research Centre (Tianjin, China); 2-mercaptopyridine (99%), 4-nitrophenylthiol (98%), 4-methyl-benzenethiol (98%) and 2-Naphthalenethiol (98%) were purchased from J&K Chemical Ltd. (Beijing, China). Melamine (99.8%) was obtained from Bodi Chemicals Inc., Ltd. (Tianjin, China) and milk powder was purchased from A. Best supermarket (Changsha, China). The other chemicals were all of analytical grade and used as received. The ultrapure water (18.32 MΩ) used was purified by Nanopure Infinity Ultrapure system (Barnstead/ thermolyne Corp, China).

2.2. Instrumentation

A Confocal Raman System Laboram 010 (Jobin Yvon Inc. USA) based on a 50 × long working-distance objective (8 mm) was used for the Raman spectra collection. A 632.8 nm He–Ne laser excitation (0.1 mW) was used as the laser source with the slit and pinhole setting at 100 and 1000 μm, respectively. The laser beam exposure time for the acquisition of SERS spectra was 15 s with 3 accumulations, which considered the balance between the signal-to-noise ratio, measurement time, and small sample volume on the SERS substrate. Usually 5–10 spots on the substrate were measured.

2.3. Preparation of silver-coated amino polystyrene microspheres

The preparation of silver coated amino polystyrene microspheres was according to the reference [30] with minor modifications: A 0.26 mL of 0.589 M silver nitrate solution was added to 47.5 mL of deionized water and stirred vigorously on a magnetic stirrer-hot plate and heated to 90–100 °C. A 5.0 ml of 5.0% (w/v) Polystyrene microspheres were added to the heated solution, and stirred for another 5 min. The initiation of the reduction reaction was initiated by the addition of 0.5 mL of 1.36 M sodium citrate solution to the mixture. The temperature was kept at 90–100 °C for different time before cooling down in ice water and the silver nanoparticles coated amino modified polystyrene microspheres (PS-NH_2/Ag NPs) were thus prepared. Different reduction time points as 1 min, 2 min, 4 min, 6 min, 8 min and 15 min were studied to optimize the silver coverage and SERS activity.

2.4. Nanojets formed between PS-NH_2/Ag NPs and smooth substrate for nondestructive detection

In order to study the influence of substrate material toward nanojets formed underneath individual PS-NH_2/Ag NPs, 2-mercaptopyridine was allowed to assembly on smooth gold or glass surface as a SERS probe layer, then PS-NH_2/Ag NPs microspheres were dispensed on top of the probe layer. For the preparation of smooth Au/2-mercaptopyridine/(PS-NH_2/Ag NPs), a polycrystalline gold (99.9%) electrode with a 2 mm diameter was first mechanically polished with 0.3 μm and 0.05 μm Al_2O_3 powder to a mirror finish, followed by ultrasonic cleaning with ethanol and ultrapure water for 2–3 times. For the preparation of glass/2-mercaptopyridine/ (PS-NH_2/Ag NPs), glass slide was washed carefully and then ultrasonic cleaning with ethanol and ultrapure water for 2–3 times. Then 10 μL 2-mercaptopyridine of 10^{-3}M was dropped onto the fresh gold or glass surface to form a probe layer. After the 2-mercaptopyridine layer was dried, 30 μL of PS-NH_2/Ag NPs (reduction time: 6min) was dropped on top of the probe layer. SERS spectra at different positions were collected to compare the enhancement effect of the substrate materials.

2.5. Preparation of surface-enhanced Raman spectroscopy encoded microspheres

A 1000 μl of silver nanoparticles coated amino modified polystyrene microspheres were mixed separately with 100 μL 10^{-9}M~10^{-3}M 2-mercaptopyridine while stirring. After self-assembly at room temperature for 12h, thin monolayer of thiol compounds would be formed on the silver surface. The thiol compounds labeled silver coated microspheres were collected by centrifuge at 4000 r/min for 3 min and the excess unassembled thiol compounds in the supernatant were discarded. The thiol compounds labeled microspheres were then washed three times with ultrapure water to remove the unabsorbed thiol compounds before resuspended into ultrapure water. For encoding, different thiol compounds as 2-mercaptopyridine (2-MP), 4-nitrobenzenethiol (4-NT), 4-methylbenzenethiol (4-MT) and 2-naphthalenethiol (2-NT) of 10^{-3}M were used to label the PS-NH_2/Ag NPs using the same method. The SERS spectra obtained from each thiol compounds labeled microspheres were detected and used for the encoding of the silver coated microspheres.

2.6. Rapid detection of melamine in milk power using PS-NH_2/Ag NPs

The stock milk powder solution was prepared by dissolving 50 mg milk powder into 10 ml of ultrapure water. The milk powder did not contain melamine, so contaminated milk powder samples were prepared by adding some melamine solution (stock solution: 1 mmol/L) into the milk powder solution. For the melamine analysis, 100 μL of contaminated milk powder solution was mixed with 100 μL of PS-NH_2/Ag NPs and then 10 μL of the mixture solution was dropped onto clean gold electrode surface for SERS detection. The final concentration of the melamine from 10^{-8}M to 10^{-3}M was detected.

3. Results and discussion

As illustrated in Fig. 1, amino group modified polystyrene microspheres were used for the deposition of silver nanoparticle in order to prepare the SERS active substrate. Using sodium citrate reducing reaction, silver nanoparticles can be easily deposited onto the amino-modified polymeric surface. The silver coverage and SERS activity can be optimized by controlling the reduction time. The silver nanoparticle surface can adsorp various small organic molecules and can achieve ultrasensitive detection as well as SERS encoded microspheres for multi-analysis.

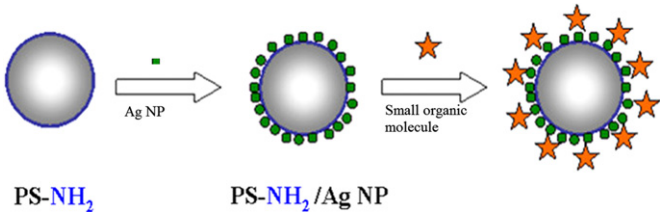

Fig. 1. Graphic illustration of the synthesis of PS-NH_2/Ag NPs SERS active substrate.

3.1. Surface morphology and SERS activity study of the PS-NH_2/Ag NPs

Fig. 2 shows the surface morphology of silver coated polystyrene microspheres prepared with different reduction times. From Fig. 2(a), one can see that very small silver nanoparticles begin to form on the microsphere surface within 1 min. However, the surface coverage of silver particles is very low and the solution displays a cream like color. As the deposition time increases, the color of the reaction solution changes from yellow-green (2 min) to light brown (4 min) and even darker after 6 min. Concurrently, the amount and the size of silver nanoparticles deposited on the microsphere surface increases gradually. By 4 min, a thin layer of silver nanoparticles covering the entire surface of the microsphere could be observed. When deposition time increases to 6 min or more, the size of the individual deposited silver nanoparticles grow larger and some silver nanoparticles aggregates into small islands.

The SERS activity of the synthesized silver nanoparticles coated amino polystyrene microspheres were measured using 2-mercaptopyridine as SERS probe. As shown in Fig. 3(A), an increasing SERS intensity of 2-mercaptopyridine is observed with increasing deposition time from 1 min to 4 min and achieved maximum at 6 min. The 1000 cm^{-1} band which belong to the ring-breathing mode of 2-mercaptopyridine is chosen to draw the SERS intensity-deposition time curve. As shown in Fig. 3(B), the SERS intensity drops after a deposition time of 6 min. It has been reported that silver nanostructures of 110–120 nm roughness presents the best enhancement effect [31]. Our result suggests that after 6 min of deposition, the microsphere surface achieve the optimal thickness of silver

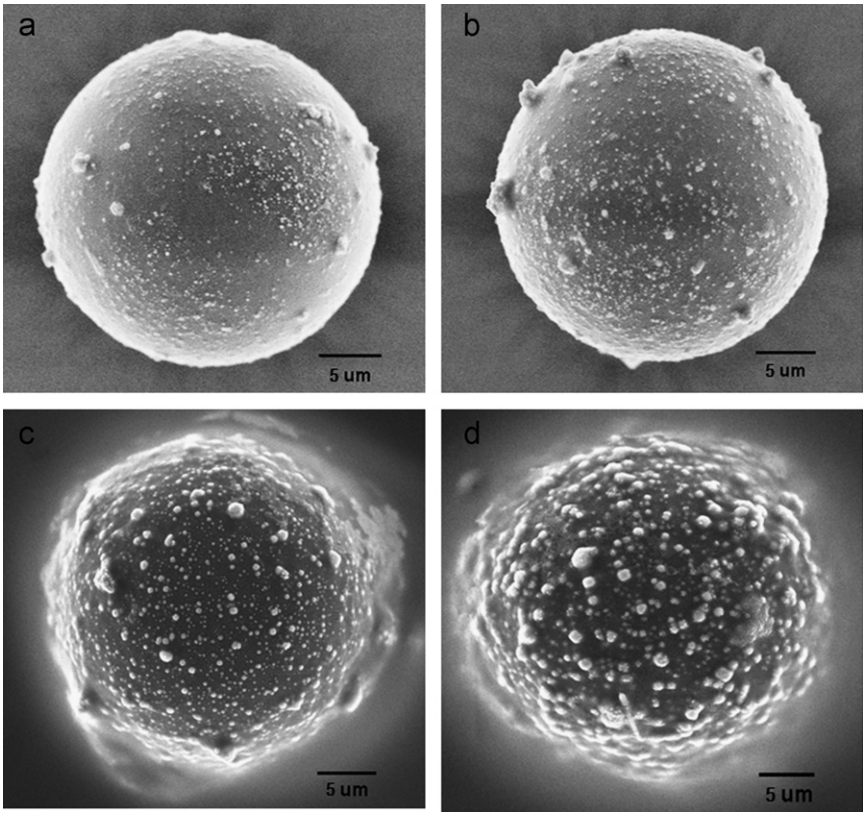

Fig. 2. PS-NH_2/Ag NPs with different silver-coating time: (a) 1 min, (b) 2 min, (c) 4 min, (d) 6 min.

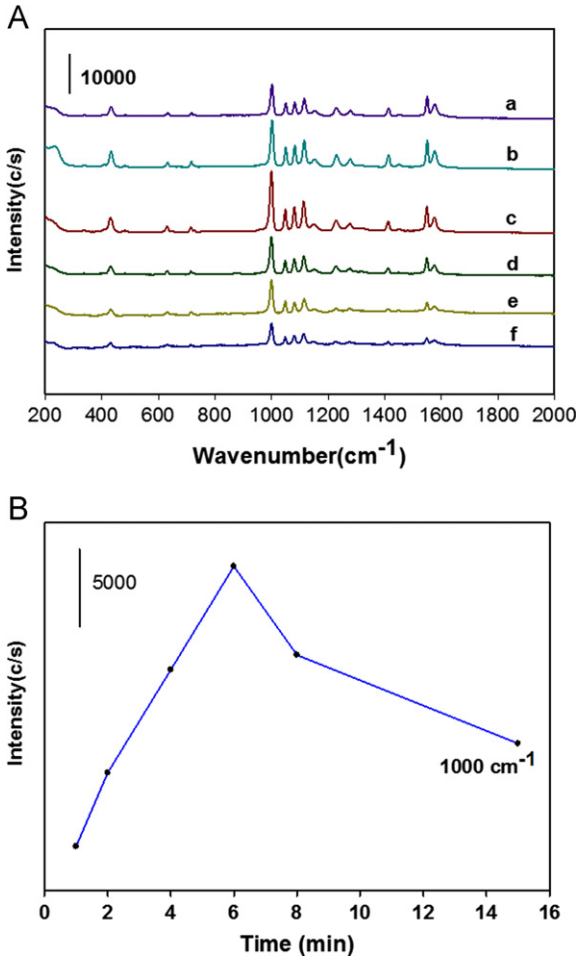

Fig. 3. (A) A representative set of SERS spectra of 2-mercaptopyridine on the PS-NH_2/Ag NPs substrate with different deposition time: (a) 15 min, (b) 8 min, (c) 6 min, (d) 4min, (e) 2 min, (f) 1 min. (B) The SERS intensity of 2-mercaptopyridine at 1000 cm^{-1} vs. deposition time.

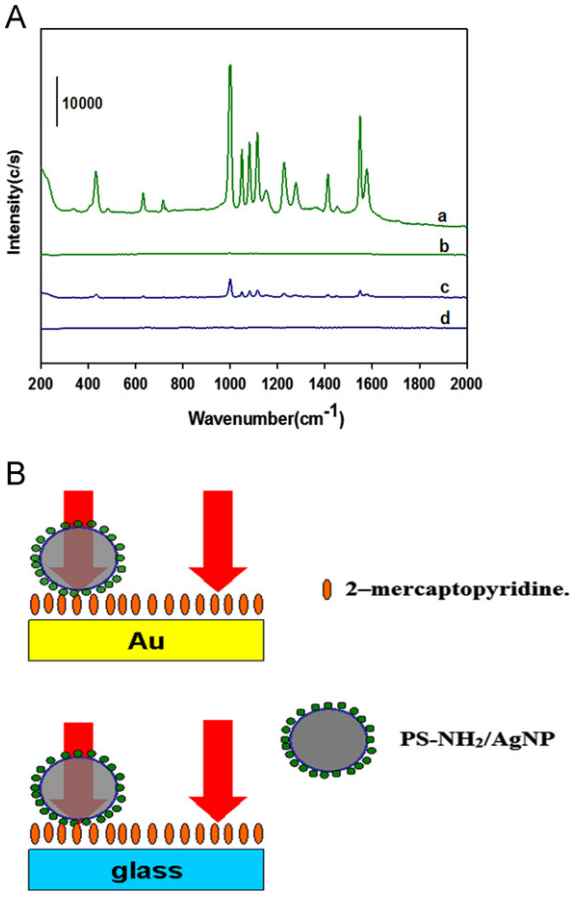

Fig. 4. (A) Raman spectra of 2-mercaptopyridine (1 mM) recorded: (a) between PS-NH_2/Ag NPs and gold substrate, (b) on gold substrate without PS-NH_2/Ag NPs microspheres, (c) between PS-NH_2/Ag NPs and glass substrate, (d) on the glass substrate without PS-NH_2/Ag NPs microspheres. (B) Graphic illustration of the assembly and detection mode.

nanoparticle coating which leads to the strongest SERS activity. The particle size is about 100 nm, which is in accordance with the reference. Certain surface coverage and particle size are important to produce plasmonic coupling effect between silver nanoparticles, and form the so called "hot spot". When the deposition time increases to more than 6 min, the silver nanoparticles aggregate into island which is unfavorable to the formation of "hot spot". Therefore, in the following study, the PS-NH_2/Ag NPs substrate used for SERS encoding and detection are prepared with a deposition time of 6min.

3.2. *Nanojets formed between PS-NH_2/Ag NPs and smooth gold or glass surface for nondestructive detection*

Some researchers found that when bare microspheres were put on smooth substrate material surface, there were some photonic nanojets (a narrow, high -intensity, non-evanescent light beam) produced underneath the microspheres [32–35]. The formation of nanojets will lead to highly localized electromagnetic field and significantly enhance the Raman intensity of molecules adsorbed on the smooth substrate [36–38]. They had shown that the waist of the nanojets was about 120 nm. The study did not use microspheres coated with silver nanoparticles, which may have produced even more favorable nanojets which could have significantly improved the Raman enhancement. Thus, in this study Raman enhancement between PS-NH_2/Ag NPs and gold or glass surface is studied. The 2-mercaptopyridine molecules are assembled onto the clean gold or glass surface followed by spreading PS-NH_2/Ag NPs on top, as illustrated in Fig. 4(B). Essentially no Raman enhancement is observed when the gold and the glass surface are layered with only 2-mercaptopyridine, as seen in Fig. 4(A). Once the PS-NH_2/Ag NPs microspheres were spread over the 2-mercaptopyridine layer, a large enhancement of Raman scattering was observed right below the microspheres; however, the area without microspheres show no SERS enhancement. This observation suggests that there are some nanojets formed underneath the PS-NH_2/Ag NPs microspheres, which enhance the localized electromagnetic field and lead to the significant enhancement of the SERS signals. By comparing the SERS spectra obtained on the gold and glass surface (a and c in Fig. 4A) it can observed that the SERS enhancement of the former is about 10 fold greater than the latter. This occurs due to the more effective coupling of surface plasmonic resonance between PS-NH_2/Ag NPs and the gold surface. According to the study, PS-NH_2/Ag NPs can also be used for nondestructive analysis of surface or interface. "Hot spot" can be formed between the PS-NH_2/Ag NPs microspheres and the surface which create SERS signals without destroying the surface.

3.3. *The SERS response of labeled molecules on the PS-NH_2/Ag NPs*

Raman tags prepared by attaching Raman active molecules on the surface of noble metal nanoparticles have been widely used in bioanalysis. In this study, thiol compounds can self-assemble onto the silver nanoparticle surface easily to form stable SERS labeled

PS-NH_2/Ag NPs microspheres. 2-Mercaptopyridine is used as a model compound to study the influence of concentration of labeled molecules on the SERS response. As shown in Fig. 5, when the concentration of 2-mercaptopyridine decreases from 10^{-3}M to 10^{-9}M, the SERS intensity decreases gradually, however, fingerprint spectra of 2-mercaptopyridine between 1000 and 1200 cm^{-1} does not change. When the concentration reduces to 10^{-9}M, 2-mercaptopyridine can still be detected. In this case, the 2-mercaptopyridine molecules on the surface of PS-NH_2/Ag NPs microspheres might be near monolayer assembly. Thus these PS-NH_2/Ag NPs microspheres are very sensitive and promising for preparing SERS tags.

3.4. The preparation of SERS encoded PS-NH_2/Ag NPs microspheres

Using different thiol compounds as 2-MP, 4-NT, 4-MT and 2-NT to modify the PS-NH_2/Ag NPs surface, one can get SERS encoded microspheres. Each microsphere displayes a unique and reproducible SERS fingerprint spectra as shown in Fig. 6. Although only four thiol molecules were used in this study, SERS encoded microspheres could also be formed using various organic compounds containing a free thiol group or amino group which can strongly adsorb onto the silver surface. $AgNO_3$ was used as a source of Ag, it is possible that NO_3^- ions may be trapped in the coating of Ag. But from Fig. 6, one can see that there is no absorbance around 1055 cm^- band, which is the vibration characteristic band of NO_3^-. So, in this study NO_3^- would not influence the SERS detection.

One advantage of these SERS encoded PS-NH_2/Ag NPs microsphere is that single wavelength excitation is needed to produce diverse SERS encoded fingerprint spectra. In addition, this SERS method has the advantage of narrow band, so spectra overlap could be effectively avoided during encoding. If some of the sites on the silver surface are replaced by functionalized DNA, antibody or antigen, these SERS encoded PS-NH_2/Ag NPs microspheres biological probes could be used for multiplex bioanalysis.

3.5. The rapid detection of melamine by SERS using PS-NH_2/Ag NPs

Monodispersed amino polystyrene microsphere with narrow size distribution is a mature commercial product. Through accurate control of deposition time, PS-NH_2/Ag NPs microspheres can be batch produced with uniform SERS activity and stored as suspension for quasi-homogenous analysis. The PS-NH_2/Ag NPs is superior to the traditional silver gel and solid nanostructure substrates as it is easier to store and has less batch-to-batch various. Through accurate control of deposition time, large amount of PS-NH_2/Ag NPs microspheres with uniform SERS activity could be produced easily. Parallel analysis of large number of samples could be realized by mixing a few microliter PS-NH_2/Ag NPs suspension with microscale samples and dispersing the mixture onto smooth gold substrate for detection. We demonstrated here the practical use of PS-NH_2/Ag NPs for the detection of melamine in milk powder.

As shown in Fig. 7, milk powder solution of 5 mg/mL has almost no background SERS interference from 600 to 1000 cm^{-1}. The 698 cm^{-1} peak, which belongs to the in-plane deformation vibration of triazine ring, increases gradually with increasing amounts of melamine. This peak is consistent with the characteristic band of melamine reported in the literature on silver substrates [39]. The standard curve of SERS intensity at 698 cm^{-1} vs. the concentration of melamine is shown in Fig. 8 for quantitative analysis of melamine in milk powder. A linear relationship is observed from 10^{-8} to 10^{-3} mol/L and the calibration curve can be expressed as follows:

$$y = 1498.6 + 187.4 \log[C]$$

In this equation, y represents the SERS intensity at 698 cm^{-1} and $[C]$ represents the concentration of melamine in milk powder solution. The correlation coefficient is calculated to be 0.9927.

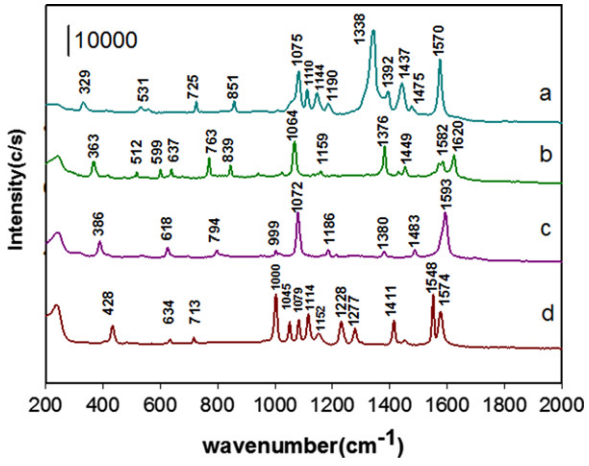

Fig. 6. The spectra of four SERS encoded PS-NH_2/Ag NPs: (a) 4-Nitrobenzenethiol, (b) 2-Naphthalenethiol, (c) 4-Methylbenzenethiol, (d) 2-Mercaptopyridine.

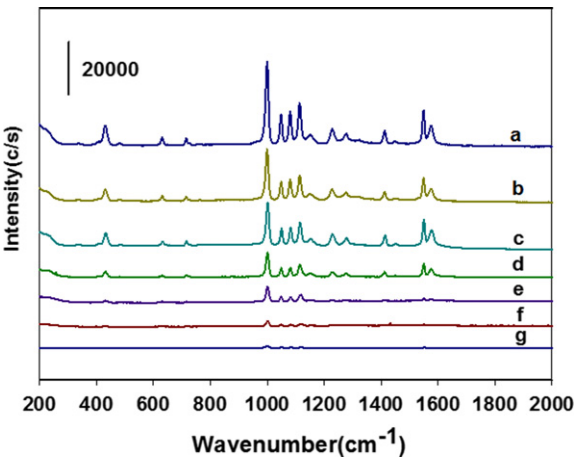

Fig. 5. SERS characteristic of different concentrations of 2-mercaptopyridine on PS-NH_2/Ag NPs subtract: (a) 10^{-3} M, (b) 10^{-4} M, (c) 10^{-5} M, (d) 10^{-6} M, (e) 10^{-7} M, (f) 10^{-8} M, (g) 10^{-9} M.

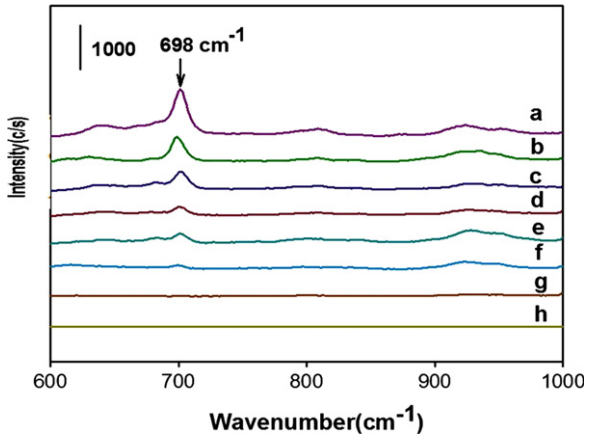

Fig. 7. SERS spectra of milk powder solution containing melamine of different concentrations: (a) 10^{-3} M, (b) 10^{-4} M, (c) 10^{-5} M, (d) 10^{-6} M, (e) 10^{-7} M, (f) 10^{-8} M, (g) 0M recorded using PS-NH_2/Ag as substrate. (h) The response of milk powder solution without PS-NH_2/Ag microspheres.

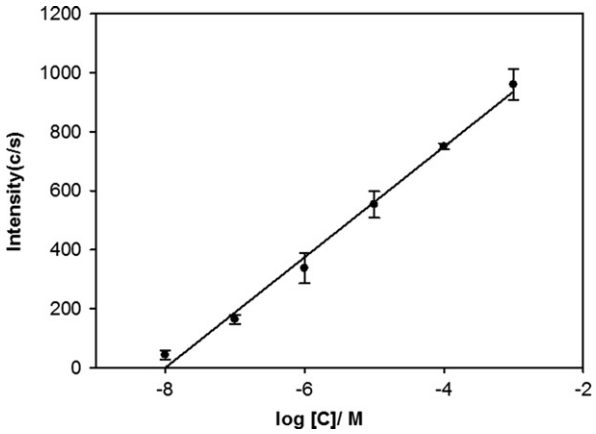

Fig. 8. The SERS intensity at 698 cm^{-1} vs. logarithm of the concentrations of melamine.

According to the rule of 3σ and also repeated SERS testing, a confidential LOD of melamine could be 1.9×10^{-8} mol/L (2.4×10^{-3} ppm), which is far lower than the standard (2.5 ppm) issued by the US Food and Drug Administration (FDA) in 2008. The safety limit of melamine in dairy products in China is 1 mg L^{-1} for infants and 2.5 mg L^{-1} for adults [40] which means a range around 10^{-5} mol/L is sufficient. Our proposed method (linear region from 10^{-3} to 10^{-8} mol/L) covers this range and it's sufficient for quantification of melamine in accordance with legal limits.

The standard method specified for melamine contamination in milk powder includes high performance liquid chromatography (HPLC), gas chromatography-mass spectrometer (GC-MS) and liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS). These methods need expensive equipment, special experimental environment and complicated sample pretreatment though the results are accurate and reliable. In recent years, some researchers began to explore the detection methods based on melamine induced aggregation between magnetic AuNPs and Raman labeled nanorods or color change of label-free gold nanoparticles (Au NPs) [40–42]. These methods showed reasonable detection limit, however, the interference from substances (even positively charged ions that are present in milk) or environmental conditions were also observed that could cause aggregation. The abovementioned PS-NH_2/Ag NPs microsphere SERS substrate, however, shows a great potential in detecting of melamine in milk powder with a simple pretreatment step, low protein background interference, characteristic fingerprint spectra identification, short detection time, low cost and capable of batch examination. The PS-NH_2/Ag NPs microsphere SERS substrate coupled with portable Raman spectrometer will certainly open some new opportunity for the rapid in-situ detection in food safety field.

4. Conclusion

In this study it was shown that silver nanoparticles could be deposited onto amino polystyrene microsphere surface easily due to the strong interaction between silver and NH_2 group. The surface morphology and SERS activity could be precisely controlled by manipulating the deposition time. Raman scattering of 2-mercaptopyridine underneath the individual PS-NH_2/Ag NPs microsphere could be enhanced greatly due to the nanojets formed between PS-NH_2/Ag NPs microspheres and the smooth gold or glass surface. The effective coupling of surface plasmonic resonance between PS-NH_2/Ag NPs and gold presented 10 fold higher enhancements than that of the glass surface. Well defined SERS spectra could be observed with 2-mercaptopyridine down to a concentration of 10^{-9} M. Different thiol compounds labeled PS-NH_2/Ag NPs microspheres were successfully synthesized for SERS encoding and each of them had unique fingerprint SERS spectra for identification. Furthermore, the PS-NH_2/Ag NPs microspheres showed great potential for the preparation of SERS encoded bioprobe for multiple bioanalysis. In this study, the primary application of the PS-NH_2/Ag NPs substrate for rapid detection of melamine in milk powder was demonstrated. A LOD of 1.9×10^{-8} mol/L melamine was achieved which meets the current standard for detection. The detection process does not need any sample pretreatment and background interference from protein is very low. The PS-NH_2/Ag NPs substrate will have potential use in food safety field in the near future.

Acknowledgments

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