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Analytical Methods

Ultra-sensitive and absolute quantitative detection of Cu^{2+} based on DNAzyme and digital PCR in water and drink samples

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ABSTRACT

Here, we developed an ultra-sensitive and absolute quantitative detection method of Cu^{2+} based on DNAzyme and digital PCR. The binding model between DNAzyme and Cu^{2+} and the influence caused by the additional primer sequence were revealed to ensure quantitation independent of standard curves. The binding model of DNAzyme and Cu^{2+} showed that one molecular DNAzyme could bind one Cu^{2+} in the biosensor step. Thus, the final quantitative results, evaluated by three parallels, showed that the limit of quantitation (LOQ) was as low as 0.5 pmol, while the sensitivity was evaluated as 50 fmol. The specificity evaluation of our methodologies shows that extremely low crossing signal is existed within the non-specific ions. Moreover, the results of practical detection have shown that the quantitative results were stable and accurate among different food substrates. In conclusion, a flexible quantitative detection method with ultra-sensitivity was developed to detect trace amounts Cu^{2+} within different substrates.

1. Introduction

Native DNA performed the genetic function for most organisms. However, the latest research has shown that single-stranded DNA in a complex hairpin structure can perform molecular recognition and catalysis. The catalysis functions of single-strand DNA have broken the common idea that all catalysis functions should be performed by proteins and has raised concern in researchers. Among different kinds of biosensors, deoxyribozyme sensors for the detection of metal ions have improved outcomes compared with normal detection methods with simpler protocols (Li, Shi, Wang, & Dong, 2009; Liu & Lu, 2007a, 2007b; Wang, Lee, & Lu, 2008). Most of them are based on complex structures caused by nuclear strand folding, similar to the tertiary structure of common enzymes. Heavy metal ions act as the co-effectors to heavy metal biosensors, while the existing ions induce a reaction between the DNAzymes and substrates. Copper, as a type of widely used heavy metal in industry, can be leaked into the environment through different methods. High copper exposure for a prolonged period may cause permanent liver and kidney damage. Although the U.S. Environmental Protection Agency (EPA) has recommended the highest concentration of copper in drinking water to be 1.3 ppm, research has shown that trace amounts of copper can induce neurotoxicity by interacting with cholesterol in the diet after a treatment with 0.06 ppm copper for 16 weeks (Lu et al., 2006). Thus, the sensitive and selective detection of copper is important for human nutrition. In the 2000s, Liu found that a specific nucleic acid sequence could lead to the cleavage of a DNA strand in the presence of Cu^{2+} (Liu & Lu, 2007a). That study showed a sensitive and selective detection method using a biosensor that increased fluorescence. However, signal amplification steps were not included for the original biosensor assay; thus, the sensitivity might not be sufficient for practical detection. On the other hand, by measuring fluorescence emission through a spectrophotometer, only qualitative detection could be achieved. This method could not determine whether the concentration of copper is above a certain value. Therefore, to promote the detection of copper by a biosensor method, signal amplification steps and quantitative detection are essential.

The most attractive aspect of heavy metal ion biosensors is that the ion signals can be transferred to nucleic acids, so that PCR methods could be introduced to improve the sensitivity and specificity. PCR is a popular tool to amplify nucleic acids. PCR can amplify nucleic acids sensitively and selectively in an exponential manner within 2 h (Bustin, Benes, & Garson, 2009). Digital PCR, a newly developed quantitative PCR-based detection method, has been regarded as the third generation of PCR technology for its better sensitivity and directly absolute quantitative detection (Miotto et al., 2014; Pinheiro et al., 2012; Sanders et al., 2011;

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Vogelstein & Kinzler, 1999). After separating the original reaction volume in a microfluidic or oil-in-water manner (Pinheiro et al., 2012; Sanders et al., 2011), a large number of separating reaction cells are generated so that the original target is also divided into each cell. Thus, the target copy number for each partition results from the Poisson distribution based on the ratio of positive partitions to total partitions. After first being reported in the 1990s (Vogelstein & Kinzler, 1999), much progress has been achieved in the field of copy number determination (Hindson, Ness, Masquelier, & Colston, 2011), single nucleotide polymorphisms and absolute quantitative detection of low-abundant target samples (Burns, Burrell, & Foy, 2010; Corbisier, Bhat, Partis, Rui Dan Xie, & Emslie, 2010; Demeke et al., 2014). Compared with real-time PCR, digital PCR can determine the copy number of a target template in the original solution without the generation of standard curves (Morisset, Štebih, Milavec, Gruden, & Žel, 2013). This feature mostly avoids the uncertainties caused by standard curves and their procedures. Thus, digital PCR is regarded as an improved quantitative detection compared with real-time PCR. In the 2000s, Bio-Rad commercialized digital PCR with the production of the Bio-Rad QX100 digital PCR system. After development for nearly a decade, this system has been regarded as the digital PCR system with better accuracy, stability and cost performance compared with other platforms by meeting the requirements.

Based on this original biosensor, different detecting methods have been combined to increase the sensitivity or the feasibility in latter researches, including the paper-based stripes (Shing, Heng, & Surif, 2013; Zhao, Ali, Aguirre, Brook, & Li, 2008), the G-quadruplex structure (Zhang, Cai, Li, Kong, & Shen, 2012; Zhang, Fan, et al., 2015) or the isothermal amplification (Li et al., 2014; Torres-Chavolla & Alocilja, 2011; Zhang, Wang, & Zhang, 2014). However, all the above researches could only achieve the quantitative detection of target ions by the analysis of standard curves through relative quantitation. This relative quantitation could be easily effected by the operation or the data analysis. Therefore, the absolute quantitation of target ion was essential to the risk assessment of heavy metal ions. For absolute quantitative detection of copper using the nucleic biosensor, understanding the binding relationship between the DNAzymes and cofactors is essential. Previous research on different types of biosensors has focused on the standard curves between the fluorescence emission and concentration of heavy metal ions (Hao et al., 2014; Li et al., 2014; Liu & Lu, 2007a). However, this method of data analysis cannot measure the absolute concentration of ions without serial dilutions of ion solutions. Therefore, the binding relationship of heavy metal ions with DNAzymes is essential for the absolute quantitation of heavy metal ions using the biosensor approach.

Considering the demand for quantitative detection with good sensitivity and specificity, we developed a detection system based on the deoxyribozyme biosensor and digital PCR. This detection system takes advantage of the nucleic acid signal translation of the biosensor and absolute quantitative detection of digital PCR. In our study, the binding model between the DNAzyme and ion was identified and validated. This knowledge is the key for achieving the absolute quantitative detection of ions by biosensor-based methods. According to the final detection results, the limit of quantitation (LOQ) was determined to be 0.5 pmol evaluating different replicates, while the sensitivity was 50 fmol as determined by the obvious difference from the negative control group.

2. Materials and methods

2.1. Oligonucleotides and reagents

All the oligonucleotides are listed in Supplementary Table 1. All the primers and probes were synthesized by Invitrogen (Life Technologies, California, US). All chemical reagents used in our assays including copper (II) chloride dihydrate, Tris base and other chemicals in the buffer for the cleavage assay were purchased from Sigma-Aldrich (St. Louis, MO). The reagents used in our experiment are all Analytically Pure. All the reagents related to the droplet-digital PCR were purchased from Bio-Rad (Pleasanton, CA, USA).

2.2. Preparation of the practical detection sample

For practical evaluation, natural water and Sprite were used as the detection objects. The natural water was directly collected from the Beijing Laboratory for Food Quality and Safety, Beijing, China. The Sprite samples were bought from Merry Market, Beijing, China. Before the detection of Sprite samples, the heat pretreatment was achieved to get rid of the CO_2 of drink substrate. Different concentrations of copper dilution were added to the experimental objects manually. The mixture was then placed on a Dynamic CM-200 mixer and shaken to achieve equal distribution.

2.3. DNAzyme cleavage assay

The self-annealing cleavage assay was conducted in cleavage buffer [50 mM HEPES (pH 7.0), 0.5 M NaCl, 0.5 M KCl, 0.5 μM ascorbate] with the Cu^{2+} ion as the cofactor of DNAzyme. The total reaction volume of cleavage assay is 50 μL. Final concentration of substrate in the reaction volume was 3 μM, while the concentration of the DNAzyme was optimized according to the different ratios. All the contents were mixed and shaken gently for uniform distribution. The mixture was heated to 95 °C for 5 min to completely denature the DNA into single strands. Then, the mixture was slowly cooled to room temperature (25 °C) for the cleavage assay. Finally, 10 μL of EDTA (10 mM) was added to terminate the reaction.

2.4. Droplet-based digital PCR assay

Before droplet digital PCR (ddPCR), the products of the cleavage assay were serially diluted to ensure the proper copy number range. The final ddPCR mixture contained 1× ddPCR Master Mix (Bio-Rad), reverse and forward primers at a final concentration of 0.24 μM and a TaqMan probe at a final concentration of 0.12 μM. The concentration of the added template was optimized for further experiments. Then, 20 μL of the above mixture was transferred to a disposable eight-channel droplet generator cartridge (Bio-Rad). A 70 μL volume of droplet generation oil (Bio-Rad) was loaded to each well. After the droplet generation step and PCR amplification, the PCR products were used in the droplet-reading step. Each droplet was separately passed through the beta-prototype droplet reader (Bio-Rad). The fluorescence of each droplet was collected.

The PCR amplification procedure consisted of pre-denaturation at 95 °C for 5 min, 50 cycles of a two-step thermal profile involving 10 s at 95 °C for denaturation and 60 s at 60 °C. Finally, a 95 °C step for 5 min was conducted to stabilize the droplet.

2.5. Real-time PCR assays

The real-time PCR was performed in Bio-Rad C1000 PCR facility. The volume and temperature cycling of the real-time PCR reaction were same as that used in the droplet-based digital PCR. The fluorescence of each tube was collected automatically to generate amplification curves. The results were analyzed according to the standard or amplification curves.

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2.6. Secondary structure determined by circular dichroism (CD)

The Chirascan™-plus CD Spectrometer (Applied Photophysics, UK) was used to measure the CD of different samples. The CD of the mixture of DNAzyme and Cu^{2+} at different concentrations was measured to evaluate the difference between the secondary structure of DNAzyme with or without copper ions. A 200 μL volume was used in these experiments. According to the official guidelines for CD measurement, the lowest suggested DNA concentration was 10 μM. In the final experimental volume, the concentration of DNAzyme was 10 μM, and 1× cleavage buffer was used as the background dilution. The final concentrations of copper ion varied among samples.

2.7. Data analysis

After fluorescence collection of all droplets, amplification heatmap was automatically generated according to the difference in fluorescence emission of positive and negative droplets. The final copy number of the original volume was calculated using the ratio of positive to total droplets by Poisson distribution. The original copy number and cleavage efficiency were calculated by the equations below (Lun et al., 2008):

$$A(\text{samples}) = -\ln[(N - X)/N] \times N \quad (1)$$

$$B(\text{negative control}) = -\ln[(N - Y)/N] \times N \quad (2)$$

For the above equations, A (samples) and B (negative control) indicate the absolute copy number of the experimental samples and negative samples, respectively. X and Y indicate the number of positive droplets in the sample groups and negative control group, respectively. N indicates the number of droplets in the reaction volume.

For the analysis of the final detection results, the cleavage rate (CR) of DNAzyme in each group was calculated to evaluate the cleavage ability. The (CR) of DNAzyme in each group was calculated by the equation below:

$$\text{Cleavage Rate} = [B(\text{Negative Control}) - A(\text{Samples})]/481.6 \quad (3)$$

The cleavage rate was manually defined as the parameter to evaluate the detection results in our study, which was shown as linear relationship with the D-value between the copy number of negative control group and sample group. In the above equation, A (samples) and B (negative control) have the same meaning as Eqs. (1) and (2). The 481.6 in the denominator represented the base of the cleavage rate based on the serial dilutions before digital PCR and the Avogadro constant (Deslattes et al., 1974). The cleavage rate was regarded as the basic index for absolute quantitation in our study.

3. Results

3.1. Principle of our detection system

The scheme of sensing system was described in Fig. 1. The whole system mainly consisted two parts, the biosensor step (Fig. 1a and b) and the digital PCR step (Fig. 1c and d). The designation of the biosensor sequences used in our study was shown in Fig. 1a. The sequence of the substrate contained both the locations of primers and the cleavage site of DNAzyme. For the first step (Fig. 1b), the single strand of the substrate could be hydrolyzed by the DNAzyme with the existence of Cu^{2+} during the proper temperature. The sequence of the DNAzyme was according to the original research (Liu & Lu, 2007a) of biosensor detection of copper (II). Three types of single strands existed after the biosensor step, the DNAzyme, the cut substrates strand and the uncut substrates. All of the products of first step were loaded on the digital PCR platform after serial dilutions, in which the different sequences were loaded into different droplets (Fig. 1c). However, only the uncut substrates could show positive signal. For the analysis of the data achieved by the ddPCR, the fluorescence threshold was set to largely distinguish the negative and the positive droplets. Therefore, during the droplets reading step after the PCR amplification (Fig. 1d), the droplets containing the sequence of DNAzyme or the cleavage substrates would show the negative signal, which was represented as the grey dots in the final hotmap. The blue dots in the hotmap represented the positive signal in the droplets containing the uncut substrates sequence. Finally, the cleavage rate was calculated according to the Eq. (3) and then was used to evaluate final results.

3.2. Tolerance evaluation of Cu^{2+} in the PCR reaction system

Heavy metal ions are regarded as inhibitors to all PCR reactions (Desai & Madamwar, 2007). To ensure the reliability of PCR-based quantitation, the effects of copper ions in a PCR reaction were evaluated. Previous research has shown that digital PCR has better tolerance for PCR inhibitors than real-time PCR (Dingle, Sedlak, Linda, & Jerome, 2013). Therefore, due to cost efficiency, real-time PCR was chosen to evaluate the effects caused by copper ions. In this part of experiments, serial dilutions of copper, which is determined with different times with magnesium, were added to the PCR volume additionally to evaluate the inhibition. The detection results (Supplementary Fig. 1) have shown that the higher concentration of the Cu^{2+} exhibited obvious inhibition to the reaction, while the lower concentration would relieve the inhibition. This indicated that the model of the copper inhibition was competitive inhibition, meaning that the side effect would be eliminated when the concentration of copper is extremely low. According to the official instructions of the real-time master mix, the final concentration of magnesium in the reaction volume was 10 mM. Thus, we considered that the highest copper concentration that would have an obvious effect on PCR reactions was 10 mM. However, for the practical biosensor detection, the cleavage product would be loaded on the digital PCR platform after serial dilutions. We considered that the effects caused by the heavy metal ion on the PCR system could be ignored.

3.3. The effect of an additional primer sequence on the DNAzyme cleavage assay

For the practical detection for the biosensor cleavage assay, we found that the additional elongation of the substrate sequence might cause uncertainty in biosensor cleavage. To evaluate the inhibitions caused by the elongation of primer sequences, we designed different primer sequences located outside of the substrate sequence. To test the most possibilities, we considered that the secondary structure of the entire substrate sequence at room temperature should be considered. Before the cleavage assay of different substrate sequences, we evaluated the secondary structure of different substrates. Combining the practical results and secondary structures (Fig. 2), we concluded that the cleavage site of the substrate sequences should not be included within the hairpin. For substrate sequences with the cleavage site within the hairpin structure obvious inhibition was observed in the cleavage assay. Therefore, the substrate that had an exposed cleavage site and higher cleavage efficiency was chosen for further experiments.

3.4. Prediction of the ion-DNAzyme binding model

To achieve absolute quantitative detection by the biosensor approach, the binding model between the ion and DNAzyme

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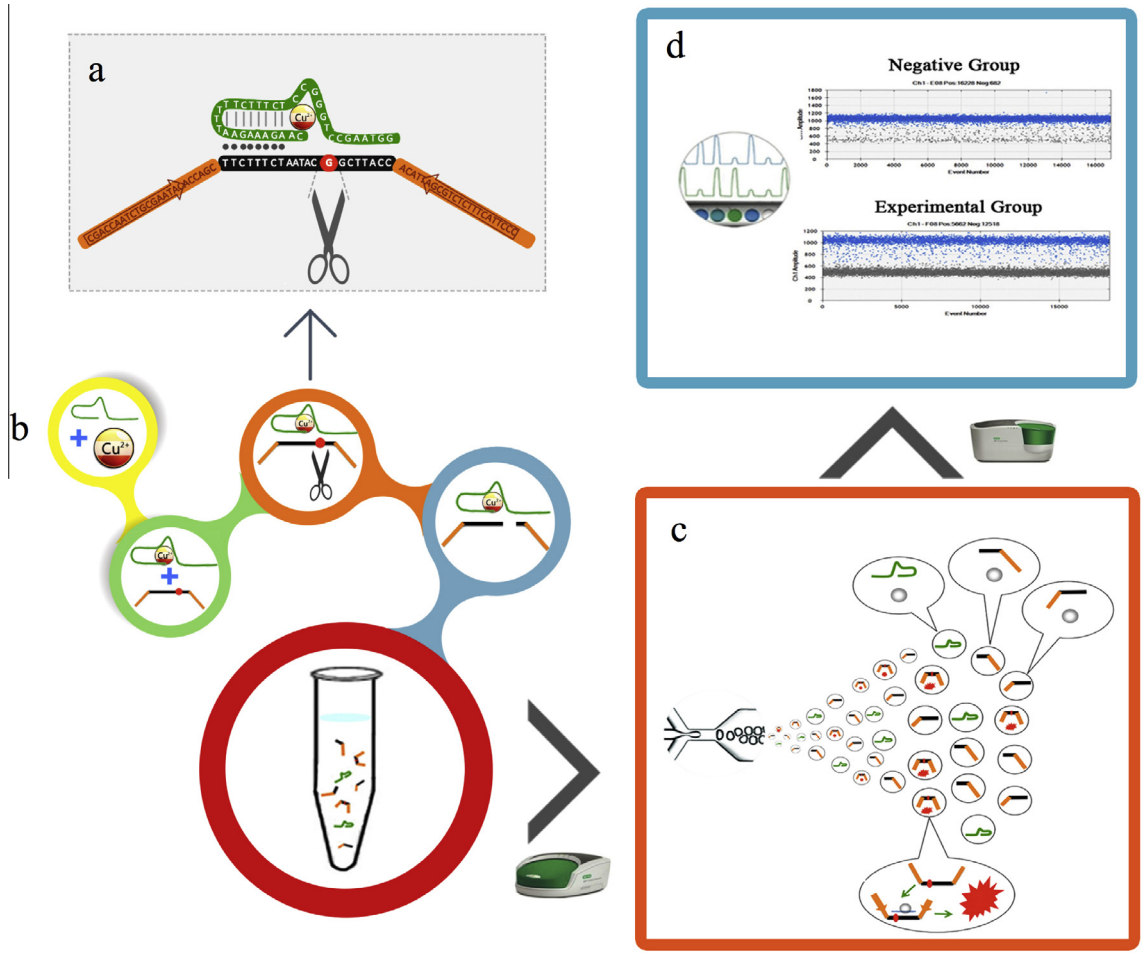

Fig. 1. Principle of DNAzyme biosensor detection system. The designation of our biosensor sequence was shown in (a). The oligonucleotide with green bases is the sequence of DNAzyme. The orange bases with the hollow arrow are the locations of primers in digital PCR step, while the bases with the blue line are the location of probe. The procedure of cleavage was shown in (b). One DNAzyme sequence binds with one copper ion (purple circle) and one substrate sequence. The cleavage would occur after the generation of the three-part structure. The procedure of digital PCR was shown in (c) and (d), which contained the droplets generation and plate reading steps. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

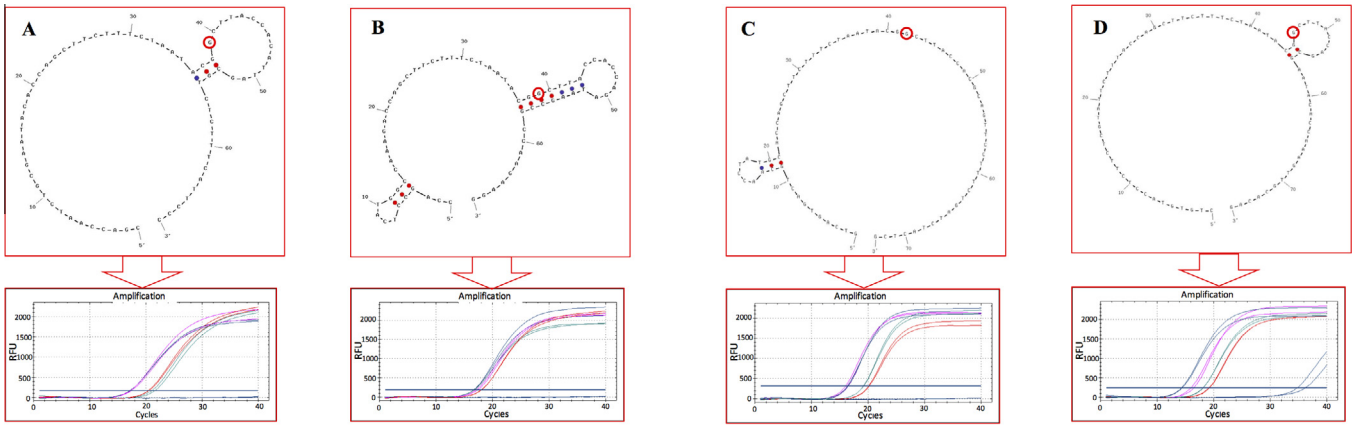

Fig. 2. Evaluation of the effects caused by additional primer sequence. This group of figures showed the hairpin structure of each substrate used in our study, as well as the cleavage results evaluated by real-time PCR. (A)–(D) represented the substrates Sub-GX1, Sub-A70, Sub-GX3 and Sub-GX2, respectively. The cleavage sites within the substrate sequence were circled in all the panels.

should be clear. However, previous studies of biosensor-based detection approaches for heavy metal ions have only focused on qualitative detection based on fluorescence emission (Liu & Lu, 2007a; Zhang, Fan, et al., 2015; Zhang, Zuo, & Ye, 2015; Zhao et al., 2008). Therefore, to achieve absolute quantitative detection, we evaluated and predicted the ion-DNAzyme binding model through CD measurements. The CD of the mixture of DNAzyme and copper diluted to concentrations was measured. According to

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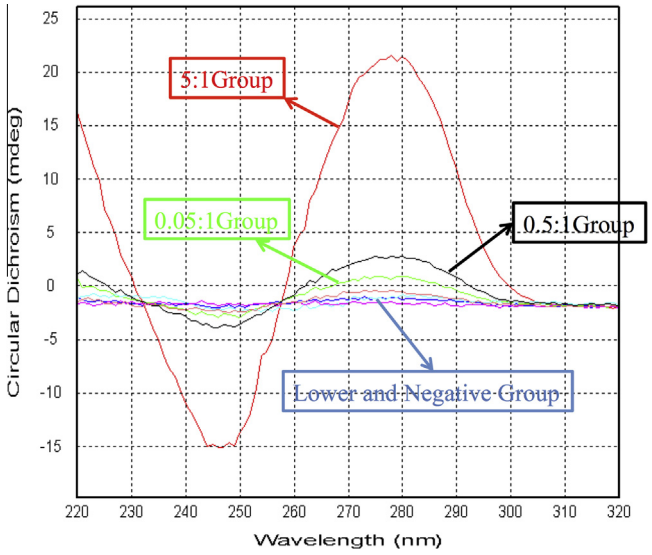

Fig. 3. Evaluation of the secondary structure predicted by CD with different ratios of Cu^{2+} to DNAzyme. All the tested groups are shown in this figure. The groups of 5:1, 0.5:1 and 0.05:1 show the single, positive characteristic peaks.

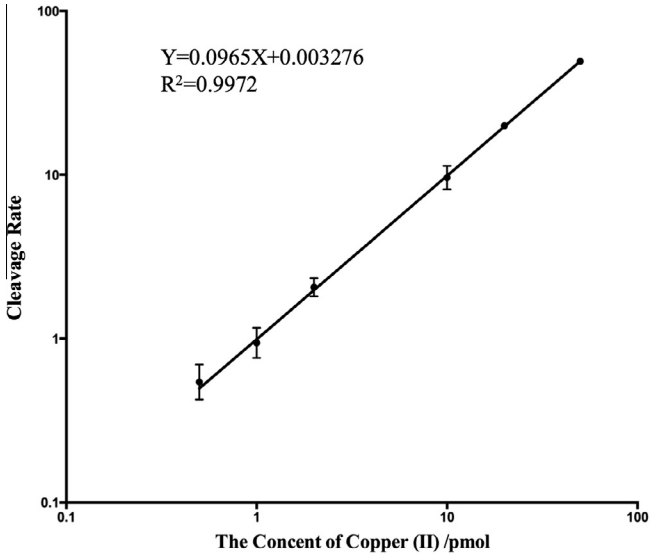

Fig. 4. Standard curve between the cleavage rate and content of copper.

the detection results for the CD test (Fig. 3), all the curves showed a unique peak for the CD of positive groups. For the group that had the lowest ratio of copper to DNAzyme of 0.05:1, the characteristic peak was the same as the groups that had higher ratios of 0.5:1 or 5:1. Therefore, we considered that a unique binding model exists between DNAzyme and copper ions. Because the characteristic peaks did not change due to increases in copper concentration, we considered the ratio of DNAzyme to copper as 1.

3.5. Optimization of the DNAzyme cleavage assay

To optimize the DNAzyme cleavage assay, we evaluated the cleavage time and ratio of DNAzyme to substrate. As the cleavage efficiency could be evaluated by the amplification signals of each group qualitatively, we used real-time PCR instead of ddPCR due to its lower cost. In optimizing the reaction time (Supplementary Fig. 2A), we determined that the cleavage reaction finished within 1 h, and the longer reaction time generated the same amplification signals as the 1-h group. The final reaction time used was 1 h. The detection results for this experiment also indicated that the copper ion has a unique binding site on the DNAzyme. Moreover, the copper ion did not fall off the DNAzyme during the cleavage assay. According to the results of varying the ratio of DNAzyme to substrate, a higher ratio had a positive effect on the final cleavage (Supplementary Fig. 2B). Therefore, to ensure complete cleavage, the ratio of DNAzyme and substrate was 10:1.

3.6. Quantitative assessment of the copper ion

The above experiments have showed that the copper ion had a unique binding site in the DNAzyme. Therefore, the cleavage rate of a serial dilution of the copper ion should show the same relationship as the content of the copper ion. In this experiment, we noticed that little cleavage occurred when the copper content was below 50 fmol. Using the DNAzyme assay and digital PCR, we generated a standard curve from the copper content and cleavage results, as shown in Fig. 4. We conclude that the cleavage rates of the DNAzyme assay have good linear relation with the copper content between 50 pmol and 0.5 pmol. This result indicated that our detection system has a dynamic range over 3 magnitudes that can achieve accurate detection of copper ions for most practical samples.

The limit of quantitation (LOQ) was defined as the lowest content to be stably quantified with its RSD of inner-group below 30% (Fu et al., 2015). To determine the LOQ, we evaluated different samples that have extremely low copper concentration. Using the detection results for these samples, we calculated the practical values according to the standard curve and relative standard deviation (RSD). Finally, the LOQ of our detection system was determined to be 0.5 pmol (Table 1). This LOQ is the lowest value compared with other methods of detecting copper ions.

3.7. Specificity assessment

The specificity of our study was evaluated by the quantitative determination of different metal ions using the biosensor detection system based on digital PCR. For practical ion detection, copper is mostly confused with other heavy metal ions, such as Ag^{+}, Ca^{2+}, Mg^{2+}, Ba^{2+}, and Fe^{3+}. To ensure that our detection system is specific for all kinds of metal ions, ions of different chemical valence were chosen to assess different types of metal ions. In our study, Ag^{+}, Ca^{2+}, Mg^{2+}, Ba^{2+} and Fe^{3+} were chosen for the specificity test. The results of the specificity tests (Supplementary Fig. 4) showed little difference within the positive droplets of the digital PCR step between the negative and experimental groups. This result indicated that our dPCR-based detection system could detect copper with extremely high specificity.

3.8. Sensitivity assessment

The sensitivity of our study was defined as the lowest content of copper (II) that could be stably detected. The sensitivity of our detection approach was evaluated by detecting serial dilutions of the original copper solution. The cleavage rate was calculated from the positive droplets in the experimental groups and negative group. As shown in Table 1, the cleavage rate of the experimental group using 50 fmol copper showed an obvious difference from the negative control, while the group of 10 fmol showed little difference. Therefore, the sensitivity of our PCR-based biosensor detection system was determined as 50 fmol. Compared with previous studies on metal ions, we have achieved the most sensitive

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Table 1
The detection results of cleavage rate of serial dilutions of copper solution.

Final concentration of copper (II)/μM	Content of copper (II)/pmol	Cleavage rate			Mean value	RSD of inner-group
2	50	48.890	47.542	51.460	49.297	4.04%
0.8	20	19.773	20.377	19.644	19.931	1.96%
0.4	10	11.229	9.873	7.758	9.620	18.18%
0.08	2	2.360	1.796	2.023	2.060	13.79%
0.04	1	1.194	0.860	0.773	0.942	23.56%
0.02	0.5	0.708	0.515	0.407	0.544	28.07%
0.004	0.1	0.105	0.196	0.192	0.165	31.21%
0.002	0.05	0.110	0.159	0.082	0.117	33.33%
0.0004	0.01	0.080	0.064	0.035	0.060	37.82%
Negative	Negative	0.062	0.042	0.075	0.060	28.20%

detection for a metal ion. Using this system, even very low concentrations of metal ions could be detected.

3.9. The application validation of our PCR-based biosensor detection approach

To ensure the fitness of our detection system for different purposes, the copper content of different samples, such as natural water and food substrate, was detected using our detection system. Copper concentrations of the negative samples of different substrates in this section were measure by ICP-MS (Fu & Wang, 2011), which was regarded as the golden standard of ion detection. The detection results are listed in Table 2. As shown in this table, the detection results for different types of food substrates show the stability of our detection system. Although background signals exist in both negative natural water and Sprite samples, the background signal is extremely low that could be ignored in the final data analysis. Furthermore, the evaluation results have showed the identical positive concentration compared with the different manually additional copper ion contents. This indicated that our detection system based on the biosensor and digital PCR has the good practicality to all the detection food substrates.

4. Discussion

In our study, we have described a quantitative detection method for copper ions based on a biosensor and digital PCR. The binding model between DNAzyme and Cu^{2+} has been revealed. According to the binding model and evaluation results, the quantitative detection system has both good quantitative and qualitative sensitivity by taking advantage of digital PCR.

To achieve absolute quantitative detection for copper by a biosensor-based method, the binding model between DNAzyme and the copper ion is necessary. According to the prediction that the secondary structure could be changed when combined with a different number of ions, the secondary structure of the DNAzyme combined with the copper ion was evaluated by CD. Based on the detection results in our study, the characteristic peaks of the combination of DNAzyme and copper ion did not change as the concentration of the copper ion increased. Therefore, this result indicated that the copper ion has a unique binding site in the DNAzyme that does not change when the ion concentration increases (Nagatoishi, Isono, Tsumoto, & Sugimoto, 2011; Nagatoishi, Tanaka, & Tsumoto, 2007). On the other hand, experiments to optimize the cleavage time have also showed that a longer cleavage time had little effect on the final cleavage. For the determination of the number of binding sites, as the CD showed a positive peak when the ratio of ion to DNAzyme was 0.05:1, we considered that the largest possible number of binding sites is 1.

Moreover, according to the standard curve generated by the digital PCR results, this curve has shown a gradient of 1, which meant the change of cleavage rate has the same relationship with the content of copper (II). This relationship was followed by the Eq. (4).

$$[CR(sample\ 1) - CR(sample\ 2)]/[n(sample\ 1) - n(sample\ 2)] \cong 1 \quad (4)$$

For the above equation, the CR (sample1) and CR (sample 2) represented the cleavage rate of two different samples. While the n (sample 1) and n (sample 2) represented the content of copper

Table 2
The practical detection results for natural water and Sprite samples.

Sample type	Cu^{2+} addition (final concentration) /μM[1]	Qualitative results			Quantitative results/μM		
		1	2	3	1	2	3
Natural water	Negative[2]	-	-	-			
	0.1	+	+	+	0.101	0.121	0.113
	1	+	+	+	1.034	1.121	1.061
Sprite sample	Negative	-	-	-			
	0.1	+	+	+	0.171	0.156	0.136
	1	+	+	+	1.102	1.341	1.253

[1]The above table has listed both the detection results for the qualitative and quantitative detection results of the practical detection. For the practical detection, the negative samples and the samples added the Cu^{2+} manually have been tested simultaneously.
[2]Negative samples have been evaluated by the ICP-MS method to measure the original concentration of negative samples. The average ICP-MS results of original natural water and Sprite samples were 0.04 ppb and 0.12 ppb.

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(II) of two samples. According to the determination of cleavage rate in the Eq. (3), we changed the CR (sample 1 and 2) to the original state. The Eq. (4) has changed to the Eq. (5).

$$\frac{\{[B(NC)-A(sample\ 1)]-[B(NC)-A(sample\ 2)]\}/481.6}{n(sample\ 1)-n(sample\ 2)} \cong 1 \quad (5)$$

In the above equation, the B (NC) represented the B (negative control) in the Eq. (3). After the equivalent transformation of the Eq. (5), the above equation has transferred to Eq. (6).

$$[A(sample\ 2)-A(sample\ 1)]/481.6[n(sample\ 1)-n(sample\ 2)] \cong 1 \quad (6)$$

As the A (sample 1) and the A (sample 2) were the detection results of digital PCR, to replace this results to the results of the cleavage step, the serial dilutions and the pipetting step should be concerned. Concerned this situation, the above equation has changed to Eq. (7).

$$\frac{10^{-8}\times\frac{2}{25}\times[Y(sample\ 2)-Y(sample\ 1)]}{481.6\left[\frac{X(sample\ 1)-X(sample\ 2)}{N_A\times10^{-12}}\right]} \cong 1 \quad (7)$$

In the above equation, Y (sample 2) and Y (sample 1) represented the copy number of the uncut substrate in the volume of biosensor cleavage step, while the X (sample 2) and X (sample 1) was the copy number of copper (II). 10^{-8} was the degree of serial dilutions of the original reaction volume. $\frac{2}{25}$, which meant 2 μL among the 25 μL, was regarded as the template of digital PCR. N_A was the Avogadro constant (Deslattes et al., 1974), which was 6.02×10^{23}. After the simplification of the above equation, we could easily lead to the below Eq. (8).

$$\frac{Y(sample\ 2)-Y(sample\ 1)}{X(sample\ 1)-X(sample\ 2)} \cong 1 \quad (8)$$

According to the meaning of Eq. (8), the decrease of the copy number of the uncut of substrate oligonucleotides in the cleavage volume was as same as increase of the absolute number of copper (II). In this manner, the evaluation results of the digital PCR have also fit the conclusion that drawn by the CD measurement that the DNAzyme has the unique binding site with the copper (II).

Using these results, we have predicted the binding model of DNAzyme and copper ions. Thus, this is the basic principle of our quantitative detection system based on DNAzyme and digital PCR.

For the practical detection of copper, quantitative detection is essential to make a final measurement. However, most detection approaches based on fluorescence emission (Liu & Lu, 2007a) or paper-based stripes (López Marzo, Pons, Blake, & Merkoçi, 2013) can only achieve qualitative detection for heavy metal ions. Using a combined DNAzyme and digital PCR procedure, we achieved absolute quantitative detection of copper in our study. Digital PCR, as an improved quantitative detection approach, can directly measure the copy number of a target sequence in the original sample. Moreover, the substrate of our detection system was extremely small oligonucleotides; thus, digital PCR is suitable for equal distribution of the original reaction volume. In our study, the cleavage rate of each group was determined by the D-value of each experimental group to the negative group. Therefore, digital PCR is the most suitable method for the quantitative detection of copper. To ensure the accuracy of our detection system for samples of different concentrations, we drew a standard curve between the cleavage rate determined by digital PCR and the copper concentration. According to the standard curve, we concluded that the cleavage rate showed good linearity with different copper concentrations; the correlation coefficient was 99.2%, even when the copper concentration was extremely low. This result indicated that the detection system could be suitable for quantitative detection for samples with very low copper concentration.

For the original copper biosensor detection approach, the authors showed sensitive biosensor detection method based on fluorescence emission. The specificity and sensitivity were evaluated in that study. This research has shown a novel idea in that a non-nucleic signal could be transferred to a nucleic signal through biosensor treatments. In previous studies on the detection of heavy metal ions, most were focused on the detection of fluorescence emission (Liu & Lu, 2007a; Zhang et al., 2012) or paper-based stripes (Zhang, Fan, et al., 2015; Zhang, Zio, et al., 2015; Zhao et al., 2008). However, the sensitivity of these studies was largely limited due to the absence or low amplification efficiency of the signal amplification steps. Therefore, to improve the sensitivity and achieve absolute quantitation of the biosensor-based methodology, we combined digital PCR with the biosensor method in our research. Digital PCR has been shown to be the most sensitive method for the detection of nucleic fragments (Fu et al., 2015). Moreover, the ability to amplify trace amounts of target sequence and the tolerance for heavy metal ions make digital PCR a potential amplification tool in biosensor-based detection. In experiments to measure the limit of our detection system, the cleavage rate of the 10 fmol copper ion group showed an obvious difference from the negative group. This detection limit in our research is the most sensitive among all the detection methods based on the sensors. Thus, our detection system is more suitable for the detection of extremely low concentration copper ions. Moreover, the detection results of different food substrates, including natural water and drinks, have shown the feasibility of our detection system among different complicated substrates.

5. Conclusion

Here, a flexible and stable quantitative detection method with ultra-sensitivity was developed by combining a biosensor and digital PCR. By revealing the binding model between DNAzyme and Cu^{2+}, we solved the relationship of the signal generation and Cu^{2+} content. Taking advantage of the absolute quantitative character of digital PCR, an absolute quantitative detection system of Cu^{2+} was established. Thus, using the ultra-sensitivity of digital PCR, we have achieved the LOQ and LOD values as low as 0.5 pmol and 50 fmol, which regarded as the lowest limits among different studies based on sensors. Finally, the evaluation results of different practical food samples have shown the good feasibility of our detection system.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.10.106.

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