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Highly sensitive and selective detection of Pb^{2+} ions using a novel and simple DNAzyme-based quartz crystal microbalance with dissipation biosensor†

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A novel, label-free DNAzyme-based quartz crystal microbalance with dissipation monitoring (QCM-D) biosensor was developed for the highly sensitive and specific detection of Pb^{2+} ions. To enhance the performance of the sensor, oligonucleotide-functionalized gold nanoparticles were used for both frequency and dissipation amplification. This sensor was developed by immobilizing Pb^{2+}-specific DNAzymes onto the QCM-D sensor surface and allowing them to hybridize with substrate-functionalized AuNPs. The DNAzyme catalyzed the cleavage of the substrate in the presence of Pb^{2+} ions, causing the cleaved substrate-functionalized AuNPs to be removed from the sensor surface. Thus, Pb^{2+} ions can be determined on-line by monitoring the change in frequency and dissipation signals. The results revealed that the sensor showed a sensitive response to Pb^{2+} ions with detection limits of 14 nM and 20 nM for frequency and dissipation, respectively. This QCM-D biosensor also exhibited excellent selectivity toward Pb^{2+} ions in the presence of other divalent metal ions. In addition, the approach was able to detect Pb^{2+} in tap water, demonstrating its great potential for monitoring drinking water quality. The proposed sensor system described here represents a new class of lead ion sensor. Its simple detection strategy makes it feasible for 'pollution-free' detection; thus, the approach could have applications in on-line water quality monitoring.

1. Introduction

Lead (Pb^{2+}) is considered an ubiquitous and major pollutant in the environment. The U.S. Environmental Protection Agency (EPA) has defined Pb^{2+} as a group B2 human carcinogen. Being exposed to high levels of Pb^{2+} can cause serious health effects.[1] Therefore, the determination of lead in the environment has been of great concern in the past and remains an issue today. As such, the invention of suitable sensors is of great interest as they offer exciting opportunities for *in situ* and real-time detection without exposing the operator to the risk of metal poisoning.

A variety of recognition elements such as proteins,[2] nucleic acids,[3,4] peptides,[5] and small ligands[6] have been employed in various chemosensors and biosensors for the specific detection of Pb^{2+} ions. Among these recognition elements, DNAzymes have been extensively studied and have attracted wide interest due to their numerous advantages.[7] Since their discovery, many DNAzyme-based sensors have been developed using various signal transduction mechanisms to detect a wide range of metal ions with high selectivity and sensitivity.[3,8–12] One of the most effective and widely used Pb^{2+}-dependent DNAzymes is the 8–17 DNAzyme reported by Li and Lu.[3] This DNAzyme may induce RNA substrate cleavage in the presence of Pb^{2+}. Several sensors based on this DNAzyme as a recognition probe have been developed. For example, Lu and co-workers reported a beacon sensor with the DNAzyme and substrate strands labeled with quencher and a fluorophore. In the presence of Pb^{2+} ions, the annealed substrate was cleaved into two pieces, separating the fluorophore and quencher.[3] To improve the sensitivity of this sensor, in a later piece of work an additional quencher was added at the other end of the substrate to lower the background fluorescence.[13] A DNAzyme-based electrochemical biosensor was also reported in which the DNAzymes were labeled with the redox-active compound methylene blue (MB).[14] Although it is selective for Pb^{2+}, the 8–17 DNAzyme is still active in the presence of Mg^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, and Ca^{2+}. Recently, Lu and co-workers found that a DNAzyme called GR-5 is more selective for Pb^{2+} than the 8–17 DNAzyme.[15] They found that Zn^{2+} was the most active interfering metal ion; although the 8-17 DNAzyme offers ~160-fold selectivity for Pb^{2+} over Zn^{2+}, the GR-5 DNAzyme is ~40 000 times more selective for Pb^{2+} than Zn^{2+}. Applications of GR-5 are so far very limited, only a fluorescent

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sensor has been recently developed based on the GR-5 DNAzyme, with the use of graphene for signal amplification.[16] Although all the above-mentioned DNAzyme-based strategies show high sensitivity, most of them operate in the solution phase and require labeling of substrate. This makes them impossible to reuse and subject to high background signal as washing to remove the cleavage product is not allowed. The need to label the substrate also makes them less cost effective. Therefore, the development of sensitive and label-free sensors is receiving more attention.

Among the label-free assay techniques, the quartz crystal microbalance (QCM) has been extensively investigated as a transducer for heavy metal detection in aqueous media. QCM is a mass-sensing device that operates based on the piezoelectric properties of quartz crystals. The change in resonant frequency is proportional to the mass of the absorbed material on the sensing area. It is a cost-effective and simple method that offers opportunities for on-line analysis and quantification of target molecules. Recently, a few QCM sensors have been developed for the detection of Hg^{2+} using thymine-rich oligonucleotides as sensing probes.[17,18] A polymer-grafted QCM chemosensor was also developed for heavy metal ions such as Pb^{2+}, Cu^{2+}, Cr^{3+}, and Cd^{2+}.[19] However, the selectivity of this sensor was poor. To the best of our knowledge, QCM sensors based on Pb^{2+}-dependent DNAzymes have not been reported thus far.

QCM with dissipation monitoring (QCM-D) could provide information on the viscoelastic properties of the bound mass by simultaneous measurements of the frequency and dissipation factor.[20] There have been relatively few efforts made to adapt QCM-D to biosensor development, especially for metal ion detection.[18] Therefore, in this work, a DNAzyme-based QCM-D sensor is developed for the detection of Pb^{2+} ions. The GR-5 DNAzyme is used as a recognition probe for this proposed sensor due to its high sensitivity and selectivity.

2. Experimental

2.1. Chemicals and materials

All oligonucleotides were purchased from Integrated DNA Technologies (IDT) and HPLC-purified. The DNAzyme sequence was 5′-HS-$(CH_2)_6$-TTTTTTTTTTTTACATCATCTCTGAAGTAG CGCCGCCGTATAGTGA-3′, and the substrate sequence was 5′-HS-$(CH_2)_6$-TTTTTTTTTTTTTCACTATrAGGAAGAGATGATGTT-3′.

Sodium citrate, hydrogen tetrachloroaurate ($HAuCl_4 \cdot 4H_2O$), HEPES, potassium phosphate monobasic, potassium phosphate dibasic, tris(2-carboxyethyl)phosphine (TCEP), 6-mercaptohexanol (MCH), lead nitrate ($Pb(NO_3)_2$) and other metal salts were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were of analytical-reagent grade. Ultra-pure water at a resistivity of 18.2 MΩ cm was used in the experiments.

2.2. Preparation of Au-nanoparticles (AuNPs) and substrate-functionalized AuNPs

AuNPs were synthesized by the citrate reduction of $HAuCl_4$. A solution of $HAuCl_4$ (100 mL, 0.01%) was heated to 100 °C for 20 min. Subsequently, 2 mL 0.1 M sodium citrate was added, and the solution was stirred rapidly as it kept boiling. After the colour change, the solution was stirred for an additional 30 min, allowed to cool to room temperature and stored at 4 °C before use. The average diameter of the gold nanoparticles (14 ± 1 nm) was confirmed by transmission electron microscopy (TEM) using a JEOL JSM-6701F (Fig. S1A†).

Substrate-functionalized gold nanoparticles (S-AuNPs) were prepared according to procedures in the literature, with some modification.[21] The thiol-modified substrates (0.4 μM, 100-fold molar excess) were mixed with 1 mL of synthesized AuNPs (≈4 nM) after pre-treatment with TCEP for 30 min at room temperature. After 16 h, the solution was added with concentrated NaCl and phosphate buffer to make the final solution containing 0.1 M NaCl and 10 mM phosphate (pH 7.4) and allowed to stand for another 40 h to increase the density of the substrates immobilized on the AuNPs. Subsequently, the solution was centrifuged at 14 000 rpm for 30 min to remove the excess free substrates. The red oily precipitate was washed and recentrifuged three times with working buffer (25 mM HEPES including 100 mM NaCl, pH 7.4) and redispersed in 1 mL of working buffer. The AuNPs were scanned using UV-visible absorption spectrometry to characterize their change in properties before and after modification by the substrate strands (Fig. S1B†).

2.3. Sensor fabrication and QCM-D detection procedure

QCM-D experiments were performed using a Q-Sense E4 QCM-D instrument (Q-Sense AB, Västra Frölunda, Sweden) with a 5 MHz AT-cut gold-coated quartz crystal. The sensors were prepared offline, and the detection procedure was monitored on-line in the flow-through model. Prior to modification, the quartz crystal was cleaned with a heated solution (30% hydrogen peroxide, 28% ammonia and deionized water in a volume ratio of 1 : 1 : 5) for 5 min, rinsed thoroughly with deionized water and dried with nitrogen gas. From our preliminary experiments, 1 μM DNAzyme was chosen as the optimum condition for sensor fabrication (see ESI Fig. S2†). Hence, the cleaned gold surface was immediately treated with a solution composed of 1 μM of DNAzyme, 20 mM phosphate buffer (pH 7.4), and 100 μM TCEP for 2 h at room temperature to allow the self-assembly of a DNA monolayer on the gold surface. TCEP was added to reduce disulfide bonds. The surface was then soaked in 1 mM ethanoic MCH for 1 h. Subsequently, the DNAzyme monolayer surface was incubated with the S-AuNPs in a 80 °C water bath for 10 min and allowed to cool slowly to room temperature over 2.5 h. The sensor was dried and placed into the flow cell.

Prior to using the prepared sensor for Pb^{2+} sensing, it was allowed to incubate in working buffer for 1 h at 37 °C in the flow cell to remove any physisorbed substrate strands. After equilibration with working buffer, the sensor surface was allowed to react with various concentrations of Pb^{2+} in working buffer for 40 min, after which the flow was switched back to the working buffer. The entire detection procedure was performed at 37 °C with a flow rate of 120 μL min^{-1}, and both frequency and dissipation measurements were recorded. The selectivity of this

sensing system was also evaluated by measuring the frequency response of the system towards other common metal ions.

2.4. Characterization of the QCM-D surface

During the modification procedures, the QCM-D sensor surface was characterized using cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). The CV and EIS measurements were performed using an Ivium Potentiostat. An Au electrode, Pt wire and Ag/AgCl (1 M KCl) electrode were used as the working electrode, counter electrode and reference electrode, respectively. The experimental details are given in Fig. S3.†

2.5. Analysis of tap water samples

The practicality of the proposed method was evaluated by analyzing tap water. Concentrated buffer was added to collected tap water samples to result in final concentrations of 25 mM HEPES including 100 mM NaCl. Subsequently, the tap water samples were spiked with different concentrations of Pb^{2+} ions. The samples were then analyzed by QCM-D using the above described method. The samples were also analyzed by ICP-MS to assess the accuracy of the proposed method.

3. Results and discussion

3.1. Sensor fabrication and design strategy

Two DNA strands are used in the sensor fabrication, GR-5 DNAzyme and the substrate (Fig. 1). One end of the GR-5 DNAzyme was modified with a thiol group to facilitate its immobilization on the gold surface of the sensor. Since the mass of DNA is quite light compared to bacteria and proteins, the mass change that arises from substrate cleavage in the presence of Pb^{2+} is negligible. Therefore, signal amplification is required to improve the sensitivity of the sensor. Gold nanoparticles have been widely used in various biosensors for signal amplification due to their desirable biocompatibilities. In this study, oligonucleotide-functionalized AuNPs were used to amplify the detection signal. The 5′-end of the substrate was extended and modified with a thiol group to bind to the AuNPs. The UV-vis absorption peak of the AuNPs experienced a slight red shift after modification with substrates (Fig. S1B†), indicating the successful conjugation of substrates to AuNPs.

The rationale of the AuNPs-enhanced QCM-D sensor for Pb^{2+} is shown in Fig. 1. GR5-E was first immobilized onto QCM-D sensor and the surface was then backfilled with blocking agent, 6-mercaptohexanol (MCH). The resulting DNAzyme monolayer was then hybridized with S-AuNPs. Each step in the above procedures was characterized by CV and EIS. As shown in Fig. S3,† the peak potential separation and Nyquist diameter increase gradually after modification with DNAzymes, MCH, and S-AuNPs. The results imply that the capability of charge transfer of the redox pair $Fe(CN)_6^{3-/4-}$ over the sensor surface is becoming difficult, indicating the successful modification of DNAzyme and S-AuNPs onto the QCM-D sensor. In the absence of Pb^{2+}, the substrates stayed intact and the entire strands and AuNPs remained on the sensor surface; hence no change in frequency and dissipation can be observed (upper route in Fig. 1). In the presence of Pb^{2+}, the substrates will undergo cleavage at rA position, causing the AuNPs attached on the cleaved-substrates to be removed from the sensor surface. The decrease in the number of AuNPs on sensor surface resulted in an increase in frequency signal and a decrease in dissipation factor (bottom route in Fig. 1). The increase in frequency is due to huge mass loss of S-AuNPs from the sensor surface, while the decrease in dissipation is due to the release of "soft" substrate chains conjugated on AuNPs and the large coupling of water molecules that are trapped in between the oligonucleotide chains. Since the amount of substrate cleavage relies on the concentration of Pb^{2+} ions, it is possible to determine the concentration of Pb^{2+} ions in the solution by monitoring the frequency and dissipation changes of the QCM-D sensor. In order to confirm that the changes in frequency and dissipation resulted from substrate cleavage, SEM images of the sensor surface before and after reaction with Pb^{2+} were compared. As shown in Fig. 2, the number of AuNPs on the sensor surface was decreased after reaction with Pb^{2+}, which was in accordance with the proposed principle. The uniform distribution of AuNPs on the sensor surface also indicated that the AuNPs were well distributed on the sensor surface without evidence of aggregation.

3.2. The performance of the DNAzyme-QCM-D sensor for Pb^{2+} detection

To study the performance of the proposed sensor, the sensors were treated with Pb^{2+} at different concentrations. The Pb^{2+}

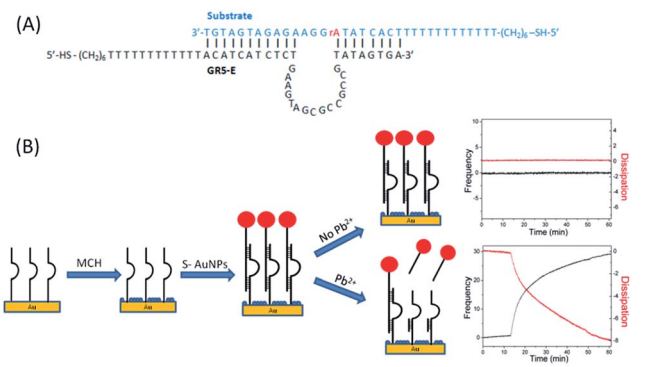

Fig. 1 Schematic of the fabrication of the QCM-D biosensor for the detection of Pb^{2+} ions based on the DNAzyme.

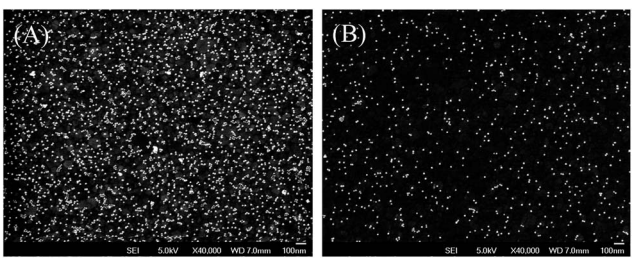

Fig. 2 SEM topographic images of QCM-D sensor before (A) and after (B) reaction with Pb^{2+} at concentration of 2000 nM.

solutions were allowed to flow through the surface of the prepared sensors on the QCM-D instrument, and the frequency and dissipation changes were monitored on-line. Based on the response profile obtained, it is apparent that a longer flow through time of the Pb^{2+} solution on the sensor surface may result in greater frequency and dissipation response as more substrates are cleaved. Hence, in this experiment, the Pb^{2+} solution was allowed to flow through the sensor surface for 40 min in order to achieve enough sensitivity within a certain period of time. Fig. 3A shows the frequency change upon detection with the Pb^{2+} concentration ranging from 0 to 3000 nM. The frequency change increased gradually with increasing Pb^{2+} concentration as an increasing number of substrates were cleaved by DNAzymes; this result is in accordance with the principle of the method. A linear relationship between frequency change and Pb^{2+} concentration was obtained from 46–3000 nM Pb^{2+} ($R^2 = 0.997$). The frequency response reached saturation at concentrations above 3000 nM as most of the substrates on the sensor were cleaved. The detection limit of the sensor was determined to be 14 nM based on 3σ/slope, which is lower than the US EPA defined toxicity level for Pb^{2+} in drinking water (72 nM); this detection limit is either comparable or better than previously reported Pb^{2+} sensors.[3,14,19,22–26] Similar to the frequency response, an obvious increase in dissipation change was observed with increasing Pb^{2+} concentration as the sensor surface became more "rigid" after the removal of the substrates of "soft" nature conjugated on AuNPs (Fig. 3B). The dissipation response was linearly related to the concentration of Pb^{2+} ions in the range of 66–3000 nM ($R^2 = 0.994$) with a detection limit of 20 nM. Herein, the dissipation change that resulted from the oligonucleotide-functionalized AuNPs was investigated to provide a novel and alternative quantification parameter for the development of oligonucleotide sensors. Compared to dissipation, the frequency response gives better sensitivity and reproducibility. Although the sensitivity of dissipation is relatively low, it can serve as a complementary method to support the accuracy of the frequency results by providing viscoelastic information about the sensor surface. This is rather important in the analysis of real water samples containing complex matrices as many external factors could give rise to false positive results. The use of dual-signal measurements could help confirm that the responses indeed arise from Pb^{2+}, eliminating the possibility of false-positives. Meanwhile, in the control experiment, no evident changes in frequency and dissipation were observed in the absence of Pb^{2+} ions (curve of 0 nM). This result further confirms that the binding of S-AuNPs with the DNAzymes is a highly specific interaction.

Many reported immobilized sensors suffer from time-consuming and multistep processing as the signal amplification step is introduced during or after the reaction with the target.[17,18,25,27] Compared to these sensors, our approach does not require lengthy hybridization procedures or the handling of DNA-containing solutions during the detection procedure; instead, our procedure can be used for direct assay detection of Pb^{2+} after sensor preparation, and the sensor can be prepared ahead of time and stored. The attachment of DNAzymes to the sensor surface also allows the regeneration of the sensor by

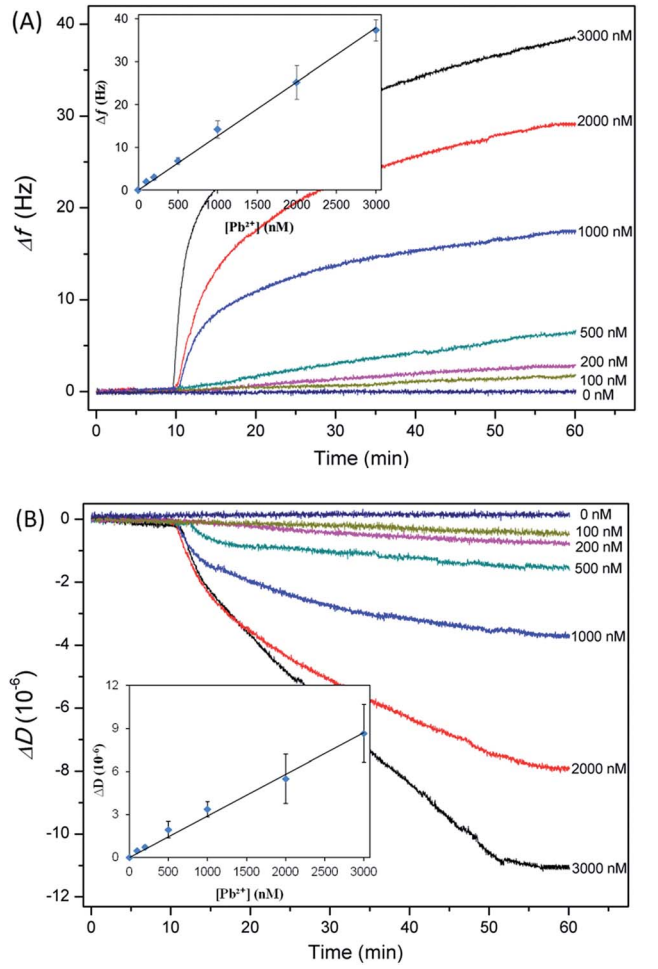

Fig. 3 On-line frequency (Δf) (A) and dissipation (ΔD) (B) responses of the QCM-D biosensor for Pb^{2+} detection at concentrations of 0, 100, 200, 500, 1000, 2000 and 3000 nM. Insets: the linear relationships between the frequency change and dissipation change against Pb^{2+} concentration. Error bars are standard deviations of three repetitive experiments.

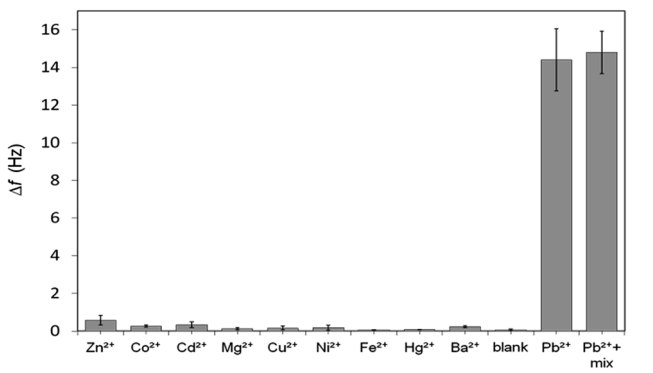

Fig. 4 Selectivity of the QCM-D sensor for Pb^{2+} detection; the frequency change between the blank and the solutions containing different metal ions. Concentration of Pb^{2+}: 1 μM, concentration of other metal ions: 10 μM. Error bars are standard deviations of three repetitive experiments.

Table 1 Determination of Pb^{2+} in tap water samples using the proposed QCM-D sensor and ICP-MS. The mean and standard deviation values are based on three repetitive experiments

Pb^{2+} concentration			
Added concentration	Measured (mean ± SD)	Recovery (%)	ICP-MS (mean ± SD)
50 nM	49.7 ± 1.6	99.5	53.0 ± 0.1
200 nM	199.7 ± 7.5	99.9	202.7 ± 0.9
500 nM	474.6 ± 15.3	94.9	476.5 ± 1.9
2000 nM	1800.4 ± 14.1	90.0	1914.3 ± 15.6

washing off the cleaved substrates and replacing them with new substrates. By changing the DNAzyme probe, the design of this sensor could also be extended to detect other metal ions such as Cu^{2+}, Cd^{2+} and Hg^{2+}.

The selectivity of this sensor for Pb^{2+} was also investigated by detecting several environmentally relevant metal ions (including Zn^{2+}, Co^{2+}, Cd^{2+}, Mg^{2+}, Cu^{2+}, Ni^{2+}, Fe^{2+}, Hg^{2+} and Ba^{2+}) at a higher concentration (10 μM) under the same conditions. As shown in Fig. 4, the frequency changes for the other metal ions were very small, even though they were present at concentrations 10 times more than Pb^{2+} (1 μM). This indicates that these metal ions have negligible effects on the proposed sensor. This result suggests that the approach offers excellent selectivity towards Pb^{2+} ions, giving it potential for future use.

3.3. Detection of Pb^{2+} in tap water

To demonstrate the practical application of this sensor in water analysis, the performance of the proposed method was evaluated by analysing tap water. Tap water samples were spiked with different concentrations of Pb^{2+} ions and analyzed by the QCM-D sensor. As tabulated in Table 1, the values obtained using the proposed method showed good agreement with the added values and good recoveries. In addition, these results showed good consistency with the ICP-MS measurements. These results demonstrate that this method has a good accuracy for the determination of Pb^{2+} ions in drinking water.

4. Conclusions

In conclusion, we have developed a simple, label-free, sensitive and selective DNAzyme-based QCM-D sensor for the rapid detection of Pb^{2+}. This method combines the high selectivity of DNAzymes with the high sensitivity of piezoelectric detection, making it possible for pollution-free detection in water. To our best knowledge, this is the first example of a DNAzyme-based QCM biosensor for Pb^{2+}. Based on this method, detection limits as low as 14 nM and 20 nM are achieved for frequency and dissipation, respectively, which are lower than the EPA regulated level for Pb^{2+} in drinking water. This sensor also offers excellent selectivity toward Pb^{2+} in relation to other potentially coexisting metal ions. The satisfactory results obtained for the analysis of tap water demonstrate the feasibility of this approach for the determination of Pb^{2+}. The proposed work extends the application of QCM-D to metal ion sensing by providing high sensitivity and dual-signal measurement. Compared to conventional techniques such as ICP, this label-free detection strategy is much more cost-effective and smaller in size, providing the possibility for on-site and real-time detection of Pb^{2+}. This simple detection strategy could find applications in on-line water quality monitoring that requires the continuous measurements of contaminants in water.

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