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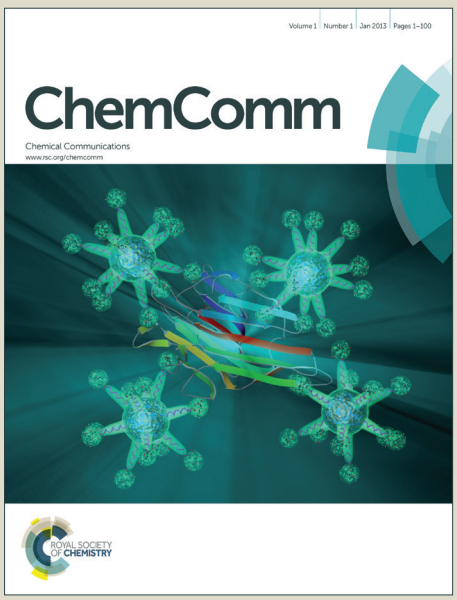

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COMMUNICATION

Femtogram Level Competitive Immunoassay of Mercury (II) Based on Surface-enhanced Raman Spectroscopy

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A femtogram level and specific surface enhanced Raman spectroscopy (SERS) based competitive immunoassay was developed to detect Hg(II) in aqueous solution for the first time. This novel approach provides an alternative, ultrasensitive and specific analytical method for the detection of Hg(II).[1]

Mercury is a toxic and widespread pollutant that originates from oceanic and volcanic emissions, and human activities.[1] Mercuric ions [Hg(II)] is the most stable form of inorganic mercury, which will damage the brain, nervous system, kidneys, and endocrine system.[2] Furthermore, microorganisms are able to biomethylate Hg(II) to methylmercury [$CH_3Hg(I)$]. Such process allows bioaccumulation and biomagnifications to occur and consequently be of concern for public health.[1,3] Therefore, it is necessary to develop mercury detectors for both environmental and biological samples.[4] Various methodologies such as sensors based on fluorophores, chromophores, foldamers, ampter, silver nanoparticles, conjugated polymers, gold nanoparticles, electroanalytical methods such as capillary electrophoresis and voltammetry, pyridylporphyrin–DNA, peptides, proteins, enzymes, oligonucleotides, and DNAzyme have been reported in the past few years.[5] Nevertheless, the above methodologies will be interfered by chemically-related metals, delayed response to Hg(II), and/or insufficient sensitivity.

Surface-enhanced Raman spectroscopy (SERS) is a general and powerful spectroscopy technique that has been generally used by lots of surface science scientists in the past few years. It provides non-destructive and ultrasensitive characterization down to single molecular level.[6] SERS has been applied to various areas such as chemical sensing, medical diagnostics, and the studies of living cells and bacteria.[7] Very recently, SERS can also be employed to determine the Hg(II) ions, mainly by the nanoparticle-based chemical approaches.[8] For example, Zhang selected appropriate organic ligands for the femtomolar detection of Hg^{2+} by a SERS chip with silver-coated gold nanoparticles. These SERS chips possess many advantages, such as excellent anti-interference ability, recycling usage, long-term stability, portable feasibility, and simple operation, it exhibited high sensitivity and specificity. [8]

The introduction of immunoassay in SERS measurements can integrate their advantages of specificity and sensitivity.[9] For instance, the strong and specific binding of an antibody to its antigen has been widely exploited in biochemical studies, clinical diagnostics, sensor design, and environmental monitoring.[10] In past years, many different approaches such as scintillation counting, fluorescence, chemiluminescence, electrochemical detection, enzymatic,[11] and surface plasmon resonance (SPR)[12] have been developed for a direct measurement of antigen-antibody binding. The expose of SERS first appeared in the enzyme immunoassay at the end of 1980s, in which Cotton et al. employed the SERRS effect to detect human thyroid simulating hormone (TSH) antigen.[13] Nevertheless, the SERS based immunoassay was usually used for macromolecule proteins using a sandwiched pattern[14]. In our previous studies, we reported the high sensitive detection and separation of antigen based on magnetic SERS immunoassay[15]. Herein, we report a femtogram level and specific competitive immunoassay for the detection of Hg(II) in aqueous solution.

Using hybridoma technique we successfully produced monoclonal antibody (mAb) against Hg(II), which was then characterized by an indirect competitive enzyme linked immunosorbent assay (ELISA).[16] The IC_{50} value of the ELISA for Hg(II) was 1.12 ng/mL (i.e. 5.6 nM) and the limit of detection (LOD) at 3 times of signal/noise was 0.08 ng/mL (i.e. 0.4 nM). Low values of IC_{50} and LOD indicated that the sensitivity of the ELISA is high. The cross-reactivity values of the mAb with Hg(II), $CH_3Hg(I)$, 6-mercaptonicotinic acid (MNA, the ligand selected for the preparation of immunogen) and CH_3Hg-MNA were 100%, 1.97%, 0.72% and 1.16%, respectively. There was no cross-reactivity of the mAb with Cu^{2+}, Cr^{3+}, Sn^{2+}, Ni^{2+}, Mn^{2+}, Pb^{2+}, Zn^{2+}, Cd^{2+}, Fe^{3+}, Co^{2+} and Mg^{2+}. The values clearly demonstrate that the mAb has highly specific recognition with Hg(II).

Combining the highly specific mAb against Hg(II) with the highly sensitive analytical method SERS, we expect that the SERS based immunoassay is able to detect Hg(II) with ultrasensitivity and specificity. In general, the antibody is immobilized on the surface of the substrate and the target analyte

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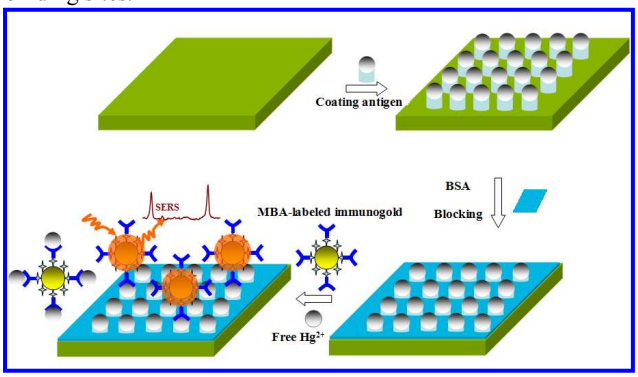
is packed by the immobilized Ab and another Ab labeled with SERS reporter, so called as sandwich immunoassay for detecting macromolecules. However, the competitive immunoassay is utilized to detect the small species that is unable to react with two antibodies simultaneously, such as Hg(II). The principle and processes of the SERS based competitive immunoassay for Hg(II) were illustrated in Scheme 1. The coating antigen (i.e. antigen-protein conjugate) is firstly immobilized on the surface of substrate, it competes the limited binding sites of the mAb with the Hg(II) in the solution. The weak SERS signal is due to the less amount of the mAb-gold nanoparticles-4-mercaptobenzoic acid (MBA) conjugate bound on the substrate which is caused by the competition of the high concentration of Hg(II) in the solution.

To establish a SERS based competitive immunoassay, besides mAb and coating antigen, another requirement is to prepare qualified MBA labeled immunogold. Firstly, we synthesized gold nanoparticles with the size of 30 nm. Then, MBA was attached onto the surface of Au nanoparticles with full coverage, and an appropriate amount of mAb was immobilized. Finally, excessive bovine serum albumin (BSA) was added to the MBA labeled immunogold to completely block nonspecific binding sites.



Scheme 1 the principle and processes of the SERS based competitive immunoassay for Hg(II)

In order to achieve high sensitivity of the assay, there are several criteria, such as maintaining consistent concentration of coating antigen (500 ng/mL) and diluting ratio of MBA labeled immunogold, have to be fulfilled. On the other hand, the possibility of SERS background signals from the coating antigen (i.e. antigen-protein conjugate) should be clarified and eliminated because some amino-acid residues are Raman active species. Fortunately, it was confirmed that there was no any background SERS signals raised from coating antigen, and all detected signals in the SERS based immunoassay were generated from MBA bound on immunogold.

Under optimal assay conditions, the standard curve for Hg(II) was constructed within the range of 10^{-5}-100 ng/mL. The procedures of the SERS based immunoassay were as followed: 25 ng of coating antigen, diluted by phosphate-buffered saline (PBS), was dropped onto the substrate. After being placed in a chamber with a relative humidity of 65%-75% overnight, the substrate was dried at room temperature for 30 minutes. It was then incubated in 5% BSA for 1 hour to block nonspecific active sites followed with the treatment by Millii-Q water and dry nitrogen gas. Then, it was exposed to a mixture of 20 μL of different concentrations of Hg(II) and 150 μL of MBA labeled immunogold in borate buffer solution for 2 hours at room temperature. It was lastly treated in the order of TBS, 0.1% Tween, TBS, ultrapure water, and dry nitrogen gas. The SERS signal of the MBA labeled immunogold immobilized on the substrate was read out by a confocal microprobe Raman system. The SERS intensity at 1077 cm^{-1} arose from Raman reporter (MBA) was used for quantitative analysis.

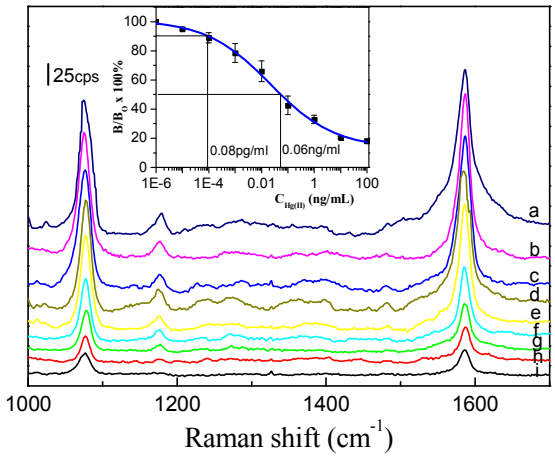


Fig 1 The SERS signals and calibration curve of the SERS based immunoassay for Hg(II) with different standard concentrations (a-i). The inserted figure is the calibration curve, where B and B_0 is the SERS signal of the analyte at the standard point and at zero concentration of the analyte, respectively.

The spectra and the calibration curve of the SERS based immunoassay for Hg(II) were shown in Figure 1. It was found that the SERS signal steadily decreased (a-i) as the concentration of Hg(II) increased,. The IC_{50} value was found to be 0.06 ng/mL (i.e. 0.3 nM) and the LOD was 8×10^{-5} ng/mL (i.e. 0.4 pM, in this case, the analyte volume for detection was 20 μL, thus the quantitative amount was 1.6 fg). The values of IC_{50} and LOD obtained in this approach are 18.7 and 1000 times respectively lower than those in ELISA using the same mAb,[16] indicating the great improvement in the sensitivity of the assay for Hg(II) when SERS was integrated. Comparing to other reports for detection of Hg(II), the proposed SERS based immunoassay is the most sensitive analytical method for Hg(II) achieved (see Table S1).

The specificity of the SERS based immunoassay was also verified. Six species including Pb(II), Cd(II), Ag(I), MNA, $CH_3Hg(I)$ and CH_3HgMNA were selected to test the cross-reactivity (CR). The standard solutions of cross-reacting species were prepared in the concentration range of 10^{-5}-100 ng/mL and applied to the procedures of the assay. CR was expressed as percent IC_{50} value based on the response of Hg(II), e.g. CR(%) = [IC_{50} for Hg(II)]/[IC_{50} for competing chemicals] × 100%. The cross-reactivity values of the mAb with Hg(II) and six species were shown in Figure 2. The original spectra were presented in Figure S4. One could conclude that no cross reactivity with Pb^{2+}, Cd^{2+}, Ag^{+}, $CH_3Hg(I)$, MNA and CH_3HgMNA was detected. It clearly demonstrated that SERS based immunoassay exhibited high specificity in detecting Hg(II).

Canal water samples collected from Suzhou city was filtrated by a nylon membrane filter (0.45 μm). The filtrates were adjusted to pH 7.0 and were analyzed by the proposed method. Then, the SERS based assay was applied to detect the filtrates which were spiked with Hg(II) at the concentrations of 0.1 ng/mL

and 0.5ng/mL. Acceptable recovery rates of 83.5-95.7% were obtained, which suggested that the method could be used for the detection of Hg(II) in real aqueous samples.

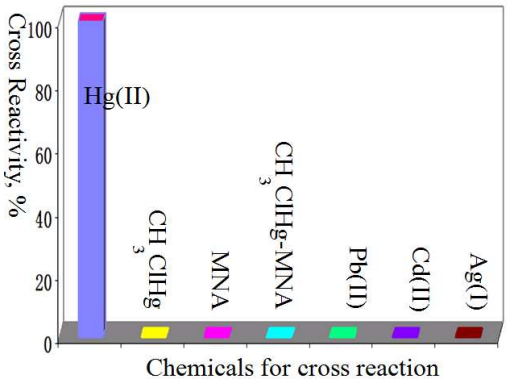


Fig 2 The cross-reactivity values of the mAb with Hg(II) and other species tested by the proposed SERS based competitive immunoassay.

Conclusions

In summary, a femtogram level and specific SERS based competitive immunoassay for the detection of Hg(II) in aqueous solution was developed successfully. The labeled immunogold was synthesized by linking MBA and mAb to the surface of gold nanoparticles. The LOD value of the assay for Hg(II) was found to be 8×10^{-5} ng/mL (i.e. 0.4 pM or 1.6 fg in 20 μL analyte solution), which was much lower compared to that in normal ELISA approach. No cross reactivity with Pb^{2+}, Cd^{2+}, Ag^{+}, MNA, CH_3Hg^{+} and CH_3Hg-MNA revealed high specificity of the assay. The proposed method was used for the detection of Hg(II) in spiking water samples with acceptable recovery rate. This novel approach not only provides an alternative, ultrasensitive and specific analytical method for the detection of Hg(II), but also can be extended as an useful model for the detection of other small molecular compounds in biological, food and environmental areas.

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Yuzhen Wang and Shuai Chen contributed equally to this work.

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