Analytical Methods

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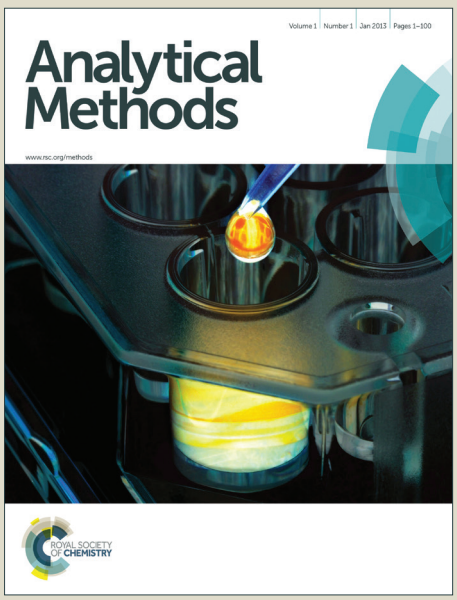

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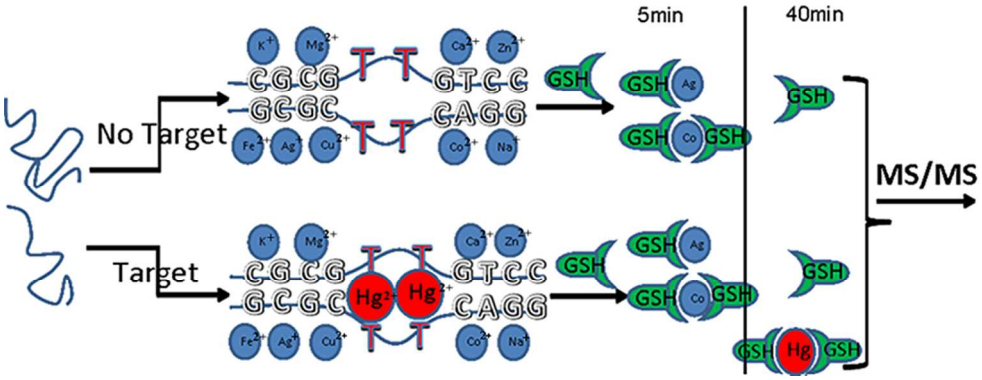

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Novel electrospray ionization-tandem mass spectrometry strategy for monitoring mercury (II) ion based on the competing system of mercury specific DNA and glutathione to mercury (II) ion

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Abstract

An electrospray ionization-tandem mass spectrometry (ESI-MS/MS) strategy was developed for the detection of mercury ion with high sensitive and selective based on a competition system of glutathione and mercury specific DNA to mercury ion. Mercury ion selectively bound to a special mismatched DNA and glutathione (GSH) as thiol-containing tripeptide can effectively sequester Hg^{2+} ion from thymine-Hg^{2+}-thymine structure. Following the competition reaction, the concentration of mercury ion in analyte was indirectly reflected by monitoring the concentration change of free GSH by ESI-MS/MS method. The electrospray ionization-mass spectrometry (ESI-MS) analysis of competition system provided the connection of mercury ion and mercury specific DNA (MSD), and also provided the process which GSH competed mercury ion from MSD. Notably, the proposed competing platform exhibits exquisite selectivity and sensitivity to Hg^{2+} ion with the detection limit of 5 nM. Furthermore, this assay design avoids the labeling of the probe, the use of the quantum dot, fluorescence dye, and heavy metals. So it is much friendly for the operators and environment. The proposed method was applied to some real samples (tap water, lake water, and fish), and the results were satisfactory.

Key words electrospray ionization-tandem mass spectrometry, mercury (II) ion, glutathione, competing system, selectivity, salt tolerance

Introduction

Mercury is a highly toxic element in ecosystems,[1-2] and it can be accumulated in human body and causes the damages of brain, nervous system, immune system, and many other organs. With the increasing threat of mercury exposure in environment from global mercury emissions as well as various forms of contaminations,[3] there has been a growing interest in the development of highly sensitive and selective analytical methods for the monitoring of mercury ion (Hg^{2+}) over the past few years.[4,5]

The most commonly analytical techniques for detecting mercury ion include atomic absorption spectrometry (AAS),[6] atomic emission spectrometry (AES),[7] inductively coupled plasma mass spectrometry (ICP-MS),[8] electrochemical method,[9] and so on. A common shortcome for all the above methods is applicable to only contain mercury ion for the detection process, and their analytical results are easily influenced by the present of other metal ions. In recent years, some new methods have been developed for monitoring Hg^{2+} ion,[10-12] such as using biosensors and chemical sensors. The detection of Hg^{2+} ion using various sensor systems based upon organic chromospheres or fluorophores,[13]conjugated polymers,[14] oligonucleotides,[15] DNAzymes,[16] proteins,[17] thin films,[18]and nanoparticles (NPs).[19] However, most of these methods suffer from low water solubility and complex synthesis procedure. Moreover, these strategies still suffer a series of problems, such as poor sensitivity to Hg^{2+} ion, cross-sensitivity to other metal ions, and incompatibility

with aqueous systems.[20] Increasing concerns over monitoring mercury ion in aqueous solution have motivated the development of new methods with high selectivity and sensitivity, healthy, harmless, and high salt tolerance.

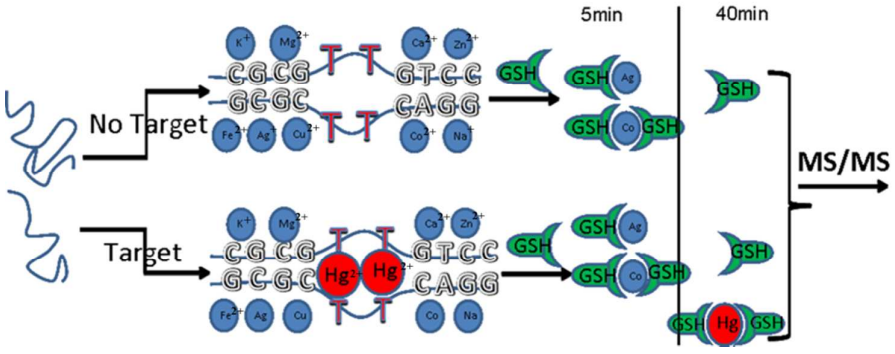
Hg^{2+} ion can bind specifically to iminonitrogen (N3) of thymine (T),[21] and possibly forms the linkage of T (N3)-Hg^{2+}-T (N3). Recently, T-Hg^{2+}-T (T=thymine) chemistry has been highlighted in the development of Hg^{2+} sensors because T-T mismatch shows high selectivity to Hg^{2+} ion against many other metal ions.[22-23] Two protocols based on the binding of Hg^{2+} ion to a mercury specific DNA(MSD) have been reported. One is based on fluorescence resonance energy transfer (FRET) between fluorescein and dabcyl which has been labeled on both ends of a MSD.[24-27] The other protocol is based on the color change of the surface functionalization gold nanoparticles (AuNps) by MSD probe which responds to Hg^{2+} ion and induced conformation altransition.[28,29] Both protocols have shown high selectivity to Hg^{2+} ion against many other metal ions. However their most disadvantage is that fluorescent materials or some potential risk materials are used.[30] So it is much harmful for operators and circumvent. Thus, a rapid, sensitive, safe, and reliable analytical method is needed to reduce the harm to the operators or environment.

Previously, many researches have shown that mercury (II) ion has an extremely high affinity for thiol-containing compounds [31,32] like cysteine, N-acetyl-cysteine, methionine, glutathione, lipoic amide and coenzyme A. Some of these studies suggested a protective effect of thiol compounds against

the mercury toxicity. It is well-known that GSH-Hg^{2+}-GSH and Cys-Hg^{2+}-Cys complexes, as well as Hg^{2+} complexes with other biothiols and nitrogen bases, have very high stability constants. Based on this principle, Lee et al [33] developed a highly sensitive and selective colorimetric detection method for Cys based upon oligonucleotide functionalized AuNP probes. When GSH or Cys was added into an analyte, it binds to Hg^{2+} ion and removes Hg^{2+} ion from thymine-Hg^{2+}-thymine complex, thereby lowering the temperature of the DNA duplex dissociation (Tm) and changing the color from purple to red. HuiXu[34] developed a "molecular beacon" method based the detection of GSH and Cys relying on Hg^{2+}-induced self-hybridization of the beacon strand. Han B[35] based on this theory developed a detecting system for biothiols in cells.

But for all of these studies, they all use the fluorescent dye, heavy metal or quantum dots. All of these were unhealthy for operators or the environment. In order to reduce the harmful effect but keep the sensitivity and selectivity, in this paper, we developed a novel ESI-MS/MS detection method based on a competing system of mercury specific DNA (MSD) and glutathione (GSH) for mercury(II) ion in analyte. The method is based on a competitive ligation of Hg^{2+} ion between GSH and thymine-thymine (T-T) mismatches in a DNA strand of the self-hybridizing beacon strand. A mercury (II) specific DNA (MSD) has been elegantly designed for Hg^{2+} ion assay, the sequences of the leading strand and the lagging strand were 5′-CGCGTTGTCC-3′ and 5′-GGACTTCGCG-3′, respectively. These sequences are reverse complement but have two mismatched T bases. It forms a double strand DNA

structure in the presence of Hg^{2+} ion, and presents a random coil form in the absence of Hg^{2+} ion. In here, we firstly investigated the specific interaction of Hg^{2+} ion with T-T mismatches and then with GSH for developing a highly sensitive ESI-MS/MS method to detect Hg^{2+} ion. In the presence of Hg^{2+} ion, it specially fills in the T-T mismatch hole and dramatically raises the Tm value of MSD. Once GSH is added into the solution, Hg^{2+} ion will come off MSD and bind to GSH due to the high binding affinity of GSH to Hg^{2+} ion. After this competing reaction, the concentration of GSH will be measured for indirect detection of Hg^{2+} ion, because the concentration of GSH is correlative with that of Hg^{2+} ion (scheme 1).The interfering effect of Na^{+} and many other metal ions was also investigated in this paper. The result shows that the good selectivity for Hg^{2+} ion can be achieved by our method, even in the case of high concentration of salts. We applied this proposed method for the detection of the concentration of mercury ion in real samples, such as in tap water, lake water, and fish. The results were further verified by ICP-MS.



Scheme 1. The mechanism of competing system for Hg^{2+} ion in this study

Materials and Methods

Reagents and Apparatus

The thymine-rich single stranded DNA (T-rich ssDNA) was purchased from TAKARA Inc. (Dalian, China). The sequences of T-rich DNA used in this work were 5′-CGCGTTGTCC-3′ and 5′-GGACTTCGCG-3′, respectively. $HgCl_2$, NaCl, $MgCl_2$, KCl, $ZnCl_2$, $CaCl_2$, $AgNO_3$, $Co(NO_3)_2 \cdot 6H_2O$, and L-Glutamic acid (Glu) as internal standard were purchased from Sigma Aldrich Chemical Co. All solutions were prepared using ultra-pure water (18.2 MΩ cm) from the Milli-Q system.

ESI-MS/MS experiments for the concentration detection of free GSH was performed using Xevo-TQ triple quadrupole tandem mass spectrometer(Waters Crop., USA)equipped with an ESI source and Waters Masslynx V4.1 workstation combined with Acquity ultra performance liquid chromatography (UPLC) system.

The automatic sampler and pump of LC apparatus were used to inject samples and send the mobile phase to mass spectrometer. The mobile phase was the mixture of 30% methanol and 70% water. The flow rate was kept at 0.2 mL/min, and 2 μL of sample was injected. The running time was 2 min. ESI-MS/MS analysis was done in positive ion mode with multiple reaction monitoring (MRM). Mass transitions of GSH and Glu (as internal standard) were optimized at 308→76 and 148→84, respectively. The cone voltage and collision energy for GSH were 12 V and 16 eV, respectively. The cone voltage and collision energy for Glu were 14 V and 12 eV, respectively. The spray voltage was 3.0 kV. The temperatures of source and desolvation were held at

120°C and 350 °C, respectively. The flow rate of desolvation gas was at 800 L/h, and the pressure of argon as the collision gas was kept at 1.4×10^{-3} mbar.

ESI-MS experiment for the structure confirmation of duplex (MSD) and complex (MSD-$2Hg^{2+}$, GSH-Hg^{2+}-GSH) was performed using Synapt G2 Q-TOF mass spectrometer (Waters Crop., USA) equipped with an ESI source. The sample analysis was performed in negative ion mode. The voltages of capillary and sample cone were at 3.0 kV and 35 V, respectively. The source temperature was at 120°C. The flow rates of cone and desolvation gas were set at 30 L/h and800 L/h, respectively. Samples were introduced directly into the mass spectrometer at a flow rate of 5 μL/min.

The UV-Vis spectra of the analytes were recorded using Carry 50 spectrophotometer in the wavelength range of 200-500 nm.

The complexes formations of MSD and GSH with Hg^{2+} ion

Three tubes of 100 nM MSD solution were mixed with 5 mM NH_4Ac and 1mM NaCl and labeled as S1, S2, and S3, respectively. Then, 100nM Hg^{2+} ion was added into S2 and S3, respectively. The three tubes were heated at 93°C for 5 min and then gradually cooled down to room temperature to facilitate the formation of double-strand DNA. The solutions of S1 and S2 were analyzed by ESI-MS on Q-TOF mass spectrometer. An additional of 400nM GSH was added into the S3 solution and the mixture was incubated at 37°C for 40 min before ESI-MS analysis. S1, S2, and S3were then analyzed by ESI-MS on a Q-TOF mass spectrometer.

Sensitivity and Standard Curve

For making standard curve of the analyte, the experiment process was implemented as following. The detection system was performed in the 5 mM ammonium acetate buffer solution with 1mM NaCl. A series of DNA (100 nM) solutions with different concentrations of Hg^{2+} ion (0, 5, 10, 20, 40, 60, 80, and100nM) were prepared. The above solutions were heated at 93°C for 5 min and then gradually cooled to room temperature. 200 nM GSH was then added, and the final solutions were incubated for 40 min in order to remove Hg^{2+} ion from T-Hg^{2+}-T base pair. The GSH stock solutions were freshly prepared on the day of use. Finally, the concentration of remaining GSH in reaction systems was detected using ESI-MS/MS on multiple reaction monitoring (MRM) mode on a triple quadrupole mass spectrometer. The free GSH concentration could indirectly reflect the concentration of Hg^{2+} ion in analyte. Due to the minor reactivity of Glu to mercury ion, we chose Glu as an internal standard in this study. ESI-MS/MS analysis was performed in positive ion mode with MRM as mentioned in section2.1.

Selectivity

To make sure the special selectivity of this detection method for Hg^{2+} ion, several solutions containing different metal ions (Na^{+}, K^{+}, Co^{2+}, Fe^{2+}, Ag^{+}, Cu^{2+}, Zn^{2+}, Mg^{2+}, and Ca^{2+}, each at 100 μM) were tested under the same conditions as the solutions only containing Hg^{2+} ion. Remarkably, no optical and thermal transition profile changes of these solutions were observed with up to

mill-molar concentrations of these metal ions. The change of free GSH concentration in solution system was also tested in the presence of other metal ions under the same condition as for Hg^{2+} ion.

Salt tolerance

To observe the influence of salt concentrations, a series of solutions containing different concentrations of NaCl (50μM, 100μM, 150μM, 200μM, 500μM, 750μM, 1mM, 5mM, and 10mM) ,100 nM MSD, and 1mM NH_4 AC were prepared. 100 nM Hg^{2+} ion was added into the solution and then heated at 93°C for 5 min. When the temperature went down to 37°C, GSH was added into the tubes. The final concentration of GSH was 400 nM. These solutions were incubated at 37°C for 40 min, and then the concentrations of free GSH were detected by ESI-MS/MS as mentioned before**.**

Method validation

Artificial samples

For the validation of the method, 200 nM, 100 nM and 50 nM of Hg^{2+} ion solution were prepared, respectively. Each of 5 samples was included in a group. The mean value was taken as the final result.

Real Samples

The applications of the proposed method were evaluated for determination of Hg^{2+} ion in both tap water and lake water samples. Lake water sample was obtained from South Lake of Changchun, Jilin province, China. Tap water and

lake water were filtered through a column packed with anionic exchange resin to remove oil and other organic and biological impurities[1].

Fish sample was collected from commercial market in Changchun, China. The frozen sample was treated as following. The fish was washed with distilled water and dried after defrosting. A portion of the edible muscle tissue was removed from the dorsal part of each fish, homogenized and stored in clean-capped glass vials and kept in a freezer until analysis. A part of the muscles was taken out quickly and dried in an oven at 70 °C for 48 h. After grinding the dry tissue, 5 g of each sample was digested with 10 mL of concentrated HNO_3 in a Teflon beaker for 4 h at 100 °C. After digestion, all of the samples were transparent solutions and pH was adjusted to 7.0. Obtained mixture was filtered into 100 mL Erlenmeyer flask and then concentrated to 5 mL[1]. The mercury ion content of the above samples was analyzed by the proposed procedure and ICP-MS, respectively.

Results and Discussion

The binding of Hg^{2+} ion to mismatched MSD and GSH

In "Reagents and Apparatus" section, the experimental process of Hg^{2+} ion binding to mismatched MSD and GSH was described. S1 contains only 100 nM MSD, S2 contains 100 nM Hg^{2+} ion and 100 nM MSD, and S3 also contains 400 nM GSH besides 100nM MSD and 100nM Hg^{2+} ion. The above samples were analyzed by using ESI-MS/MS in negative ion mode on a Q-TOF mass spectrometer. The results showed that the MSD containing two mismatch base-pairs (5′-CGCGTTGTCC-3′,

5′-GGACTTCGCG-3′) was significantly stabilized for S1 sample without Hg^{2+} ion (Figure 1a), and Hg^{2+} ions bound to MSD in S2 sample under the presence of Hg^{2+} ion (Figure 1b). The formation of T-Hg^{2+}-T pair (MSD+ $2Hg^{2+}$) in S2 sample was also detected by ESI-MS method (Figure 1b). This indicates that T-Hg^{2+}-T pair (MSD+$2Hg^{2+}$) was stable enough to give out quasi-molecular ion peaks of the corresponding duplex with multi-charges. For S3 sample, only $[M-H]^-$ ion at *m/z* 813.0897 for GSH-Hg^{2+}-GSH was observed (Figure 1c). Although the concentration of GSH was rather low, the binding of two GSH molecules to one Hg^{2+} ion was still observed. While the relative abundance of MSD+$2Hg^{2+}$ ion started to decrease and eventually disappear when increasing the concentration of GSH gradually. This result indicates the binding affinity of GSH to Hg^{2+} ion is far greater than MSD in the competition system of MSD and GSH to Hg^{2+} ion. When the concentration of GSH reached to twice amount of Hg^{2+} ion, $[M-H]^-$ ion at m/z306.0681 of GSH started to appear in mass spectrum as shown in Figure 1c, indicating overdose of GSH. Figure1c shows $[(GSH-Hg^{2+}-GSH)-H]^-$ ion (major species), as well as $[GSH-H]^-$ (minor species), and the inset little figure shows the experimental isotopic profile of the GSH-Hg^{2+}-GSH with one charge, which is consistent with its theoretic value.

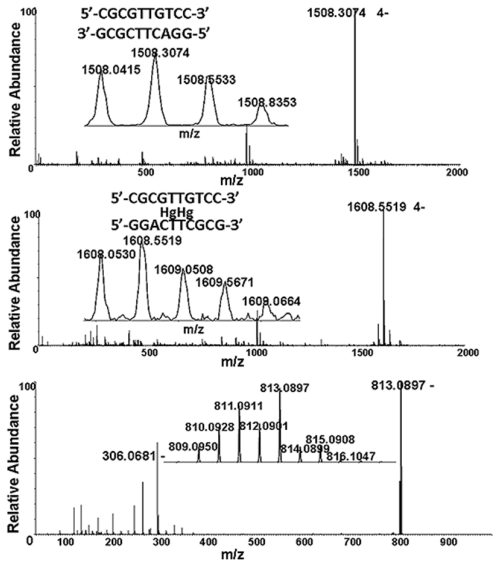


Figure 1. The ESI-MS spectra of MSD(a), MSD with two Hg^{2+} ion (b) and GSH-Hg^{2+}-GSH complex (c) in negative ion mode.

Figure 2a shows the UV absorption spectra of duplex in the presence of different concentrations of Hg^{2+} ion and Figure 2b shows the UV absorption spectra of T-Hg^{2+}-T in the presence of different concentration of GSH (Figure 2b). In Figure 2a, we can see that the optical density decreases with the increasing of Hg^{2+} concentration. A clear transition point at around 1:2 of (Hg^{2+}:thymine residues) was revealed by plotting the optical density against the Hg^{2+} concentration (Figure 2a), indicating the formation of complexes containing one Hg^{2+} ion and two thymine residues (T-Hg^{2+}-T pair). In Figure 2b, we can see that the optical density increases as GSH concentration increasing, and the clear transition point is at around 1:2 ratio of Hg^{2+} ion to GSH.

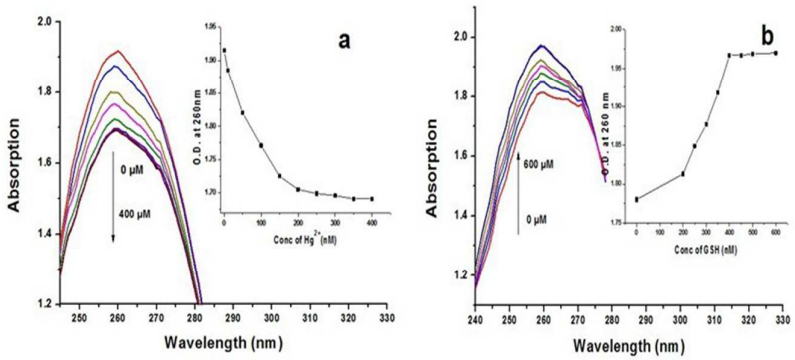


Figure 2. The UV absorption of MSD in presence of different concentration of Hg^{2+} ion (a), and the UV absorption of MSD+2Hg^{2+} in presence of different concentration of GSH (b).

Consequently, the above results indicate that a double helical structure containing only T-Hg^{2+}-T pair was formed and its concentration was affected by GSH concentration in analytes. The ESI-MS results (Figures 1a, 1b) also indicate the formation of the double helical structure. The best concentration of GSH in competing system was double concentration of Hg^{2+} ion.

Sensitivity

Figure 3 shows the optimized mass transitions of 308→76 (a) for GSH as analytical target and 148→84 (b) for Glu as an internal standard in positive ion ESI-MS/MS analysis on MRM mode.

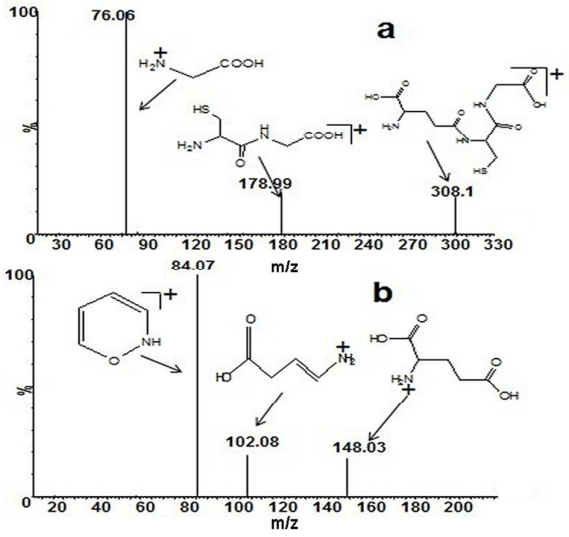


Figure 3. The MS/MS product ion spectra of GSH(a) and Glu as internal standard (b) on positive ion mode.

In the standard curve (Figure 4) of analyte, Y axis is the ratio of the concentration difference of GSH in 5min and 40min and Glu (as internal standard), X axis is the concentration of Hg^{2+} ion. A liner relation was y = 0.0596x + 0.7234 with R^2 = 0.9472. The limit of detection (S/N > 3) was 5 nM/mL. We also changed GSH to cystine in the monitoring system, but it didn't show better liner relation. It might because GSH binds to Hg^{2+} ion more strongly and quickly than cystine, which has been reported by HuiXu previously[34]. By changing the concentration of GSH and MSD in the competition system, we can also monitor the concentration of Hg^{2+} ion out of the liner range (5nM-100nM). When the concentration of GSH is 40 μM and the concentration of MSD is 10 μM, the linear range of Hg^{2+} ion is 10 μM to 200 μM.

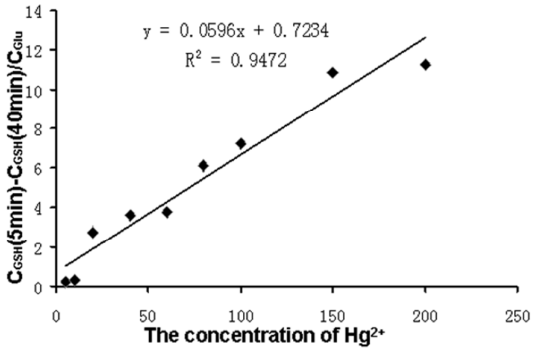


Figure 4. The calibration curve of concentration ratio of free GSH to Glu and concentration of Hg^{2+} solution in ESI-MS/MS analysis on positive ion mode. 100 nM MSD was added in to a series of Hg^{2+} solutions (0, 5, 10, 20, 40, 60, 80, 100, 150 and 200nM) respectively. After the heated and cooled process then added 400 nM GSH detected the concentration of GSH in 5 min and 40 min)

Selectivity

As shown in Figure 5, the addition of different metal ions, such as Na^{+}, K^{+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, Mg^{2+}, and Ca^{2+}, does not change the concentration of free GSH significantly. However, in the cases of Co^{2+} and Ag^{+}, some reactions with GSH were observed (Figure 5).To distinguish the difference of Hg^{2+} with other ions, we investigated the process of GSH competing Hg^{2+} ion with thymine-mismatched DNA. As shown in Figure 5, the reaction in the first 10 minutes is slow, but it is fast in the following 20 minutes and nearly completes after 40 minutes. It is because GSH can sequester Hg^{2+} ion from thymine-Hg^{2+}-thymine (T-Hg^{2+}-T) structure. But for other ions, they cannot bind to the mismatched MSD but bind to GSH directly. Therefore, the concentration of Hg^{2+} ion captured by MSD can be reflected by the concentration

difference of GSH in 5 min and 40 min. Moreover, this method can dramatically differentiate the mercury ion from other metal ions (Figure 6).

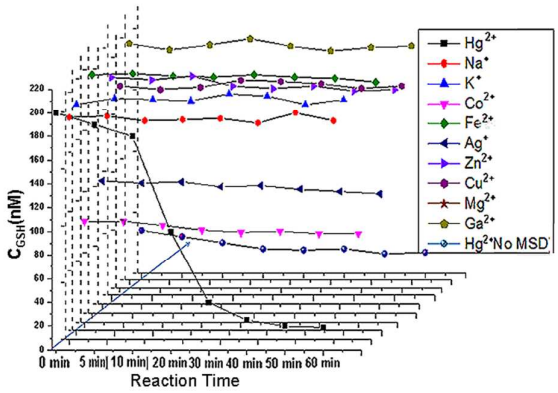


Figure 5. The change of free GSH concentration in competing reaction system with different metal ions solution.(The 50 nM MSD was heated and cooled in 100 nM different ions solution and then added 200 nM GSH , detected the concentration of GSH in 0 min,5 min, 10 min, 20 min, 30 min, 40 min, 50 min and 60 min)

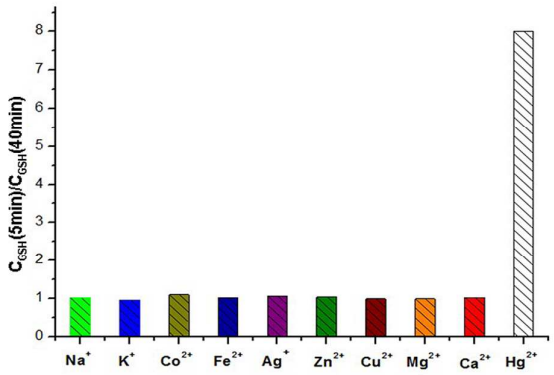


Figure 6. The ratio of free GSH in 5 min and 40 min in competing reaction system with different metal ion solutions. (MSD added to different metal solutions then heated and cooled, and then added GSH, detected the concentration of free GSH

during this action, and then calculate the ratio of the concentration of GSH before and after reaction to distinguish the Hg^{2+} ion)

Salt tolerance

Figure 7 shows the influence of different salt concentrations on the detection of free GSH. The result shows that even with 5×10^4 times higher concentration of salt to Hg^{2+} ion, only minor influence of the detection sensitivity can be observed. In addition, the concentration of salt only affects the Tm value of MSD, but not the detection sensitivity for Hg^{2+} ion.

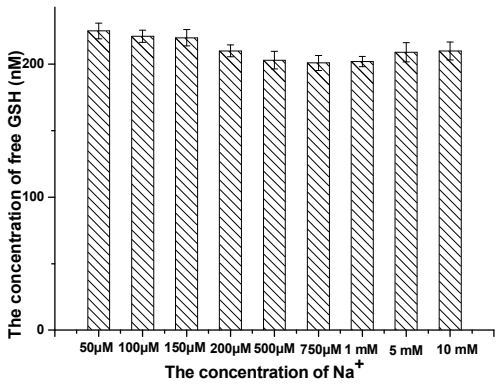


Figure 7. The analysis of free GSH in competing reaction system with deferent concentration of Na^+ solution.(n=3) (100 nM MSD was added into different salt solution to detected 100 nM Hg^{2+} ion, after heated and cooled process then added 400 nM GSH, these was the concentration of GSH after the reaction)

Method validation

The overall mean precision defines by RSD (n=5) is no higher than 8.5. Analytical accuracy, expressed as the percentage difference of the mean

observed values compared with the known concentration, is also no more than 8.5. The result indicates that the precision and accuracy of this method are in the perfect range (Table1).

Table 1. The precision and accuracy in different concentration of Hg^{2+} solutions.

Nominal concentration (nM)	Observed concentration (nM)	Precision (RSD, %)	Accuracy (%)
50	47.56±2.6	5.5	95.12%
100	110.91±5.56	5.1	110.90%
200	186.90±13.6	8.5	93.45%

Real samples

The proposed method was applied for determination of mercury ion content in real samples. For this purpose, the amount of Hg^{2+} ion was determined in tap water, lake water and fish, and the results of this study are listed in Table 2. The U.S. environmental Protection Agency has set the maximum allowable level of Hg^{2+} ion in drinking water at 2 ppb, [36] and maximum lever of 0.3 μg g^{-1} for fish tissue (wet weight) . [37,38] As show in Table 2, the concentrations of Hg^{2+} ion are not detected in tap water and lake water, and for fish, the concentration of Hg^{2+} ion is 0.15 μmol L^{-1}, i.e., 0.03 ppm, after extraction process and concentrated. The concentration of Hg^{2+} ion in the real samples obtained from proposed procedure agrees well with that of ICP-MS method, demonstrating the potential of this Hg^{2+} ion monitoring method for sample analysis.

Table 2. Determination of Hg^{2+} ion in real samples (n=3) using the proposed method and ICP-MS.

Real sample	Added (μmol L^{-1})	Proposed method mean[a] + RSD[b]	ICP-MS Mean + RSD
tap water	0	0	0
	0.5	0.47±0.005	0.51±0.024
Lake water	0	0	0
	0.5	0.48±0.018	0.54±0.036
Fish sample	0	0.15±0.024	0.17±0.037
	0.5	0.69±0.028	0.73±0.031

[a] Mean of three separated measurements.
[b] RSD, relative standard deviation.

Conclusions

In this paper, we developed a novel method for detecting mercury ion in solution system based on "mercury specific DNA" (MSD) and a competing reaction system to Hg^{2+} ion . In which, ESI-MS/MS strategy combined with the competing reaction system of GSH and MSD to Hg^{2+} ion was used to detect the concentration of mercury ion. In this method, ESI-MS/MS technique possesses high sensitivity and the competing reaction systems of GSH and -MSD to Hg^{2+} ion provided high selectivity. Compared with other detection methods, our method has following advantages. (1) Under the condition in this study, the concentration of Hg^{2+} ion changes from 5 to 100 nM with an LOD of 5 nM, i.e., the higher sensitivity of sample analysis can be achieved. (2) During the detection process, only some common materials are needed, thus harmful materials such as the fluorescent dyes, quantum dots, gold nanoparticles, etc., can be avoided. Obviously, there are no harmful effects for the operators and circumvent. (3) In the presence of high concentration salts, such as a 5mM

buffer solution containing 10 mM NaCl, this method is also sensitive enough. It is noted that, in this situation, the concentration of salt is 5×10^4 times than that of Hg^{2+} ion, however, the selectivity is only slightly affected by other ions. (4)This proposed method can also accurately detect the concentration of Hg^{2+} ion in tap water, lake water and fish.

Acknowledgements

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