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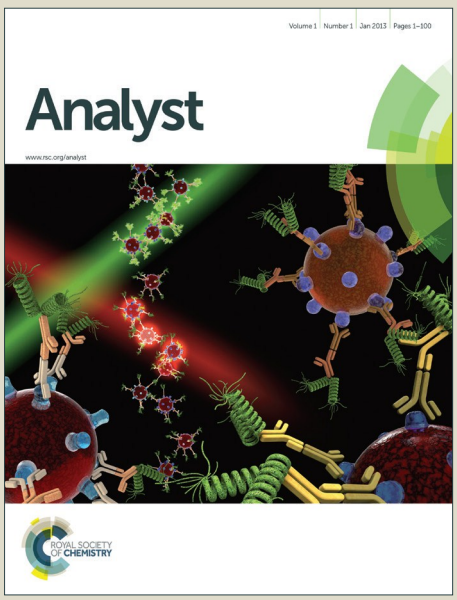

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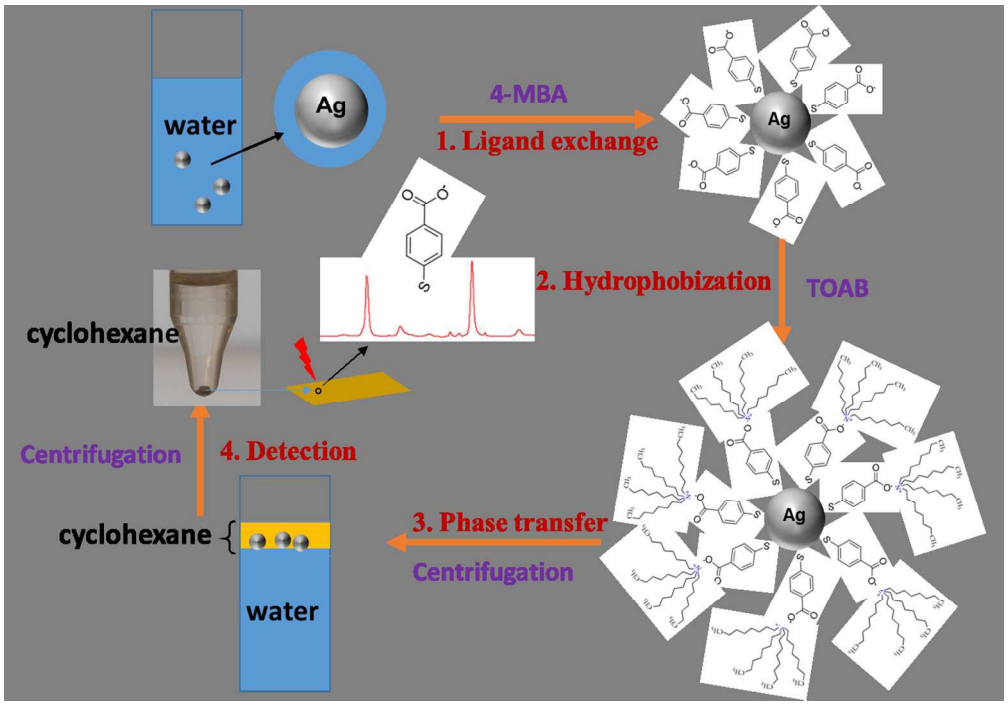

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COMMUNICATION

Ultra-sensitive Determination of Silver Nanoparticles by Surface-enhanced Raman Spectroscopy after Hydrophobization-Mediated Extraction

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An innovative and ultra-sensitive surface-enhanced Raman spectroscopic (SERS) method that uses a triple-functional surfactant ligand for nanoparticle surface binding, phase transfer and SERS signal reporting was developed for silver nanoparticles (AgNPs) detection. The method was able to detect 100 ng/L of AgNPs in aqueous samples and 2 µg/g AgNPs in wheat plants.

Among all nanomaterials, silver nanoparticles (AgNPs) have attracted particular attention from both industry and the research community due to the particles unique properties and widespread uses. One major exposure route of AgNPs is the wide application in consumer products as antimicrobial agents, with subsequent intentional or unintentional discharge into the environmental systems[1]. AgNPs can also be naturally formed from metallic ions in the presence of reductive components in the environment[2]. For example, natural organic matter (NOM) has been reported to facilitate the reduction of silver ions to AgNPs[3–6]. Many plants and microorganisms have also been shown to reduce silver ions to AgNPs, both *in vivo* and *in vitro*[7–11]. Given the wide application and natural formation of AgNPs, the environmental and biological fate and toxicity of AgNPs should be investigated comprehensively, including if AgNPs transfer through different trophic levels and impact food chains. Prior to any such effort, one needs a robust and accurate platform to detect and quantity AgNPs in the environment and biota. Two major challenges facing for the accurate evaluation of AgNPs fate and toxicity are the low concentrations of particles in these samples and the need to screen a large number of samples in a variety of complex matrices. As such, the need to develop sensitive and analytically robust techniques to identify and quantify AgNPs in complex samples is great. Conventional techniques for analyzing AgNPs have 3 major limitations: complex sample preparation that alters the particles, matrix interference and inability to speciate[12,13]. Advanced techniques such as synchrotron X-ray absorption near-edge spectroscopy (XANES), single particle inductively coupled plasma mass spectrometry (sp-ICP-MS) and field flow fractionation ICP-MS (FFF-ICP-MS) are promising techniques that can address some of these issues. However, these techniques also have limitations. For example, XANES is a qualitative method and single particle ICP-MS (sp-ICP-MS) requires monodisperse and spherical NPs with size > 20 nm to produce reliable data[14]. FFF–ICP–MS is able to characterize and quantify AgNPs in different media[15,16]. However, the method is sophisticated and time-consuming, and also requires a large dataset for method optimization[14]. In summary, the lack of methodology for effectively analyzing AgNPs in complex matrices has been a major hindrance in trying to accurately assess the environmental and biological fate and impacts associated with AgNPs exposure.

Previously, we reported that surface-enhanced Raman spectroscopy (SERS) has many advantages over conventional techniques and can specifically detect AgNPs owing to the NP-specific enhancement effect [17]. SERS has been used to detect AgNPs in consumer products, environmental surface water and spinach extract[17,18]. However, the analytical sensitivity (>0.1 mg/L) is insufficient given the predicted environmental levels (ng/L to µg/L)[19,20]. To improve method sensitivity, a filtration-enabled SERS method has been developed that can detect AgNPs at concentrations as low as 10 µg/L in environmental water[21]. However, the filtration-based SERS method is subject to matrix effects due to clogging of membrane pores and interference of the AgNP signal. Therefore, efforts were focused on how to simultaneously minimize matrix interference and achieve ultrasensitive detection for AgNP in complex samples. In this study, we developed an ultra-sensitive approach by using surface modification and microextraction to separate AgNPs from matrix components and then detected the concentrated AgNPs in the water-cyclohexane interlayer using

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SERS. The innovation of this method is the use of a triple-functional surfactant ligand that will: 1) bind to the AgNP surface strongly to replace existing adsorbents, 2) modify the surface hydrophobicity of the bound AgNPs so that they can be extracted by an organic solvent, and 3) produce a strong and distinct SERS signal for detection and quantification of the extracted AgNPs. The key features of the molecular structure of this binding compound includes a thiol group that is used for binding with AgNPs, a carbon chain at the other end that can increase the hydrophobicity of AgNPs and facilitate the extraction into the interface, and a Raman active group such as an aromatic ring.

Here, we demonstrate the use of 4-mercaptobenzoic acid (4-MBA) and tetraoctylammoniumbromide (TOAB) to form a triple functional ligand. As shown in Fig. 1, 4-MBA (dissolved in methanol) is mixed with aqueous solutions of citrate-AgNPs at a volume ratio of 1:10. The concentration of 4-MBA we used was 0.5 mM, which was reported to be the optimum concentration to sufficiently modify the surface of 1 mg/L AgNPs through ligand exchange in a previous study[13]. The concentrations of AgNPs we used were no more than 1 mg/L, which means that 4-MBA at a concentration of 0.5 mM is high enough to saturate AgNPs. In this study, 4-MBA serves three functions. First, the molecule modifies the surface of the AgNPs through displacing the coated citrate with thiol group. Second, the 4-MBA forms acid-base pairs with TOAB through electrostatic attraction, which significantly increases AgNPs hydrophobicity. Last, 4-MBA has distinct SERS peaks at approximately 1080 and 1590 cm^{-1}, effectively serving as an AgNP signal reporter. To facilitate the adsorption of 4-MBA to AgNPs, the mixture was bath ultrasonicated (130 W) for 3 min and further shaken for 2 h at 150 rpm on a platform shaker (Innova 2100, Eppendorf). Then, 1 mL of cyclohexane containing TOAB was added to the suspension to enhance the surface hydrophobicity of AgNPs and facilitate extraction from the aqueous phase. After 10 min of incubation on the shaker, the mixture was centrifuged at 3 000 rpm for 10 min. Phase separation is clearly evident, with the cyclohexane layer on the top.

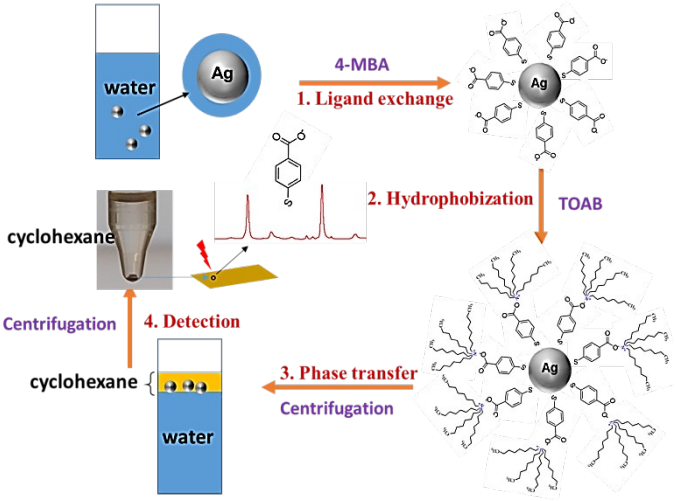

Fig. 1 Schematic diagram illustrating the determination of AgNPs by SERS after hydrophobization-mediated extraction.

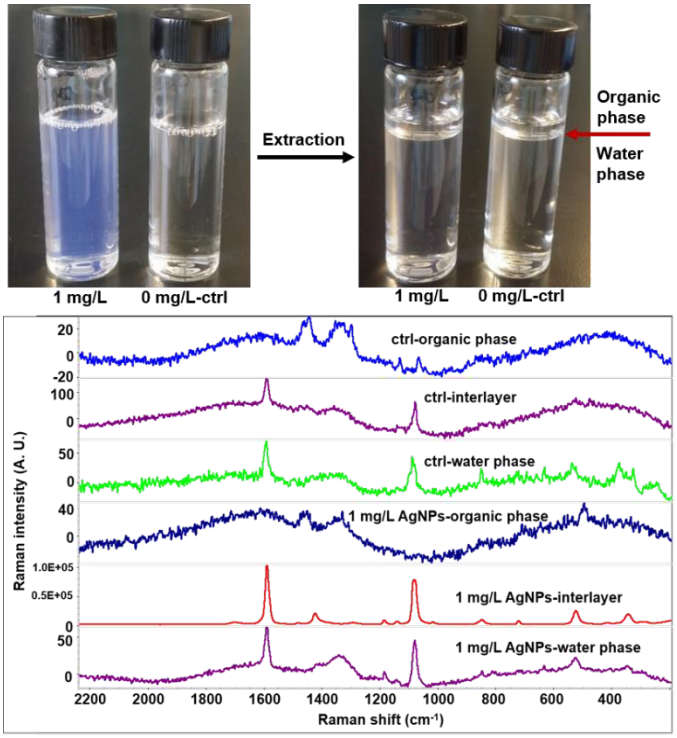

Fig. 2 Phase separation and comparison of the SERS intensities of organic phase, interlayer and water phase after extraction.

As evident in Fig. S1, color disappearance in water phase is indicative of extraction efficiency and this increased with increasing TOAB concentration from 0.05 mM to 0.5 mM as measured by UV-vis absorbance of the aqueous AgNPs. The SERS spectra show that the signal intensity increased with TOAB concentration, reaching a plateau at 0.25 mM (Fig. S2), which is consistent with UV-vis data. Therefore, 0.25 mM TOAB was chosen for further experiments.

After phase separation, location of the AgNPs is critical. We sampled the organic phase (0.5 mL), interphase (0.5 mL, half organic half water) and aqueous phase (0.5 mL) for SERS analysis after centrifugation (13 300 rpm, 5 min). The weak SERS signals for water phase at 1 mg/L and similarity in response to the control demonstrate that the majority of AgNPs were extracted from the water (Fig. 2). In fact, as determined by ICP-MS (Agilent 7500ce, Santa Clara, CA), the AgNPs in the water phase was decreased by 93.0 ± 0.8% after extraction. This finding also explains the color difference in aqueous phase before and after extraction. Subsequent analysis focused on determining whether the AgNPs were extracted into the cyclohexane or at the interface. As evident in Fig. 2, only the interlayer shows greatly enhanced 4-MBA peaks, clearly indicating that the extracted AgNPs concentrate in this phase. It has been reported that ionic surfactant-assisted phase transfer of nanoparticles into organic phase can be problematic when the particle size is greater than 10 nm[22–24]. For example, Cheng and Wang used TOAB as a transfer agent and demonstrated that nearly all ~10 nm gold nanoparticles were extracted into the interface, leaving the organic layer transparent[22]. This is consistent with our result using 60 nm AgNPs and can be

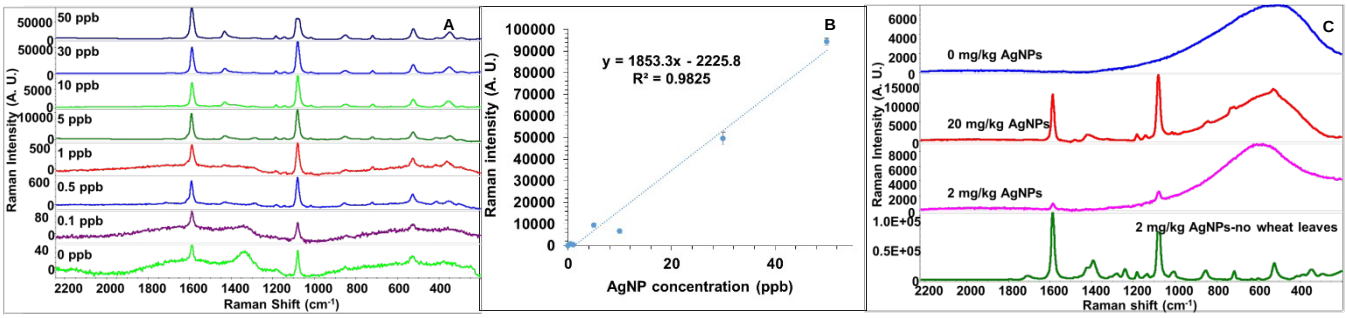

Fig. 3 Concentration-dependent SERS response to AgNPs via hydrophobization-mediated extraction assisted SERS (A). A linear relationship (B) was constructed between Raman intensity and AgNP concentration. The error bars represent the standard errors of ten parallel SERS measurements. The developed method was applied to detect AgNPs in wheat leaves (C).

explained by the surface-area-ratio effects. The larger the particle size, the lower the surface-area ratio, which leads to a lower hydrophobic ligands/NPs ratio and weaker hydrophobic extraction force[22]. The fact that the AgNPs accumulated in the interlayer is favorable for SERS detection because of the resulting enrichment and aggregation effect, as well as purification from interfering substances in the sample matrix. In addition to using SERS to demonstrate the extracted AgNPs were concentrated in the interlayer, we used ICP-MS to measure the extraction efficiency, which was 90.0 ± 1.0% based on the mass percentage of AgNPs extracted into the interlayer. Further we confirmed the reliability of our analysis with the results showing that the recovery was 76.7 ± 1.8%, which was measured by using a calibration standard and calculated as analytical result/theoretical result × 100.

To obtain AgNPs extracted in the interlayer, the organic phase and approximately 0.5 mL of the top layer of water phase were collected. The mixture solution was placed at -20 °C with the tubes inverted for 5 min. Due to the freezing point difference between water (0 °C) and cyclohexane (6.55 °C), the cyclohexane layer was frozen while the water phase was still a liquid that could be readily decanted. The remaining solution was centrifuged at 13 300 rpm for 5 min to concentrate the AgNPs to the surface of a water drop at the bottom of the tube, which was then placed on a clean surface of a gold slide and dried in a fume hood. After drying, the samples were immediately analyzed by a DXR Raman Spectro-microscope (Thermo Scientific, Madison, WI) that consisted of a 780-nm laser with an output power of 5 mW, a 20 × confocal microscope objective with 1.9 µm spot diameter, as well as a 50 µm slit width for 2 s integration time. The detection process was monitored using the OMNIC™ software (version 9.1). Ten spectra from each sample were chosen and averaged to a final spectrum using TQ Analyst software (version 8.0, Thermo Scientific).

As shown in Fig. 3A, the hydrophobization-mediated extraction assisted SERS can detect AgNPs as low as 100 ng/L, which approximates the predicted environmentally relevant levels of AgNPs[19,20]. In addition, Raman intensity and AgNP concentration display a linear relationship (Fig. 3B), which shows the potential of the developed method to quantify AgNPs under some environmentally relevant conditions. Although a large number of studies have used water-to-organic phase transfer to purify and separate synthetic AgNPs[25], few have investigated the potential of liquid-phase extraction to facilitate AgNPs detection and quantification. Majedi *et al.* previously reported that AgNPs with surface modification by mercaptoundecanoic acid and octadecylamine were able to be extracted by cyclohexane and detected by ICP-MS[13]. However, the authors only measured the aqueous and organic phase, without analyzing the interlayer. In addition, the AgNP analysis was confounded by silver ion interference unless masking agents (e.g., $Na_2S_2O_3$) were used. In the current study, instead of extracting AgNPs into the organic phase, we took advantage of water-to-interlayer extraction to achieve ultra-sensitive measurement of AgNPs resulting from the high enrichment and isolation effect. To our knowledge, this is the first time that interlayer separation and enrichment were used to screen and detect AgNPs. Importantly, our previous work demonstrated that SERS selectively detects AgNPs due to the NPs-specific enhancement effect[17], effectively excluding interference from other silver species.

The method was then challenged with complex biological tissues; wheat plants were grown for three weeks in a greenhouse (25 ℃ with 16h/8h (light/dark) cycle, light intensity ~750 µmol m^{-2} s^{-1}). The harvested leaves were separated and ground under liquid nitrogen. AgNPs colloids (60 nm, citrate coated) were added to the homogenized wheat leaf liquid at the final concentrations of 20 and 2 µg AgNPs/g wheat leaves, respectively. After incubation with 4-MBA, the mixture was centrifuged at low speed (3 000 rpm, 5 min) to remove solid plant residues. The supernatant was directly used without further pre-treatment for extraction and detection using the methods described above.

Using the developed method, we were able to detect AgNPs in wheat leaves with limited sample pre-treatment. As shown in Fig. 3C, the negative control (wheat leaves alone) has broad background noise (200-1000 cm^{-1}), which may arise from fluorescence interference produced by pigments in the leaves[26]. Fortunately, the reporter (4-MBA) we used has strong peaks at wavelengths outside the 200-1000 cm^{-1} range. Also, the presence of AgNPs can partially quench this fluorescence[27]. The wheat leaves with AgNPs showed the enhanced Raman peaks of 4-MBA, which are similar to the positive control without plant tissues. Moreover, the signal intensity was dependent on AgNP concentration. Using the developed

method, 20 and 2 µg AgNPs/g wheat leaves were detected based on the characteristic peaks of 4-MBA at 1590 cm^{-1} and 1080 cm^{-1}. To date, little information is available in the literature about the dynamics of AgNPs and ion uptake, dissolution, and/or reduction within plants due to technical barriers associated with detection and quantification of the individual species. Clearly and importantly, our method can serve as a starting point for *in vivo* studies detecting AgNPs in biota. In comparison to the no leaves control, it is obvious that AgNPs was subject to significant matrix interference. In the future, we will optimize sample pre-treatment to further reduce matrix effects. In addition, the fluorescence interference may be further minimized through the use of alternative radiation lasers in near-infrared region[28].

In conclusion, an ultra-sensitive method for detecting AgNPs was developed by combining hydrophobization-mediated extraction with SERS. The 4-MBA modified AgNPs were extracted into the water/cyclohexane interlayer after hydrophobization by TOAB. After optimizing the TOAB concentration, sensitivity was improved to 100 ng/L, which enables AgNPs detection at environmentally relevant levels[19,20]. Moreover, this work provides a promising approach for determining the AgNPs in plant tissues and other biota. Additional topics worthy of future investigation include determining the effects of AgNP coating and size on the efficacy of water-to-interlayer extraction and associated SERS response. In addition, further evaluation and optimization is needed for a much broader range of sample matrices.

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