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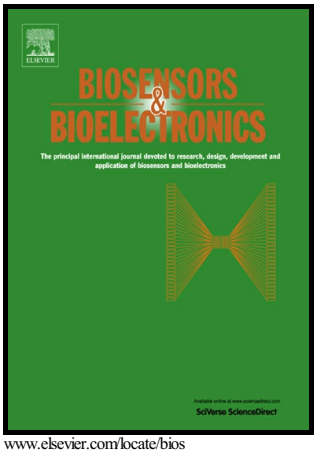

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Visually multiplexed quantitation of heavy metal ions in water using volumetric bar-chart chip

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Abstract

Heavy metal ions monitoring in water is practically significant for the environment and the human health. In this work, a lab-on-a-chip biosensor was developed for multiplexed quantitation of heavy metal ions by the integration of triple-channel volumetric bar-chart chip with DNA-nanoparticle probes. This method possesses the capability for rapid detection of Cu^{2+}, Pb^{2+} and Hg^{2+} simultaneously with high sensitivity, selectivity and accuracy. Due to the highly catalase-like activity of the

urchin-like platinum nanoparticles, this sensor exhibits high sensitivity with the detection limits of 1.0 nM, 1.0 n M and 1.8 nM for sensing of Cu^{2+}, Pb^{2+} and Hg^{2+} respectively, which are much lower than the requirement of the United States Environmental Protection Agency (EPA) in drink water and superior to the colorimetric method based on the metal ion-specific DNA assay. The accuracy and reliability were verified by our Atomic Absorption Spectrometry (AAS) and Atomic Fluorescence Spectroscopy (AFS) measurements of real water sample. As the results can be directly read out without the need of the external instruments or complicate data processing, this sensor is extremely practical for environmental applications.

Keywords: microfluidics, heavy metal ions, biosensor, platinum nanoparticles

1. Introduction

Heavy metals pollution is a great threat to the environment and the human health. Heavy metals are toxic that can damage nervous system, blood composition and other vital organs, including lungs, kidneys, liver. Among these heavy metals, copper, lead and mercury are the common contaminations in water, which can cause a series of diseases, such as Wilson's disease, irreversible brain damage, kidney failure and so on (Needleman 2004; Schell et al. 2009; Tchounwou et al. 2003). Copper is a human

essential metal for body functions, but are toxic in excess. Lead and mercury can cause severe disease or even death at low-level exposure (Cui et al. 2016). According to the United States Environmental Protection Agency (EPA), the maximum allowable levels of Cu^{2+}, Hg^{2+}, Pb^{2+} are 20 μM, 72.4 nM and 10 nM respectively. Therefore, quantitative and selective determination of the heavy metal ions is practically significant for prevention and control of the water pollution. Although the conventional instruments, including inductively coupled plasma mass spectrometry (ICP-MS) (Corte Rodríguez et al. 2017), inductively coupled plasma optical emission spectrometry (ICP-OES) (Hall et al. 2017), atomic absorption spectrometry (AAS) (Resano et al. 2014) and atomic fluorescent spectrometry (AFS) (Liu et al. 2012) are capable of the multiple and quantitative determination of metal ions, they require sophisticated apparatus, high cost and skilled personnel (Taylor et al. 2018). For these reasons, these instruments are impractical for on-site, real-time detection in low-resource settings.

The DNA-based biosensors have been developed for detection of heavy metal ions in recent years (Huang and Liu 2016; Li et al. 2014; Lu 2003, 2004, 2007; Xiang and Lu 2013a; Yuen et al. 2014), which are attributed to the remarkable characteristic of the metal binding affinity and specificity of some DNA sequences (Zhang et al. 2011). These DNA sequences are obtained through the systematic evolution of ligands by exponential enrichment (SELEX) (Huang et al. 2014a; Xiang and Lu 2011; Zhou et al. 2017). The DNA-based biosensors show the remarkable advantages over the traditional methods: First, the 3D binding pocket mode for specific metal coordination

is versatile and easier to achieve in comparison with the rational ligand design. Second, with the high stability, DNA could be renatured after denaturation and doesn't lose its affinity. Third, the DNA sensors show the features such as design flexibility, facility for modification and low-cost chemical synthesis (Zhou et al. 2017).

To detect the heavy metal ions in water, the DNA-based biosensors can be designed for colorimetric assay in low-resource settings, while most of them can only achieve qualitative or semi-quantitative analysis. Also, it is difficult to acquire such low detection limits for drinking water monitoring without the assistance of instrument. Currently, only a few visualized sensors without sophisticated instruments can meet the required detection limits of the EPA. Yang et al. utilized DNA enzyme cross-linked hydrogel for visual quantitative detection of Pb^{2+} with a detection limit of 2.5 nM (Huang et al. 2014b). Lu et al. designed a Pb^{2+}-specific DNA enzyme sensors, using a personal glucose meter (PGM) for quantification with a detection limit of 16 nM (Xiang and Lu 2011). Fu et al developed a portable DNA-gated mesoporous silica nanoparticles sensor to quantify Hg^{2+} by PGM with a detection limit of 0.1 nM (Xiang and Lu 2013b). However, these visual methods only involve single metal ion detection, which limit their wide applications in water pollution. Therefore, it still need a sensor for on-site and real-time multi-metal ions detection with accurate quantification and high selectivity.

In this work, we proposed a visually multiplexed quantitation method for detection of heavy metal ions in water based on our previously reported volumetric

bar-chart chip (V-Chip) technology. V-Chip can present an on-chip visualized ink bar chart, which relies on the measurement of the production of O_2 from H_2O_2 catalyzed by catalase or platinum nanoparticles (Song et al. 2014). The results can be directly read out without the need of external instruments, data processing or graphic plotting (Song et al. 2013). An enzyme-linked immunosorbent assay (ELISA) reaction (Song et al. 2012), DNA hybridization and hybridization chain reaction (HCR) have been integrated with V-Chip for the measurement of biomarker, target protein, nucleic acid and cancer cell in a quantitative and multiplexed manner (Wang et al. 2016). With the features of visualized readout and multiplexed measurement, V-Chip would supply a portable sensor for rapid, low-cost, on-site and real-time detection of a broad range of targets in the field.

Herein a triple-channel V-Chip (TV-Chip) is fabricated to integrate with DNA-nanoparticle probes for quantitative detection of Cu^{2+}, Pb^{2+} and Hg^{2+} simultaneously. Firstly, we prepared the Cu^{2+}, Pb^{2+} and Hg^{2+} probes respectively. Each probe consisted of the magnetic silica nanoparticles (MSNPs) functionalization with amino-modified nucleic acid and the urchin-like platinum nanoparticles (PtNPs) probe functionalization with thiol-modified nucleic acid. Then the MSNP-DNA and the PtNP-DNA were assembled to form the complex probes. In the presence of the target metal ions, the nucleic acid strands attached to MSNPs will be cleaving or structure-switching, which leads to the decreased affinities between the two hybridized nucleic acid strands and make the complex probes unstable. As a result, the PtNP probes dissociate from the MSNP probes. After magnetic separation, the

released PtNP probes in the solution can be collected for V-chip measurement respectively. Through sliding the chip, the PtNPs were brought in contact with H_2O_2 to produce O_2 to push the preloaded ink bars movement. As the distances of ink bars movement were proportional to the concentration of the metal ions, it can be used to visually quantitate Cu^{2+}, Pb^{2+} and Hg^{2+} in a chip simultaneously.

2. Experimental

2.1 Materials and chemicals

Materials for TV-Chip fabrication, preparation of MSNPs and PtNPs list in the Supporting Information. All the metal ions were prepared in 10 mM stock solution at 4 °C refrigerator. Oligonucleotides were synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China) and used as received:

Name	Sequences of oligonucleotide
Cu-Sub	5'-NH_2-$(CH_2)_6$-AAAAAAAAAGCTTCTTTCTAATACGGCTTACC-3'
Cu-Enz	5'-SH-$(CH_2)_6$-AAAAAAAAAGCTTCTTTCTAATACGGCTTACC-3'
Pb-Sub	5'-NH_2-$(CH_2)_6$-AAAAAAAAAGTAGAGAAGGrATATCACTCA-3'
Pb-Enz	5'-SH-$(CH_2)_6$TGAGTGATAAAGCTGGCCGAGCCTCTTCTCTAC-3'
Hg-T	5'-NH_2-$(CH_2)_6$TCATGTTTGTTGGCCCCCCTTCTTTCTTA-3'
Hg-C	5'-SH-$(CH_2)_6$-5'ACAAACATGA-3'

2.2 Characterization and measurement

The urchin-like PtNPs were characterized using JEM-200CX Transmission Electron Microscope (JEOL Company, Japan) and MSNPs were characterized using

S-3400N II Eletron Scanning Microscope (Hitachi, Japan) respectively. PtNPs and MSNPs are 19 nm±3.6 and 47 nm ±7.8 in diameter respectively (Fig.1). The concentrations of Cu^{2+} and Pb^{2+} in water sample were tested using AA240 Duo Atomic Absorption Spectrometry (Varian Company, USA). The concentration of Hg^{2+} in water sample was measured using AF-610 Atomic Fluorescence Spectroscopy (Rui Li Analytical Instrument Co., Ltd, China)

2.3 TV- Chip fabrication

TV-Chip was designed using the Auto CAD software and firstly fabricated with the standard photolithography and wet etching. The structures could be produced with a depth of approximately 50 μm at 40 °C in 40 min etching. Access holes were prepared with a diamond drill of 1.0 mm diameter. The glass plates were cleaned using acetone, isopropanol solution and oxygen plasma. Then the glass plates were modified with 1H,1H,2H,2H-perfluorooctyltrichlorosilane to give a surface hydrophobic modification. Finally, the top plate (Fig.S1c) was "face-to-face" aligned with the bottom plate (Fig.S1d) with the spreading of 2-methyl silicon oil between the two glass slides to assist the device assembly as shown in Fig. S2. The ultrathin oil layer served as a lubricant and prevents O_2 leakage during operation.

2.4 Preparation of the MSNP probes

For Cu^{2+} sensor, the nucleic acid modified MSNP probes were carried out by adding 1 ml 0.1 mg/mL MSNPs solution, 1 μL of 1 μM Cu-Sub and 4 mL of S1 buffer (25 mM Tris-acetate buffer, 150 mM $NaNO_3$, pH 8.2). The mixture stirred in the dark at 20℃ for 12 h. For Pb^{2+} and Hg^{2+} sensor, 1μL of 1 μM Pb-Sub and 1 μL of

1 μM Hg-T were added respectively, the other experimental conditions are the same for preparation of the MSNP probes in Cu^{2+} sensor. The MSNP probes were magnetically collected and the washing procedure with S1 buffer was repeated 5 times to remove the unbound nucleic acid.

2.5 Preparation of the PtNP probes

Thiol-modified DNA was activated by incubating with TCEP. 1 μL of 10 μM Thiol-Cu-Enz in Millipore water was incubated with 2 μL of 30 μM freshly prepared TCEP and 2μL of 10 mM acetate buffer pH 5.5 at room temperature for 1 hour. 0.1 mL of 1 mg/mL PtNPs aqueous solution and TECP-treated thiol DNA were mixed in 4 mL Milli-Q water and stirred at 4 ℃ for 10 h. The mixture was then centrifuged at 5000 rpm for 5 min, and washing procedure with Milli-Q water was repeated 4 times to remove the unbound DNA. Then the PtNP probes were dispersed in 0.1 mL Milli-Q water. 1 μL of 1 μM Thiol-Pb-Enz and 1 μL of 1 μM Thiol-Hg-C were respectively for preparation the PtNP probe of Pb^{2+} and Hg^{2+} sensor and the procedure was the same as that for Cu^{2+} sensor.

2.6 Preparation of complex probe

30 μL PtNP probes and the collected MSNP probes were added to 4 mL S1 buffer for incubation at room temperature. After 4 h, the complex probes were magnetically collected and the washing procedure with S1 buffer was repeated more times to remove the excess PtNP probes. The complex probes were suspended in 30 μL S1 buffer and kept in the dark at 4 °C.

2.7 TV-Chip assay

5 μL of different concentrations of Cu^{2+}, Pb^{2+} and Hg^{2+} solution were added to 25 μL of their respective complex probes in S1 buffer. The reaction was allowed to take place with shaking in dark place for 30 min. After magnetic separation, the supernatants and the reagents were transferred to their respective wells of TV-chip, using 20 μL pipette. The TV-chip was horizontally slid by moving the top plate against the bottom plate. The released PtNP probes were mixed with 30 % H_2O_2. Then the generation of O_2 pushed the ink to move in the small channel. The distances of ink bar movement were recorded at 10 min used for analysis, according to the scales beside the channels on the TV-chip. Primary data were transferred to the graphics program Origin for plotting and analysis (Song et al. 2010; Wang et al. 2017) .

3. Results and discussion

3.1 Design and fabrication of TV-Chip

The TV-Chip was designed and fabricated according to our previous procedures (Wang et al. 2016). Two 75×50×1.5 mm glass slides were used to fabricate TV-Chip. Channel patterns were first drawn using AutoCAD software and fabricated onto the glass surfaces by standard photolithography and wet etching protocols. A detailed protocol is provided in the Experimental section. The device shows large reagents loading volume compared with our previous multiplexed V-Chip design, which improves the sensitivity and is suitable for detection of various samples. Moreover, the horizontal slide operation in TV-chip to mix the reagents and samples makes it easier than the previous oblique slide design. The typical finished top and bottom

glass plates were presented in Fig. S1c and S1d. After the chip was assembled in the presence of silicon oil, the ink, samples and H_2O_2 can be loaded in one drilled hole to their respective wells of TV-Chip by pushing the pipette manually (Fig. S2). Three detection units share one channel for ink loading. A horizontal slide will make the H_2O_2 mixed with the sample. If the sample wells contain the PtNPs, which will efficiently catalyze the H_2O_2 to O_2, the produced O_2 pressure will push the ink into the small channels to form ink bars. The micro-channels are vented to atmospheric pressure, thus the ink bars will keep moving until the produced O_2 is not sufficient to push them.

3.2 Principles of the DNA-nanoparticle probes

As shown in Scheme 1, the PtNP probes will dissociate from the MSNP probes in the presence of Cu^{2+}, Pb^{2+} or Hg^{2+}. After magnetic separation, the released PtNP probes can be collected for TV-chip measurement respectively. For the Cu^{2+} sensor, the complex probes consist of the Cu-Enz modified PtNP probes and the Cu-Sub modified MSNP probes. It can be seen in Fig. 2a, the 5'-portion of the Cu-Enz hybrids the Cu-Sub by Watson-Crick base pairs and the 3'-region through formation of a DNA triplex. In the presence of Cu^{2+}, the Cu-Enz can irreversibly cleave the Cu-Sub into two segments at the cleavage site.

For the Pb^{2+} sensor, the secondary structure of Pb^{2+}- dependent DNAzyme system is shown in Fig. 2(b), the complex probes contain the Pb-Enz modified PtNP probes and the Pb-Sub modified MSNP probes. The Pb-Sub binds to the Pb-Enz through Watson-Crick base pairing reigns on the two ends. As the cofactor (Pb^{2+}) is present,

Pb-Enz possesses the RNA nuclease activity to cleave the ribonucleotide in the Pb-Sub (all other nucleosides are deoxy-ribonucleotides). The cleavage site is indicated by a red arrow in Fig 2b.

Hg^{2+} can induce one single T-rich DNA strand (named Hg-T) to fold into a hairpin structure via T–Hg–T formation. This specific interaction can stabilize T–T mismatches in a DNA duplex. The remaining base pairs between two the hybridized strands are not long enough to keep both strands stable, resulting in another partial complementary DNA strand (named Hg-C) released from the Hg-T. So Hg^{2+} sensor was designed as the Hg-T modified MSNP probes hybridization with the Hg-C modified PtNP probe (Fig. 2c).

3.3 Optimizing the sensitivity of the TV-Chip sensor

In our experiment, we found the amounts of MSNPs, PtNPs and nucleic acids in preparation of the probes were all affect the assay sensitivity. To obtain the best sensitivity, the concentrations of the probe components were carefully evaluated by measuring the corresponding distances of the ink bar. We first evaluated the concentration of MSNPs. 1, 0.1, 0.05 and 0.01 mg/mL MSNPs were added into 1μM nucleic acids to prepare the MSNP probes. When 0.05 and 0.01 mg/mL MSNPs solution were employed, it suffered obvious product loss in the separation steps. In the subsequent detection, the result of 1 mg/mL MSNPs solution showed very low sensitivity, the reason may be owing to the highly concentrated MSNPs solution would increase the aggregation of the complex probes. As a result, it need much more metal ions to dissociate the complex probes, which would be adverse to improve the

sensitivity. Therefore, 0.1 mg/mL MSNPs was selected to prepare of the MSNP probes.

It is also very important to optimize the amount of nucleic acids attached to the MSNPs. The insufficient or excessive amount of nucleic acid can influence the detection sensitivity. Different concentrations of Cu-Sub were mixed with 0.1 mg/mL MSNPs to evaluate influence of the concentration of nucleic acids attached to the MSNPs. As shown in Fig.S3, as the Cu-Sub concentration was 1 μM, it provided the furthest ink advancement. However, the higher concentration of nucleic acids could not improve the sensitivity, which may be due to the formation of mutual cross-linked complex leading to the decreased sensitivity. Thus 1 μM Cu-Sub, Pb-Sub and Hg-T was used to prepare the MSNP probes for Cu^{2+}, Pb^{2+} and Hg^{2+} sensing.

In addition to the MSNP probes, the PtNP probes also affect the sensitivity. In our experiment, the optimized concentration of PtNPs for preparation the complex probes was 1 mg/mL. Under this condition, the amounts of DNA bind to the PtNPs were evaluated. The DNA bound to the PtNPs involved in the formation of the complex probe and the PtNPs dissociation from the MSNP probes in the presence of metal ions. Therefore, the amounts of DNA bound to the PtNPs showed the effect on the efficiency of the cleavage of the Pb-, Cu-Sub and the dehybridization of the Hg-C. The ink movement distances of the different sensors were tested at 1, 10 and 100 μM DNA respectively. As shown in Fig.S4, it was found that 10 μM Cu-Enz, 1 μM Pb-Enz and 1 μM Hg-C showed the furthest ink advancements.

An optimized pH value for DNA cleaving or structure-switching and the assay

time are also important for the sensitivity. In our experiment, the optimal condition was pH 8.2 for PtNPs dissociation in the presence of the metal ions (Fig. S5). To optimize the assay time, the time dependence of the ink advancement of Cu^{2+}, Pb^{2+} and Hg^{2+} were recorded respectively (Fig. 3a). The results showed that the distance increased markedly as the time increased from 0 to 10 min, and it reached a plateau after 10 min. Therefore, all the test results were recorded at 10 min for the point-of-care detection of the metal ions.

3.4 TV-Chip for detection of Cu^{2+}, Pb^{2+} and Hg^{2+}

As the produced oxygen gas are directly associated with the amounts of the released PtNP probes, the distances of ink movement propelled by the oxygen gas are proportional to the concentration of metal ions. As shown in Fig. 2d, 2e and 2f, when different concentrations of Cu^{2+}, Pb^{2+} and Hg^{2+} were tested in the TV-Chip, the ink bar charts showed that advancements were depended on the concentration of the metal ions. Upon increasing of concentration of metal ions, the ink bar chart exhibited a gradient of distances. Fig. 3b, 3c and 3d show the calibration curves corresponding to ink advancements at 10 min for Cu^{2+}, Pb^{2+} and Hg^{2+} detection, which suggested that the TV-Chip was able to quantitate the Cu^{2+}, Pb^{2+} and Hg^{2+}. Accordingly, the detection limit for Cu^{2+}, Pb^{2+} and Hg^{2+} are 1.0 nM, 1.0 nM and 1.8 nM respectively, which are calculated by the average signal of the background plus three times standard deviation. These detection limits compared favorably with fluorescent assay (Cu^{2+}, 9.2 nM; Pb^{2+}, 9.3 nM; Hg^{2+}, 3.2 nM)(Wang et al. 2008; Zuo et al. 2009) and are superior to colorimetric method based on the same metal ion-specific DNA assay.

This high sensitivity may be attributed to the reasonable design of TV-Chip and high catalase-activity of urchin-like PtNPs, in which the platinum structures give a highly efficient activity.

To evaluate the selectivity of the TV-Chip assays, the target metal ions were tested in the presence of 1 μM Ca^{2+}, Mn^{2+}, Cd^{2+}, Mg^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}. The results in Fig. 4a, 4b and 4c showed that no significant distance change was observed for detection of Cu^{2+}, Pb^{2+} and Hg^{2+} in the absence and presence of other metal mixtures, which indicated that the signal response to Cu^{2+}, Pb^{2+} and Hg^{2+} was not affected by other metal ions. Therefore, the method is suitable for selectively monitoring Cu^{2+}, Pb^{2+} and Hg^{2+} in the presence of other metal ions.

Multiplexed assays are crucial for the real sample detection. The proposed method was employed for multiplexed analyzing the river water samples. The collected water samples were spiked with Pb^{2+} and Hg^{2+} and filtered through a 0.22 μm filter membrane to remove the insoluble substance. The Cu^{2+}, Pb^{2+} and Hg^{2+} were simultaneously measured in a TV-Chip and the results were recorded (Fig. 4d). As in Fig. 4e, our results are in a good agreement with the results of AAS and AFS. Therefore, the TV-Chip is capable for multiplexed detection of Cu^{2+}, Pb^{2+} and Hg^{2+} in real samples with good accuracy.

Conclusions

In summary, we have developed a visually quantitative method based on TV-Chip for multiplexed detection of Cu^{2+}, Pb^{2+} and Hg^{2+} in water without the need

of the external instruments or complicate data processing. This method exhibited high sensitivity and selectivity for detection of Cu^{2+}, Pb^{2+} and Hg^{2+}. The detection limits are much lower than the requirement of EPA in drink water, which compare favorably with the fluorescent method and are superior to the colorimetric sensor based on the metal ion-specific DNA assay. This method also demonstrated good accuracy and reliability in real sample assays that were verified by AAS and AFS instruments. The design principle of this work can be generally applied to a broad range of targets by changing the metal ion-responsive DNA substitutes. This sensitive, portable, low-cost, and user-friendly platform may be integrated with the smartphone for real-time environment monitoring in the future.

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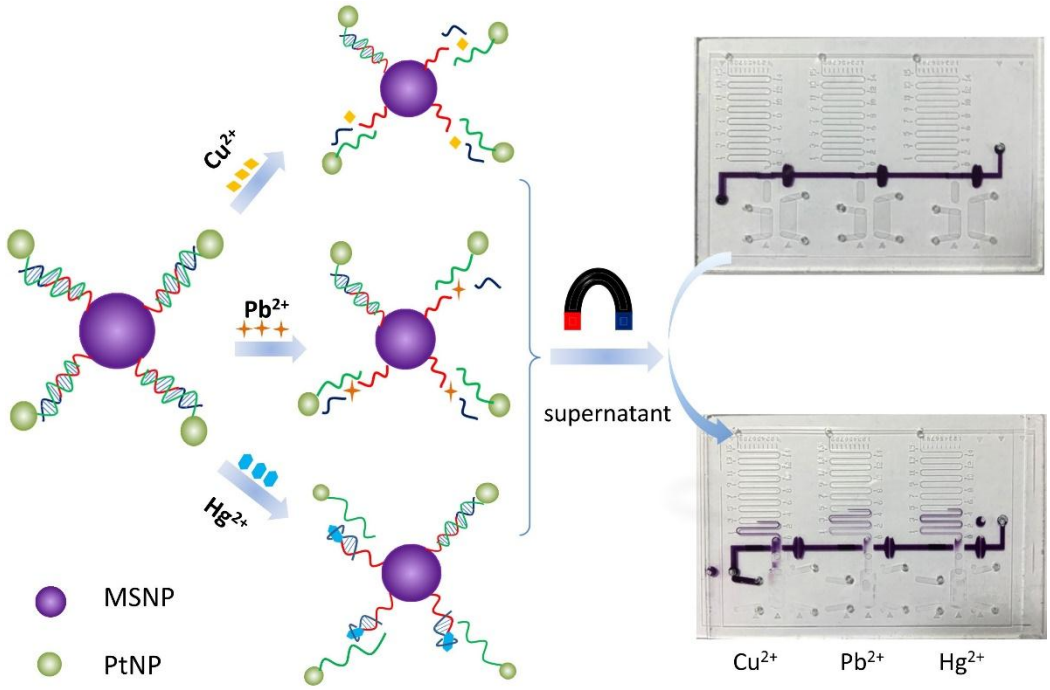


Scheme 1. The working principle for the detection of Cu^{2+}, Pb^{2+} and Hg^{2+} using TV-Chip integrated with DNA-nanoparticle probes.

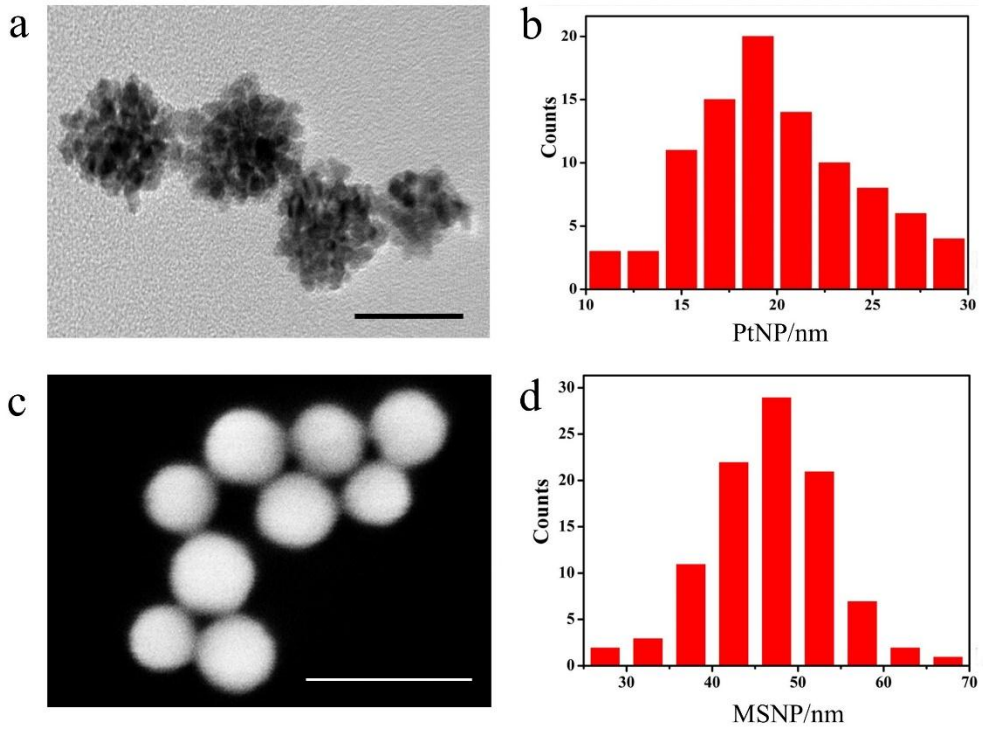


Fig. 1. Characterization of the nanoparticles. (a) TEM image and (b) size distribution of urchin-like platinum nanoparticles (PtNPs). The scale bar is 20 nm. (c) SEM image and (d) size distribution of magnetic silica nanoparticles (MSNPs). The scale bar is 100 nm.

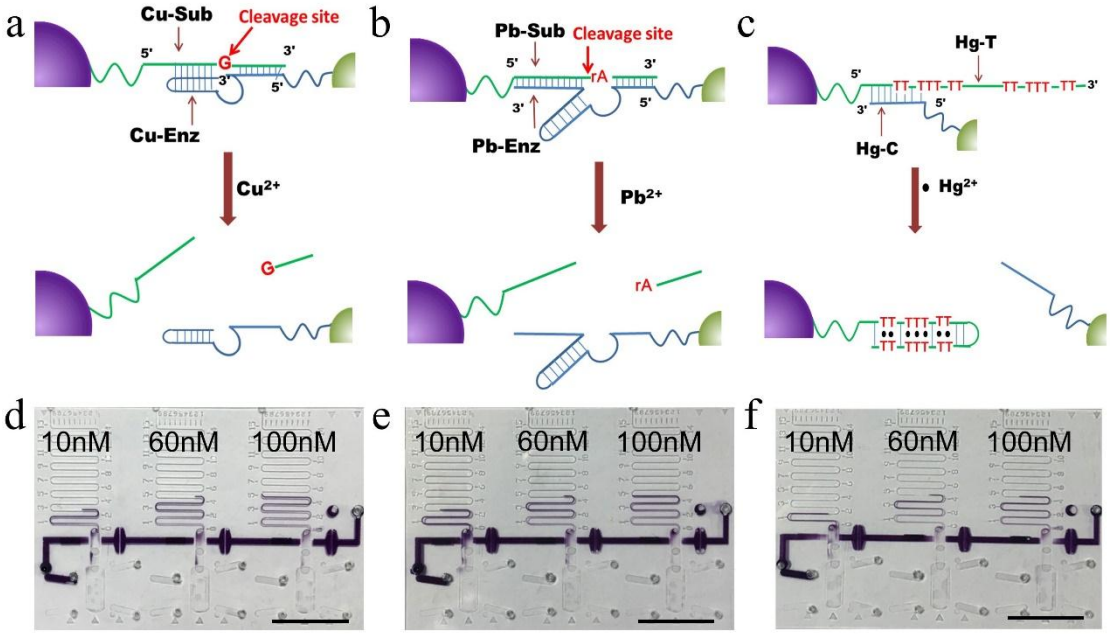


Fig. 2. Basic principles of DNA-nanoparticle probes. (a) Cu^{2+}-induced cleavage of the Cu^{2+} complex probe. (b) Pb^{2+}-induced cleavage of the Pb^{2+} complex probe. (c) Hg^{2+}-induced Hg-T allosteric probe. Detection of (d) Cu^{2+}, (e) Pb^{2+}, (f) Hg^{2+} by integration TV-Chip with DNA-nanoparticle probes. The concentration is 10, 60, 100 nM from left to right respectively. The scale bars are 1.5 cm.

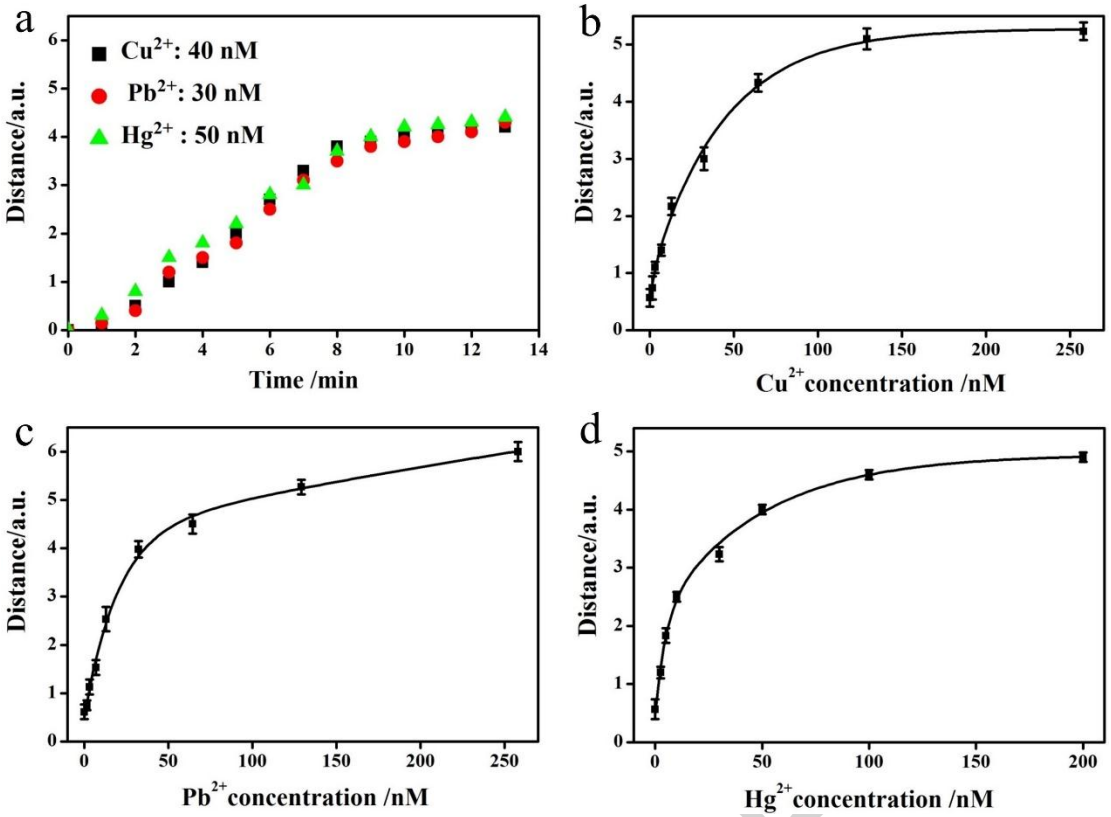
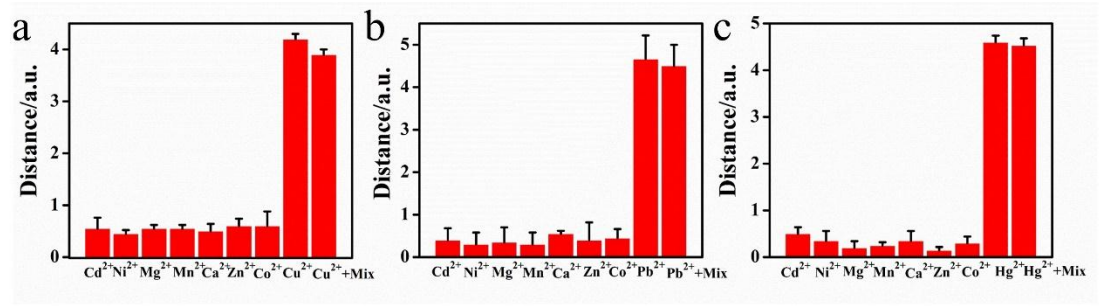


Fig.3. Detection of the three heavy metal ions using TV-Chip. (a) Time-dependent ink advancements with the detection of different concentrations of Cu^{2+}, Pb^{2+} and Hg^{2+}. Plot of TV-Chip bar distances against (b) Cu^{2+} concentration from 1.5 nM to 258 nM . (c) Pb^{2+} concentration from 1.5 nM to 258 nM. (d) Hg^{2+} concentration from 2 nM to 200 nM (d). All the detections were performed in S1 buffer at 10 min, The error bars represent the s.d. of three measurements.



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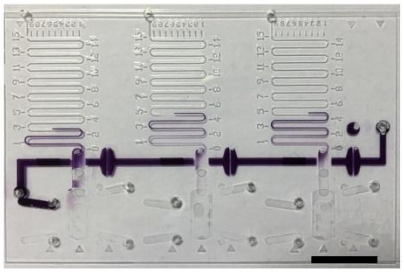

e

	Add/nM	TV-Chip	AAS	AFS
Cu^{2+}	0	23.1±0.6	20.5±0.1	—
Pb^{2+}	50	57.9±2	55.5±0.2	—
Hg^{2+}	80	82±5	—	71.8±0.5

Fig. 4. Selectivity of the method for the detection of (a) 60 nM Cu^{2+}, (b) 60 nM Pb^{2+}, (c)100 nM Hg^{2+} in the mixed solution with 1μM interference ions. (d) The TV-Chip readout of the heavy metal ions in river water sample. The scale bar is 1.5 cm. (e) The results of TV- Chip, AAS and AFS for analyzing the river water sample. The river sample was spiked with Pb^{2+} and Hg^{2+}.

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Highlights

- Triple-channel volumetric bar-chart chip was integrated with DNA-nanoparticle probes for visually multiplexed quantitation of Cu^{2+}, Pb^{2+} and Hg^{2+}.
- Urchin-like platinum nanoparticles exhibit excellent catalase-like activity and were introduced in the microfluidic device for highly sensitive detection of heavy metal ions.
- A lab-on-a-chip biosensor was developed for on-site and real-time monitoring heavy metal ions in water.