Microchimica Acta (2021) 188:176
https://doi.org/10.1007/s00604-021-04822-0

ORIGINAL PAPER

Microfluidic particle accumulation for visual quantitation of copper ions

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Received: 14 January 2021 / Accepted: 5 April 2021

Abstract

A portable biosensor has been developed based on microfluidic particle accumulation for visual quantification of copper ions. A copper-dependent DNAzyme is used to connect magnetic microparticles (MMPs) and polystyrene microparticles (PMPs), forming "MMPs-DNAzyme-PMPs." When copper ions are present, the DNAzyme is cleaved, allowing free PMPs to be released from the MMPs-DNAzyme-PMP complex. Using a capillary-flow-based microfluidic device, the MMPs-DNAzyme-PMPs are first separated by a magnetic chamber, allowing the free PMPs to continue flowing until being trapped at a particle dam with a narrowing nozzle. Therefore, as a thermometer-like display, the copper level can be visually quantified by the accumulation length of the free PMPs in the trapping microchannel. The limit of detection (LOD) is 33 nM determined by the linear range of 25–100 nM, which is 900 times lower than the prevalent standard (~30 μM) in Hong Kong. The system shows excellent selectivity (> 1000-folds) against other heavy metal ions and abilities to adapt to multiple water environmental conditions. Tests on tap water samples and three local natural water sources in Hong Kong manifest that the device can effectively monitor the quality of freshwater with >70% recovery and 26.16% RSD.

Keywords Copper ion · DNAzyme · Microfluidics · Microparticle · Visual detection

Introduction

Heavy metal ion in fresh water has always been a threat to the environment. When uncontrolled, such water pollution may create severe damage to human health through contaminating soil, water, or enter the food chain [1]. Copper ion (Cu^{2+}) is an essential nutrient and a cofactor supporting manifold enzyme reactions in vivo. However, excessive amount of copper ion conduces to adverse gastrointestinal reactions, kidney or liver injury, and neurodegenerative diseases [2, 3]. To safeguard it, the limit of the Cu^{2+} in drinking water has been set to 2000 μg·L^{-1} (~31.47 μM) in Hong Kong [4]. Conventionally, atomic fluorescence spectrometry (AFS) [5], atomic absorption spectrometry (AAS) [6], or plasma mass spectrometry (ICP-MS) [7] are employed to analyze the water sample. However, these approaches rely on sophisticated equipment and time-consuming sample-preparation procedures, which is not suitable for regular end users.

In recent years, benefiting from the development of microfluidic technologies, numerous microsensors based on electrochemical, colourimetric, or fluorescent methods have emerged for miniaturized copper detection [8–12]. DNAzyme is a group of functional nucleic acids optimized by iterative selection using polymerase chain reaction [13]. After selection, the DNAzyme can be sensitive and selective toward a particular metal ion, showing great potential in detection of metal ions. Yi Lu et al. [14] published a "turn-on" fluorescent sensor design by modifying Breaker's original Cu-induced DNAzyme design [15]. In the presence of Cu^{2+}, the DNAzyme was activated, and the substrate strand was cleaved into three pieces. As a result, a fluorescent signal was generated because a fragment with fluorophore was released from a quencher. With appropriate chemical modifications, gold nanoparticles (AuNPs) [16–18], gold nanorods (AuRNs) [19], quantum dots (Q.D.) [20, 21], fluorescent copper nanoparticles (CuNPs) [22], and graphene [23] were applied in conjunction with DNAzyme to display the changes of Cu^{2+} concentration. Additionally, hybridization chain reactions (HCR) [24], split Mg^{2+}-dependent DNAzyme subunits [25],

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Published online: 27 April 2021

or Hemin/G-quadruplex-assembled DNAzyme concatamers [26] were also used for signal amplification.

While versatile applications have been demonstrated, quantification of fluorescent/colorimetric signal heavily relies on bulky spectrophotometer [14, 24], which are inconvenient for non-professional users [23]. Fang et al. [18] published lateral flow nucleic acid biosensors (LFNABs) for the copper ions detection based on copper-dependent DNAzyme and AuNPs. The visual detection of Cu^{2+} was achieved by capturing AuNPs on the test and control zones of the nitrocellulose membrane. With the presence of Cu^{2+}, a red band whose darkness is proportional to the Cu^{2+} concentration was generated. By employing a handheld strip reader, the intensity of this red band can be semi-quantitatively recorded. However, a reader is still unavoidable.

Here, we report a portable biosensor based on microfluidic particle accumulation for visual quantification of the copper ion in fresh water. A Cu-dependent DNAzyme is designed with two termini that can hybridize with the single-strand DNA oligonucleotides on MMPs and PMPs to constitute the "MMPs-DNAzyme-PMPs" structure (Fig. 1). Once the DNAzyme is cleaved by Cu^{2+}, free PMPs can be released to reflect the amount of copper ions. To quantify such cleavage, the particle solution is loaded into a capillary-flow-driven microfluidic device, where the MMPs and MMPs-DNAzyme-PMPs are first separated by a magnetic chamber, allowing free PMPs to flow and accumulate at a particle dam with a narrowing nozzle. In this regard, the level of Cu^{2+} can be interpreted by the length of PMP accumulation which is immediately visible by the naked eye inspection. After determinating the LOD, the tolerance against harsh water conditions and application on the freshwater sample were explored to demonstrate its potential as daily routines for determining Cu^{2+} level in the natural water sources.

Materials and methods

Preparation of DNA oligonucleotides

Details of oligonucleotide sequence are illustrated in Table S1. The copper-dependent DNAzyme is composed of a substrate strand (Cu-sub, labeled green in Fig. 1) and an enzyme strand (Cu-enzyme, labeled black in Fig. 1) (BGI BIO-Solutions HONGKONG Co., Ltd.). The Cu-sub was designed to with

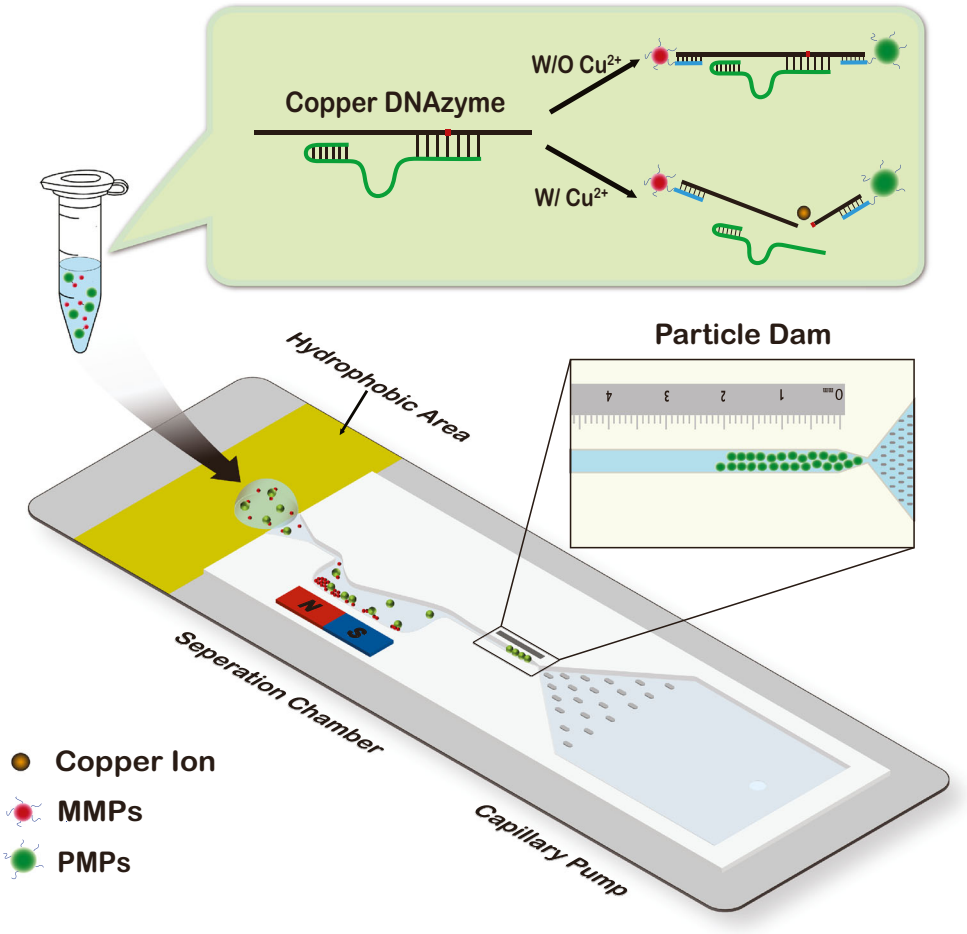

Fig. 1 The schematic of the working principle. The copper DNAzyme formed by Cu-sub (black) and Cu-enzyme (green) can connect magnetic microparticles (MMPs) and polystyrene microparticles (PMPs). With the presence of Cu^{2+}, the DNAzyme is cleaved, resulting in disconnection MMPs and PMPs. To detect it, the particle solution is loaded into a capillary-flow-based microfluidic device, where the MMPs and MMPs-DNAzyme-PMPs are first separated by a magnetic chamber, allowing the free PMPs to continue flowing until being trapped at a particle dam. As a thermometer-like display, the length of PMP accumulation can quantitatively reflect the Cu^{2+} concentration by visual inspection

12-base of extensions on 5′ end and 11-base extensions on 3′ end with sequence complementary to oligonucleotides Probes 1 on MMPs and Probes 2 on PMPs. As such, the DNAzyme can serve as a bridge to connect MMPs and PMPs. Upon addition of Cu^{2+}, the Cu-sub is cleaved at the cleavage point (labeled red in the Table S1), resulting in disconnection of MMPs and PMPs. The 100-μM standard stock solution of DNAzyme was prepared by dissolving the oligonucleotide powder into DEPC (diethyl pyrocarbonate)-treated water (Thermo Fisher Scientific) and stored at 4 °C. For the DNAzyme formation, 2 μL of Cu-sub and 4 μL of Cu-enzyme strand standard stock (100 μM) were prepared at a concentration ratio of 1:2 in 94 μL PBS-DNAzyme buffer (0.5 M NaCl, 50 mM phosphate buffer saline, all from Sigma-Aldrich, USA). After mixing, the solution was heated at 95 °C for 10 mins, followed by cooling down at room temperature for 1 h for annealing the DNAzyme at 2 μM in volume of 100 μL. This DNAzyme solution is further diluted in PBS-DNAzyme buffer according to the required DNAzyme concentrations.

Fluorescence resonance energy transfer assay

The Cy3 and Cy5 cyanine dye were labeled on the thymine of Cu-sub strand and the 5′ end of the Cu-enzyme strand, respectively (Table S1 and Fig. 2a). Thus, the fluorescence resonance energy transfer (FRET) signal can be generated by excitation of Cy3 fluorescein at wavelength of 550 nm and measured by emission light of Cy5 with wavelength of 667 nm. The Cu^{2+} solution was first diluted in deionized (DI) water (Milli-Q Plus system, with a resistivity of 18.2 MΩ·cm) from Copper AA standard solution (1000 $\mu g \cdot mL^{-1}$ from AccuStandard, USA) at 0 nM, 100 nM, 500 nM, 1 μM, and 5 μM. Next, 2 μL Cu^{2+} solution was added with 2-μL DNAzyme diluted in PBS-DNAzyme buffer together with 96 μL of PBS-reaction buffer (50 mM NaCl, 50 mM phosphate buffer saline) to make the DNAzyme final concentration as 25 nM in the total volume of 100 μL. After reaction for 30 min, the samples were tested by the fluorescence intensity acquired by a fluorescence spectrophotometer (Fluromax-4 from HORIBA scientific).

Magnetophoresis assay

The biotinylated oligonucleotides Probe 1 and Probe 2 were immobilized onto the MMPs (0.86 μm, CME0101, streptavidin coated, 1.827×10^{10} microspheres mL^{-1}, Bangs Laboratories, Inc., USA) and PMPs (0.989 μm, P01004, streptavidin coated, 1.557×10^{10} microspheres mL^{-1}, Bangs Laboratories, Inc., USA) respectively. The detailed steps are described in electronic supplementary material (ESM). After immobilization, 5 μL of Cu^{2+} solution diluted in DI water was

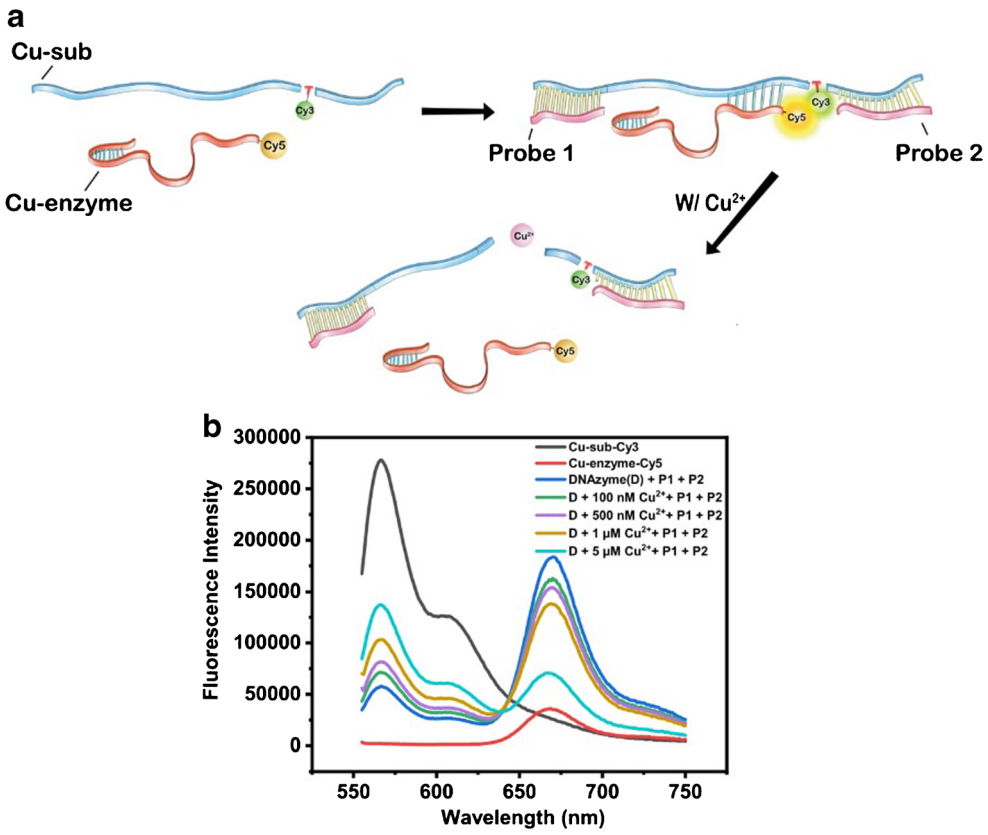

Fig. 2 Evaluation of DNAzyme cleavage using FRET assays. **a** The schematic of the FRET assay. The Cu-sub and Cu-enzyme are labeled with Cy3 and Cy5, respectively. Thus, the FRET signal produced by proximity of Cy3 and Cy5 reflects the formation/cleavage of DNAzyme. **b** The fluorescence intensity of the FRET assay

mixed with 5 μL of DNAzyme solution (40, 100, 200 nM diluted in PBS-DNAzyme buffer) with addition of 3-μL sodium ascorbate (1.333 mM, diluted in PBS-reaction buffer, J&K scientific), which is to transfer Cu^{2+} to Cu^{+} to make it more reactive to this DNAzyme [14]. To finish the reaction, the sample stood still at room temperature for 30 min. Next, 3.5 μL of Probes 1 modified MMPs and 3.5 μL of Probes 2 modified PMPs with original concentration were added into the 13-μL mixture containing 5-μL Cu^{2+}, 5-μL DNAzyme, and 3-μL sodium ascorbate to create a 20-μL system. After gently shaking for 30 min, the solution was placed on a magnetic rack where MMPs and the connected PMPs can be pulled to the tube wall, therefore reducing the absorbance of the supernatant solution which can be quantitatively determined by a UV-vis spectrophotometer with the wavelength of 400 nm (BioDrop μLITE, UK).

Detection in microchip

The details of the microchip fabrication process and a completed device (Fig. S1) are depicted in the ESM [27, 28]. For detection in microchip. The Probe 1-modified MMPs (0.24 μm diameter, SVM-025-5H, streptavidin coated, 5 mg mL^{-1}, Spherotech. Inc) and Probe 2-modified PMPs (15.3 μm diameter, CP01008, streptavidin coated, 5.033×10^6 microspheres mL^{-1}, Bangs Laboratories, Inc., USA) were first prepared (details in ESM). Next, 2 μL of Cu^{2+} solution with varied concentration was mixed with 2 μL of DNAzyme solution (75, 187.5, 375, or 750 nM diluted in PBS-DNAzyme buffer) with addition of 1 μL sodium ascorbate (3 mM, J&K scientific) in PBS-reaction buffer, and the sample stood still at room temperature for 30 min to finish the reaction. Finally, 5 μL of MMPs (diluted to 1 mg mL^{-1}) and 5 μL of PMPs (5.033×10^6 microspheres mL^{-1}) were added to make the total volume at 15 μL. A sample droplet (3 μL) extracted from the 15-μL reaction solution described above was placed onto the hydrophobic sample-loading area such that the solution can wick into the microchannel by capillary attraction. After magnetic separation, the free PMPs of 15.3 μm diameter can be trapped at the particle dam with nozzle width of 8 μm. Due to the height limitation of the microchannel (25 μm), the PMPs can accumulate into a monolayer visible by microscope or the naked eye.

Results and discussions

FRET assay

We first applied a FRET assay to ensure the enzymatic cleavage of DNAzyme (Fig. 2a). Before the formation of DNAzyme, a clear fluorescence signal of Cu-sub-Cy3 and Cu-enzyme-Cy5 was seen, but the FRET signal remained low. After the formation of DNAzyme with addition of Probe 1 and Probe 2, which best simulates the situation of microparticle connection, the proximity of Cy3 and Cy5 generates strong FRET signal as seen by the appearance of peak at 667 nm when excited by wavelength of 550 nm (Fig. 2b). Interestingly, with the increment of Cu^{2+} concentration, the signal intensity at 667 nm gradually decreased, while the intensity of Cy3 peak at 565 nm resumed, representing the cleavage of DNAzyme by the Cu^{2+} which dissociates the FRET pair. The results prove that the DNAzyme can be cleaved with the presence of copper ions.

Magnetophoresis assay

Before applying to the microchip, a magnetophoresis assay was used to evaluate particle connection in response to the copper-dependent cleavage of DNAzyme. MMPs and PMPs were linked by the DNAzyme with two termini that can hybridize to Probe 1 and Probe 2 on microparticles. After formation of MMPs-DNAzyme-PMPs, an external magnetic field was applied, which pulled MMPs and MMPs-DNAzyme-PMPs to the sidewall of the test tube, leaving only PMPs freely suspending in the solution. Thus, the turbidity of supernatant with free PMPs can be observed by the naked eye or quantified by absorbance measurement using a UV-Vis spectrometer with the wavelength of 400 nm.

Using copper concentration of 0 nM, 500 nM, or 5 μM (in 2 μL of copper sample solution), we found that the 10 nM DNAzyme (in final mixture of 20 μL) is insufficient as seen by the overall high absorbance values, especially in the blank control (Fig. 3a). For DNAzyme concentration of 25 nM and 50 nM, 25 nM DNAzyme appears to be better as high signal-to-noise ratio. Next, based on the 25 nM DNAzyme with varied concentration of copper solution, the LOD of 414 nM was determined from the linear region (0–1 μM) of the calibration model using least-squares regression model incorporating the variance of model parameters (details in ESM) (Fig. 3b and c).

Next, we explored different combination of DNAzyme concentration and particle dilution for the on-chip experiment. For detection on chip, the PMPs were changed to 15.3 μm diameter such that they can be trapped at the particle dam with nozzle width of 8 μm. In addition, the MMPs were also changed to 0.24 μm diameter such that it requires less DNAzyme for sufficient particle connections. Initially, a volume ratio of 1:1 between MMPs (0.24 μm diameter) and PMPs (15.3 μm diameter) was adopted to connect various concentrations of DNAzyme (25 nM, 50 nM, 100 nM, and 300 nM, in final mixture of 15 μL) without adding Cu^{2+}. The results showed that the use of 100 nM DNAzyme led to the shortest PMP accumulation at 0.9 mm (Fig. S2). Next, to further reduce the amount of DNAzyme required for particle connection, the number of MMPs were reduced while maintaining similar proportion of DNAzyme to MMPs, i.e., 2X

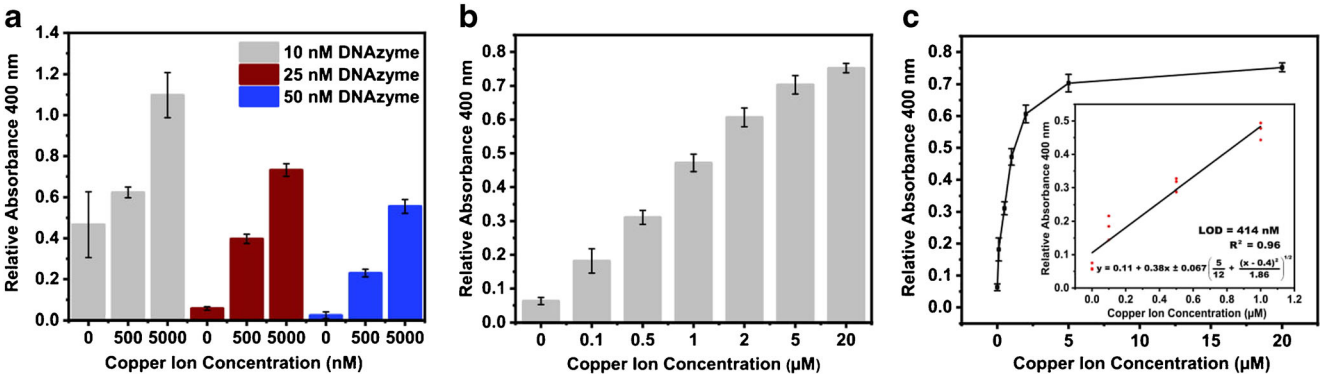

Fig. 3 Evaluation of particle connection using magnetophoresis assay. **a** Optimization of DNAyzme concentration. **b** The relative UV-Vis spectral absorbance at the wavelength of 400 nm with varying concentration of Cu^{2+} when using the DNAzyme concentration of 25 nM. **c** The calibration model of the absorbance value in **b**. Inset: linear regression from 0 to 1 μM of Cu^{2+} (mean ± max deviation, $n = 3$)

dilution MMPs +50 nM DNAzyme, 4X dilution of MMPs +25 nM DNAzyme, 5X dilution of MMPs +25 nM DNAzyme, and 10X dilution of MMPs +10 nM. After comparison, 5X dilution with 25 nM DNAzyme seem to be enough for sufficient particle connection (Fig. S3). Thus, the microparticle volume ratio of MMPs and PMPs was set as 1:5 while using Cu-dependent DNAzyme concentration of 25 nM.

LOD

Based on the optimized particle ratio between MMPs and PMPs (1:5) and concentration of DNAzyme (25 nM), we next determined the LOD by testing a series of different concentrations of Cu^{2+} on microfluidic chips (Fig. 4). Remarkably, the increase of free PMPs with the increase of Cu^{2+} concentration results in the longer trapping length of PMPs, which can be visually quantified by the eye inspection (Fig. 4a–b). According to the cumulative length and the linear regression equation, a LOD of 33 nM is achieved, which is calculated by a least-squares regression model incorporating the variance of model parameters (details in ESM) (Fig. 4c). This performance is excellent in the view of prescribed drinking watering standard (~30 μM in Hong Kong, ~20 μM from United States Environmental Protection Agency, and ~15 μM in China mainland).

Tolerance to environmental interference

To investigate tolerance to environmental interference, we next studied the selectivity of this assay against other common metal ions. We applied samples containing calcium (Ca^{2+}), cadmium (Cd^{2+}), mercury (Hg^{2+}), lead (Pb^{2+}), manganese (Mn^{2+}), cobalt (Co^{2+}), iron (Fe^{2+}), and barium (Ba^{2+}). When Cu^{2+} was added (100 nM), the trapping length was 3.3 mm in average. In contrast, when other heavy metal ions were applied (100 μM for all other metal ions), the PMP accumulation was similar to that of the blank sample (Fig. 5a) even though higher concentration was used. The results demonstrate the excellent selectivity (> 1000-folds to other heavy metal ions) of our Cu^{2+} detection.

We next investigated the tolerance to the water with different pH and hardness. Two hundred nM of Cu^{2+} was added into water sample with pH value 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5 (Fig. 5b). By comparing the results, the performance was stable in the selected pH range. On the other hand, four water samples with different water hardness, including soft (55 mg·L^{-1}), moderate (107.5 mg·L^{-1}), hard (157.7 mg·L^{-1}), and very

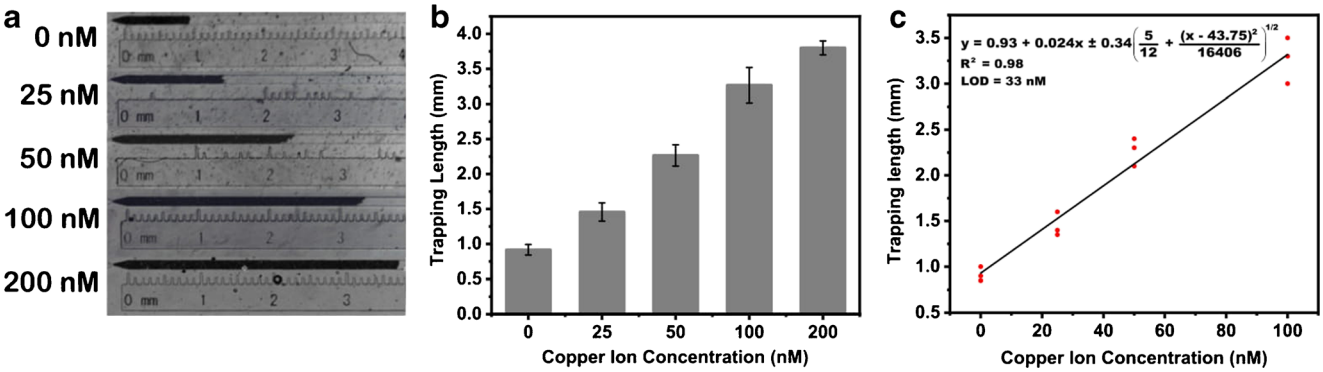

Fig. 4 Detection of copper ion on the microfluidic chip. **a** The photograph of the PMP accumulation reflecting the concentration of copper ions. **b** The trapping length of PMP accumulation. **c** The linear regression equation of the trapping length relating to **b** (mean ± max deviation, $n = 3$)

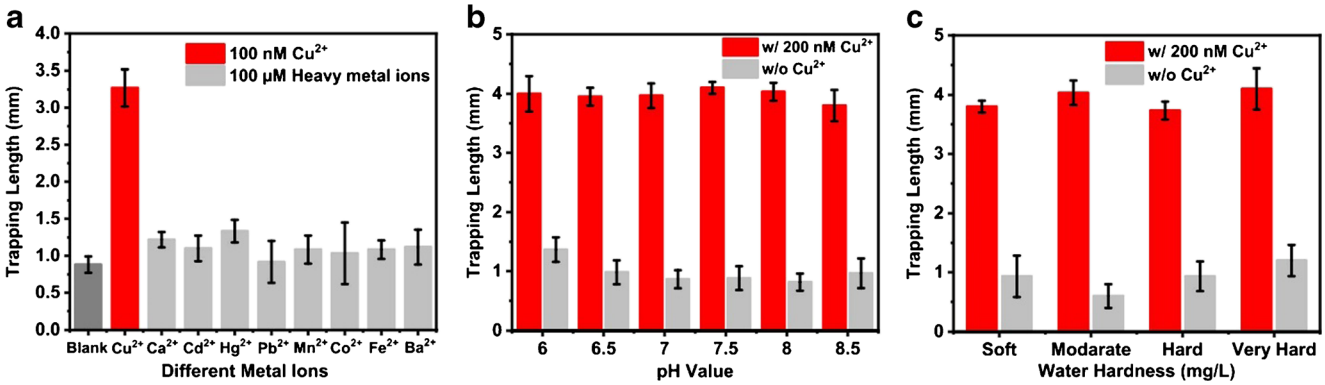

Fig. 5 The tolerance to environmental interference. **a** The selectivity of Cu^{2+} against other metal ions with a much higher concentration (1000-fold). **b** The tolerance to pH value (pH range 6–8.5). **c** The tolerance to four water samples with different water hardness (soft 55 mg·L^{-1}, moderate 107.5 mg·L^{-1}, hard 157.7 mg·L^{-1}, and very hard 318.3 mg·L^{-1}) (mean ± max deviation, $n = 3$)

hard (318.3 mg·L^{-1}) according to the water hardness standard from the World Health Organizations, were added with 200-nM Cu^{2+} (Fig. 5c). The results demonstrate that under different water hardness conditions, there is still a distinct difference of the trapping length between samples with and without Cu^{2+}. The method developed shows excellent selectivity (> 1000-folds to other heavy metal ions) and tolerance to multiple water conditions such as environmental acidity/basicity and water hardness.

Detection in fresh water

Finally, we tested the water sample of real world. Tap water is the most common source of water for urban residents. Because copper is a widely used plumbing material, tap water is susceptible to pollution from copper pipelines, which poses a threat to drinking water safety. Analyzed by ICP-MS (Envirolabs. Ltd., Hong Kong), the Cu^{2+} level in the tap water sample is 25 μg·L^{-1} (393 nM). Before testing, the tap water was diluted five times using DEPC-treated water (Thermo Fisher Scientific). The trapping length on-chip was 2.4 ± 0.45 mm, which can be calculated as 61.38 ± 9.33 nM using inverse regression of the calibration model in Fig. 4c (details in ESM). After conversion based on the dilution factor, the copper concentration can be determined as 306.9 ± 46.63 nM, which shows a recovery rate of 78.1%, demonstrating the compatibility to the tap water environment (Table 1).

Furthermore, we have extended our application to freshwater samples from different sources (Table 1). Three local natural water source in Hong Kong were sampled and measured, including the mountain water in Ma Tai stream, the agricultural water in Yau Tam Mei Tsuen village, and the surface storage water in Tai Mo Shan near a closed lead-copper mine. For agricultural water in Yau Tam Mei Tsuen village and the mountain water in Tai Mo Shan, measurement of ICP-MS shows that the copper concentration is 46 nM and 67.5 nM, respectively. Notably, we have again achieved a recovery rate of 71.2% and 70.2%, respectively. In contrast, for fresh water from the Ma Tai stream, the recovery rate was 133%, which might be because the actual copper concentration is below the LOD.

Together, we achieved the microchip with a thermometer-like display for visual quantitation. Notably, while the LOD is not superiorly better than other copper detection methods (Table 2), it is still much below the prescribed drinking watering standard (~30 μM in Hong Kong). In fact, the higher LOD is a compromise of the power-free device, enzyme-free reaction, and visual quantitation without any special reader, which, we believe, are the more essential properties for on-site and portable detection for resource-limited regions. Also, the linear dynamic range of 25–100 nM is relatively short, making the dilution process required before applying it to samples with a high concentration of copper, such as tap water. Further improvement of the LOD and dynamic concentration range will be explored by redesigning the oligonucleotide reactions, using a more Cu^{2+}-sensitive DNAzyme sequence or integrating signal amplification techniques.

Table 1 The detection in the freshwater sample

Sample Name	ICP-MS (nM)	Microchip (nM)	Recovery rate (%)
Tap water	393	306.9 ± 46.63	78.1%
Ma Tai stream	16.8	22.33 ± 9.42	133%
Yau Tam Mei Tsuen village	46	32.79 ± 9.20	71.2%
Tai Mo Shan	67.5	47.44 ± 9.13	70.2%

Table 2 An overview of recently reported nanomaterial-based methods for the Cu^{2+}determination

Materials used	Method	Sample matrix	LOD	Quantitation	Ref
Graphene quantum dots (GQDs)	Electrochemical	Deionized water	0.05 nM	Amperometer	[11]
Carbon nanoparticles	Electrochemical	Tap water	0.5 pp (7.9 nM)	Amperometer	[12]
Horseradish peroxidase (HRP) concatemers	Colourimetric	Lake water/sewage water	8 nM	UV-Vis absorption	[24]
Hydrogel and fluorescent CuNPs	Fluorescence	Tap water/ river water/lake water	20 μM	Fluorescence spectrometry	[22]
AuNPs	Lateral flow	Ultrapure water	10 nM	Stripe reader	[18]
Microfluidic Particle Accumulation	PMP accumulation	Tap water/freshwater samples	33 nM	The naked eye	This work

Conclusion

In this study, we demonstrate a portable biosensor based on microfluidic particle accumulation for visual quantification of the copper ions in fresh water. With the use of MMPs and PMPs in a microfluidic particle dam, the concentration of Cu^{2+} can be directly read by the trapping length of PMPs accumulation in the trapping channel. Our device has demonstrated sufficient LOD, high selectivity (> 1000-folds), and tolerance to multiple water environmental conditions, allowing tests on tap water samples and three local natural water sources in Hong Kong with a reasonable recovery rate. Compared to other existing methods, our biosensor is not the most sensitive one, but provides advantages of the power-free device, enzyme-free reaction, and visual quantitation without any special reader, which suggests a robust, low-cost, and user-friendly solution as daily routines to determine Cu^{2+} level in the drinking or natural water sources.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00604-021-04822-0.

Funding We are pleased to acknowledge the funding support from the General Research Fund (project nos. 11217217, 11277516, and 11217820) and NSFC/RGC Joint Research Scheme (N_CityU119/19) from the Hong Kong Research Grant Council, and internal grants from City University of Hong Kong (6000720).

Declarations

Conflict of interest The authors declare no competing interests.

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