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Spatiotemporal Regulation of Metal Ions in the Polymerase Chain Reaction

Xianjing Zhang, Jun Guo, Bo Song, and Feng Zhang*

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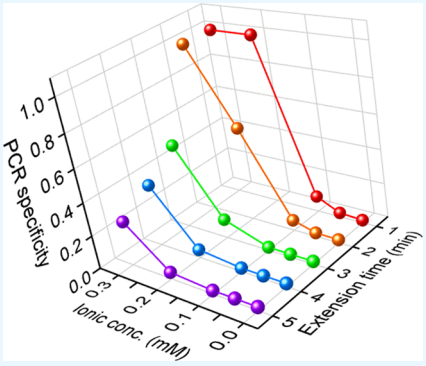
ABSTRACT: The polymerase chain reaction (PCR) has been widely used in medical diagnosis and forensic identification due to its ultrahigh sensitivity and signal amplification. Metal ions (i.e., Cu^{2+}, Zn^{2+}) have been considered PCR inhibitors and rarely shown their positive roles in PCR amplification until our report, in which we discovered that metal ions can significantly improve the PCR specificity and the yield of target DNA sequences. For an in-depth investigation with taking copper ions as a typical model, here we found an interesting spatiotemporal regulation mechanism of metal ions in PCR. The ionic concentration window for improving PCR specificity not only was independent of annealing temperature but also can be well regulated by both the annealing time and extension time. Using the ionic concentration window as a measure, the time affects either the amount or the sequence length of nonspecific amplicons in the space. The mechanism proposed in this work will deepen our understanding of the unneglectable roles of metal ions in DNA replication and meanwhile provide a new strategy for designing regulation kits for PCR-based biomedical applications.



1. INTRODUCTION

The polymerase chain reaction (PCR) has been widely used in clinical diagnosis and microorganism detection due to its unsurpassable sensitivity,[1−5] since its first invention in 1980s.[6] However, in some cases where low templates are required for continuous amplification, such as forensic identification, PCR products exhibit severe tailing bands, i.e., smear, which makes it difficult to obtain clear results.[7,8] The solution to these cases is to improve the specificity of PCR. Considerable efforts have been made to enhance PCR specificity by adding the organic molecules including betaine,[9] polyethyleneimine (PEI) derivatives,[10] polyamide-based dendrimers.[11] Besides these, it has been reported that nanostructures composed of gold,[8,12−14] carbon,[15] cadmium selenide,[16,17] and even graphene[18,19] can also significantly improve the specificity of PCR. However, it is still a challenge but worth finding new economical materials with broader applicability to improve PCR specificity.

Metal ions participate in the regulation of various bioactivities in organisms. For example, Mg^{2+}, Cu^{2+}, and Fe^{2+} are cofactors of many proteins and are involved in many enzyme-catalyzed biological reactions.[6,20,21] Biological samples collected for PCR normally contain metal ions, either from the sample itself or from the environment surrounding the sample. For example, biological samples collected from bone and blood contain endogenous calcium and iron ions, respectively.[22,23] Forensic biological samples also include DNA extraction from the surface of some metal objects, such as improvised explosive devices.[24] Metal ions (except for Mg^{2+}) contained in PCR amplification are generally considered PCR inhibitors, which inhibit the amplification of target DNA sequences[25,26] and significantly delay the cycle threshold (Ct) of real-time quantitative PCR (RT-qPCR).[27] Recently, it has been reported that inorganic ions (i.e., La ions) can significantly improve the specificity of PCR,[28] suggesting the positive role of metal ions in PCR amplification. However, the spatiotemporal regulation role of metal ions in PCR amplification has not been systematically investigated.

Here, using copper ions as a model, we employed typical PCR systems to investigate the positive role of metal ions in PCR amplification in terms of spatiotemporal regulation. We found that the appropriate concentration of copper ions significantly improved the specificity of PCR and the yield of target DNA sequences. Anion species and annealing temperature did not significantly affect the regulation effects. Further, we found that inreasing both the annealing and extension time of the PCR program resulted in a shift of the concentration window of copper ions to the higher range, indicating that the spatiotemporal regulation of copper ions was related to both the amount and sequence length of nonspecific amplicons. Due to the increase of nonspecific amplicons in the space, more copper ions are required to improve PCR specificity. Our research could help understand the important spatiotemporal

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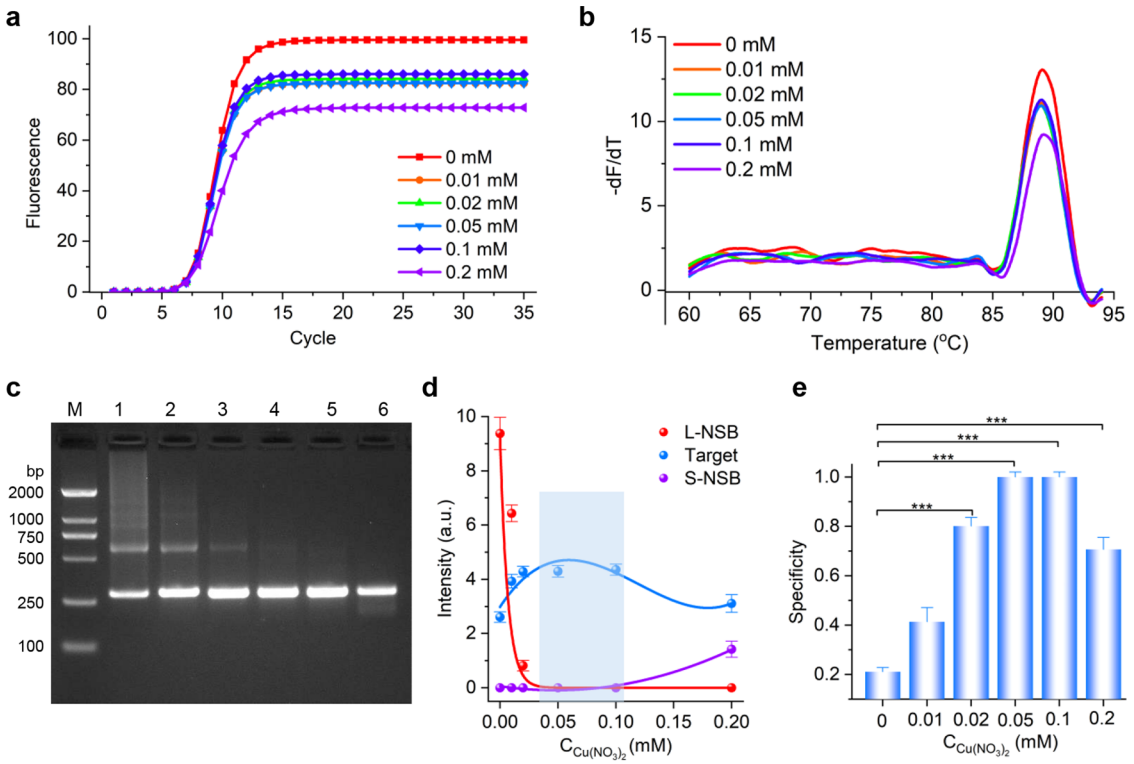

Figure 1. Copper nitrate can improve the RT-qPCR specificity. (a, b) The effect of copper nitrate on the amplification curve (a) and melting curve (b) in the RT-qPCR system. (c) The agarose gel shows the RT-qPCR products from (a). Lane M represents the DNA marker (BM2000). From lane 1 to lane 6, the final concentration of copper nitrate is 0, 0.01, 0.02, 0.05, 0.1, and 0.2 mM, respectively. (d) The yield of specific and nonspecific bands against the concentration of copper nitrate from (c). The blue rectangle represents the optimal concentration window for copper nitrate to improve the yield of target bands. L-NSB, Target, and S-NSB represent long nonspecific bands, target bands, and short nonspecific bands, respectively. (e) Plots of PCR specificity at different concentrations of copper nitrate from (c). All quantitative data are three repeated measurements, and error bars represent standard deviation (***$p < 0.001$).

regulation role of metal ions in life science and also facilitate the development of PCR additives with higher sensitivity and wider applicability.

2. RESULTS AND DISCUSSION

2.1. Copper Ions Significantly Improve the Specificity and Efficiency of PCR. An RT-qPCR system can display the amplification curve in real time and shorten the running time, which has become an important technology in biomedical, food, and other industrial fields. To investigate the positive effect of copper ions on PCR amplification, we first investigated the effect of copper nitrate on an RT-qPCR system. The addition of copper nitrate slightly inhibited the fluorescence intensity of amplification curves (Figure 1a) and had no obvious effect on the peak of melting curves (Figure 1b). Generally speaking, a single peak in the melting curve indicates that the PCR product is a single band. However, PCR products were smeared in the agarose gel (Figure 1c). When copper nitrate was added to an RT-qPCR system, nonspecific bands above the target band were significantly inhibited, and the yield of target bands and PCR specificity were improved. By quantifying the yield of bands in the gel, the yield of target bands increased when nonspecific bands were completely inhibited by copper nitrate (Figure 1d). Therefore, the decrease in the fluorescence intensity of amplification curves is attributable to the inhibition of nonspecific bands by copper nitrate. By quantifying the PCR specificity in Figure 1c, the appropriate concentration of copper nitrate can improve the specificity of RT-qPCR by five times (Figure 1e). In addition, cupric nitrate can also improve the specificity of PCR systems involving different polymerases (Figure S1), indicating that cupric nitrate can be widely applied to improve PCR specificity.

Continuous amplification of the low template usually leads to the disappearance of target bands and the appearance of severe smear. To investigate whether copper salts can eliminate severe smears, we employed an error-prone two-round PCR system to investigate the effect of copper salts on improving PCR specificity. As the number of amplification rounds increased, the nonspecific amplification products appearing in the previous round were accumulated in the latter round, and PCR products in the agarose gel exhibited a smear (Figure S2). When copper salts containing different anions were added to the PCR system, four copper salts can completely eliminate the smear and significantly improve PCR specificity and the yield of target bands in an error-prone two-round PCR system (Figure 2). Further, EDTA can reverse the effect of copper salts (Figure S3), which confirms that the role in improving PCR specificity is due to copper ions. Copper ions in PCR reactions can not only improve the specificity of PCR to obtain a single target sequence but also not affect downstream reactions (Figure S4). Gold nanoparticles have an excellent effect on improving PCR specificity.[8] The effect of copper ions on improving PCR specificity is completely comparable to that of gold nanoparticles (Figure S5) and has the advantages of simple operation, good stability, and low cost.

2.2. How Annealing Conditions Determine the Cu^{2+} Concentration Window for Enhancing PCR. From Figure

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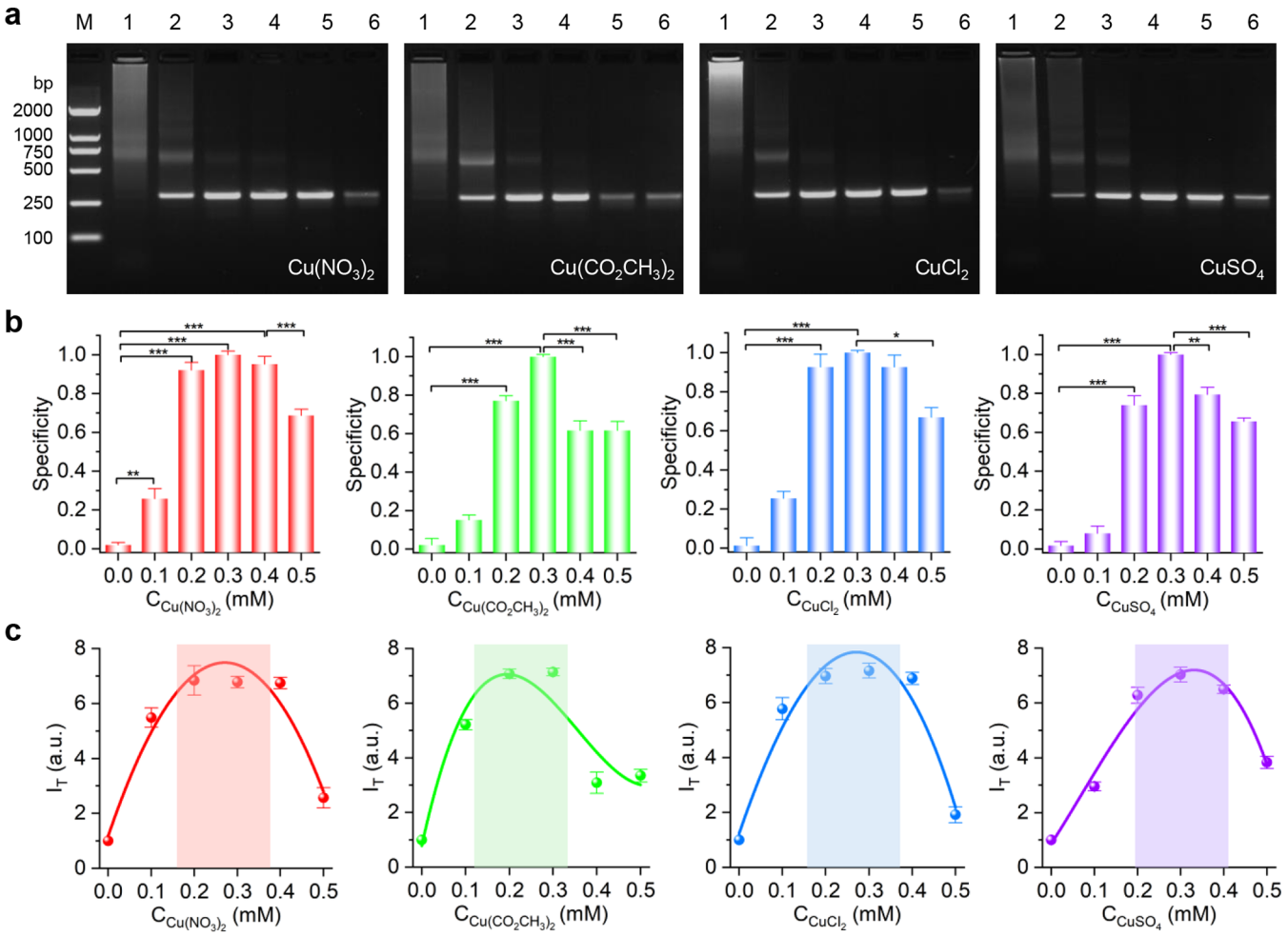

Figure 2. Copper ions improve the specificity of an error-prone two-round PCR system. (a) Gel electrophoresis analysis of different copper salts on the effect of an error-prone PCR system. For all lanes, lane M represents the DNA marker (BM2000); from lane 1 to lane 6, the final concentrations of copper ions in copper salts are 0, 0.1, 0.2, 0.3, 0.4, and 0.5 mM, respectively. (b) Plots of the PCR specificity at different concentrations of copper ions from (a). (c) Plots of the intensities of target bands (I_T) at different concentrations of copper ions from (a). The red, green, blue, and purple rectangles represent the concentration window of copper ions under different conditions, respectively. All quantitative data are three repeated measurements, and error bars represent standard deviation (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

2, we can conclude that there is a concentration window for copper ions to effectively enhance PCR. In principle, PCR performance could be very sensitive to any condition parameters such as temperature and time in the program configuration. Among all of the parameters, the annealing condition should be the most important one to be investigated. To this end, we first explored the effect of annealing temperature on the ionic concentration window in the error-prone two-round PCR system. The annealing temperature is one of the most important parameters, at which 50% of primers bind to the complementary template regions after PCR denaturation. The annealing temperature is generally between 45 and 65 °C. Using a low annealing temperature will increase the possibility of nonspecific PCR products, and increasing the annealing temperature can improve the specificity of PCR to a certain extent.[29] However, for severe smear phenomena in an error-prone two-round PCR system, merely increasing the annealing temperature cannot effectively improve PCR specificity (Figure S6). The excellent effect of copper ions can completely eliminate the smear, despite a 30 °C change in annealing temperature (Figure 3a). Moreover, changes in annealing temperature did not affect the concentration window for copper ions to improve PCR specificity. Interestingly, we found that the concentration window of copper ions can be slightly affected by the annealing time but not by room temperature (RT) incubation (Figure 3b,c). When the annealing time was increased from 1 to 4.5 min, the concentration window of copper ions shifted slightly toward the higher concentration. However, incubating PCR components containing copper ions for 1 h at RT did not affect the effect and concentration window of copper ions. Compared to the RT incubation, although the annealing temperature is not the optimal extension temperature for DNA polymerases, DNA polymerases can still exhibit polymerization activities. The increase in annealing time may increase the binding of nonspecific amplicons, ultimately leading to the increase of nonspecific amplicons in space. Therefore, the Cu^{2+} concentration window slightly shifts to the higher range to maintain their enhancing effect on PCR specificity.

2.3. Extension Time Significantly Affects the Concentration Window of Copper Ions. The extension time is also one of the most important parameters in PCR program configuration, which determines the extension length of amplicons. Therefore, we investigated the effect of the

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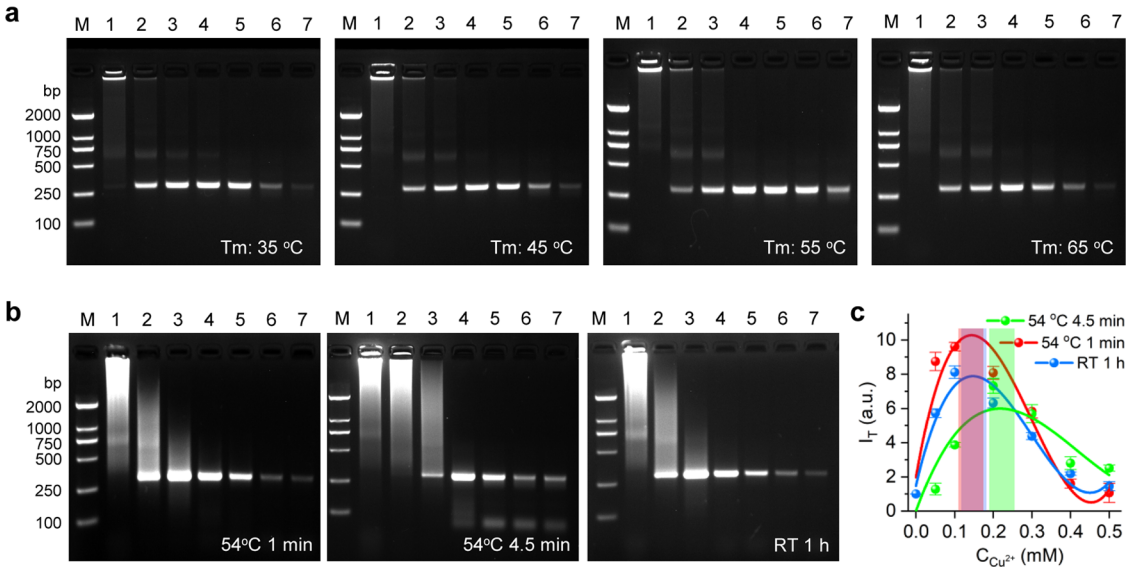

Figure 3. Effect of annealing conditions on the concentration window of copper ions. (a, b) Agarose gels show the effect of annealing temperature (a) and annealing time (b) on the concentration window of copper ions. For all gel images, lane M represents the DNA marker (BM2000); from lane 1 to lane 7, the final concentration of copper ions is 0, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 mM, respectively. Tm: annealing temperature. (c) Plots of the intensities of target bands (I_T) at different concentrations of copper ions from (b). The red, green, and blue rectangles represent the concentration window of copper ions under different conditions, respectively. All quantitative data are three repeated measurements, and error bars represent standard deviation.

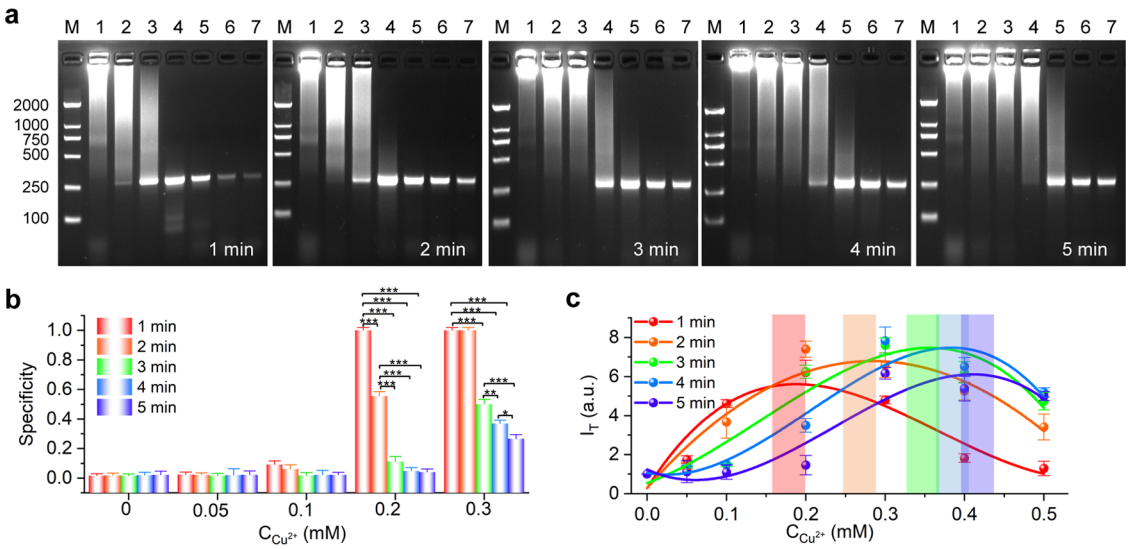

Figure 4. Increasing the extension time significantly affects the concentration window of copper ions. (a) Agarose gels of the effect of extension time on the concentration window of copper ions. For all gel images, lane M represents the DNA marker (BM2000); from lane 1 to lane 7, the final concentration of copper ions is 0, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 mM, respectively. 1−5 min represents extension times of the PCR program. (b) Plots of the PCR specificity at different concentrations of copper ions from (a). (c) Plots of the intensities of target bands (I_T) at different concentrations of copper ions from (a). Different colored rectangles represent the concentration window of copper ions under different extension times. All quantitative data are three repeated measurements, and error bars represent standard deviation (*p < 0.05, **p < 0.01, and ***p < 0.001).

extension time on the concentration window of copper ions to effectively improve PCR specificity. When the extension time is set as 1 min, smear phenomena disappeared completely after the addition of 0.2 mM copper ions (Figure 4a, 1 min, lane 4). However, when the extension time was increased to 2 min and with the addition of 0.2 mM copper ions, PCR products still showed weak nonspecific bands above the target band (Figure 4a, 2 min, lane 4). Then, the extension time was further increased to 3 min, the smear phenomena still existed, and the yield of target bands decreased significantly (Figure 4a, 3 min, lane 4). When the extension time was increased to 4−5 min, the target band disappeared until the concentration of added copper ions was increased up to 0.3 mM (Figure 4a, 4−5 min, lane 5). The concentration window of copper ions to improve PCR specificity and the yield of target bands under different extension times were quantitatively calculated (Figure 4b,c). An increase in extension time results in an increase in the sequence length of nonspecific amplicons in space. Therefore, increasing the extension time causes the optimized concentration window of copper ions to shift to a higher range. The

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concentration window of copper ions changes with reaction time, which becomes a smart method to detect the spatiotemporal effect of copper ions in PCR.

2.4. Possible Mechanisms of Copper Ions Improving PCR Specificity. The components of PCR reactions such as DNA polymerases, templates, and primers all carry negative charges. Therefore, copper ions added to the PCR system bind to PCR components. A possible mechanism is that copper ions compete with magnesium ions to bind to DNA polymerases, thereby inhibiting the activity of DNA polymerases. However, the reduction in the concentration of magnesium ions did not eliminate smears (Figure S7a), and adding additional magnesium ions also did not reverse the effect of copper ions (Figure S7b,c), suggesting that the enhancement of PCR specificity by copper ions should not be through enzymatic activity. However, adding nontarget DNA to the PCR system containing copper ions reversed the effect of copper ions (Figure S8). Therefore, we propose that copper ions in the PCR system mainly interact with DNA. The optimal concentration of copper ions is related to the amount of DNA, which may support the interaction of copper ions with DNA. Copper ions (0.05 mM) can improve the PCR specificity of samples with low templates. However, the same concentration of copper ions was not enough to improve the PCR specificity of samples with high template amounts (Figure S9).

In addition, we also found that Cd^{2+} and Zn^{2+} can also improve PCR specificity (Figure S10). Divalent transition metal ions have similar properties, and they can bind to phosphate groups and bases on DNA, resulting in a condensed state of ions−DNA complexes.[30,31] Therefore, the possible mechanism of copper ions improving PCR specificity is that the condensed state of copper ions−DNA complexes interferes with the binding of templates and primers or hinders the extension process of DNA polymerases. The nonspecific band was above the target band in gels, indicating that the sequence length of nonspecific amplicons was longer than that of target amplicons. We suspect that the longer amplicon binds more copper ions, leading to stronger condensation. Therefore, the amplification of long (nonspecific) amplicons is preferentially inhibited, while short amplicons (target) continue to amplify. Copper ions preferentially inhibited the amplification of long amplicons in both the nonspecific PCR system without a target and the duplex PCR system (Figures S11 and S12), which also confirmed the previous hypothesis. Therefore, copper ions may enhance PCR specificity by preferentially inhibiting amplification of long/nonspecific amplicons. Moreover, copper ions have spatiotemporal effects in regulating PCR specificity. The concentration window of copper ions is related to the amount and sequence length of amplicons in space. The increase in annealing time and extension time resulted in an increase in the amount and sequence length of amplicons within the space. To inhibit more nonspecific amplicons in space, more copper ions are required to improve PCR specificity.

3. CONCLUSIONS

In summary, we have demonstrated the spatiotemporal regulation role of metal ions in PCR amplification. Copper ions act as a model metal ion which can significantly improve PCR specificity and the yield of target DNA sequences, and the concentration window is closely related to the PCR program configuration. The concentration window of copper ions can be used as a measure to detect the spatiotemporal effects in regulating PCR specificity. The annealing time of PCR programs has a slight effect on the concentration window of copper ions. However, increasing the extension time resulted in an obvious shift of the concentration window of copper ions to a higher range. Given that metal ions can effectively improve PCR specificity and the yield of targets, this work provides a new strategy to developeconomical PCR additives.

4. MATERIALS AND METHODS

4.1. Materials. $Cu(NO_3)_2{\cdot}3H_2O$, $CuCl_2{\cdot}2H_2O$, $CuSO_4{\cdot}5H_2O$, $Cu(CH_3COO)_2$, $CdCl_2$, $ZnCl_2$, and EDTA-Na_2 were purchased from Aladdin (Shanghai, China). The fluorescent label reagent (GelRed) and DNA Markers (BM2000) were purchased from Biomed (Beijing, China). All primers were synthesized by Sangon Biotech (Shanghai, China). Lambda DNA (λDNA), premix Taq (Ex Taq Version 2.0 plus dye), and Ex Taq DNA Polymerase were purchased from TaKaRa (Dalian, China). Pfu DNA polymerase and Taq DNA polymerase were purchased from Beyotime (Shanghai, China). SYBR Green I was purchased from Biosharp (Shanghai, China). The plasmid of Pet28a-EGFP was purchased from HonorGene (Hunan, China). Gold nanoparticles with a diameter of 15 nm were purchased from BBI Solutions (Shanghai, China).

4.2. PCR Amplification. The RT-qPCR system was constructed as follows: 50 μL of the RT-qPCR system included 0.8 ng/μL λDNA, primers (0.2 μM each), 1.25 U DNA polymerase, 5 μL of 10 × PCR buffer, dNTP mixture (0.4 mM each), 1 μL of SYBR Green I, copper salts, and the remaining volume was filled with deionized water. The sequences of λDNA primers were 5′-GGCTTCGGTCCCTTCTGT-3′ (forward primer) and 5′-CACCACCTGTTCAAACTCTGC-3′ (reverse primer). Deionized water was used to dissolve and dilute the primers. Copper salts were dissolved in 10 mM Tris−HCl buffer (pH = 6.5), and the pH of copper salt solutions was adjusted to 6.5. The RT-qPCR procedure was as follows: a predenaturation process at 95 °C lasted for 2 min, followed by 35 cycles that each included a denaturation process at 94 °C for 30 s, an annealing process at 54 °C for 30 s, and an extension process at 72 °C for 60 s. The program of the melting curve was as follows: from 60 to 95 °C, increasing by 1 °C at each step with the continuous recording of fluorescence. The entire RT-qPCR program was performed on a 96-well thermal cycling instrument (LightCycler 96 system).

An error-prone two-round PCR system was constructed as follows:[8] λDNA was used as the template for the first-round amplification. The first-round PCR products were then diluted 400 times and used as the template for the second-round amplification. The second-round PCR amplification was performed using the same primers and under the same conditions as the first-round PCR amplification until PCR products showed smears in agarose gel electrophoresis; 25 μL of PCR components included 0.8 ng/μL λDNA, primers (0.2 μM each), 12.5 μL of premix Taq (Ex Taq Version 2.0 plus dye), copper salts, and the remaining volume was filled with deionized water. The PCR procedure was as follows: a predenaturation process at 95 °C lasted for 2 min, followed by 35 cycles that each including a denaturation process at 94 °C for 30 s, an annealing process at 54 °C for 30 s, and an extension process at 72 °C for 60 s. The last extension step at 72 °C lasted for 10 min. The sequencing of PCR products

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containing copper ions was performed by Sangon Biotech (Shanghai, China).

The duplex PCR system was constructed as follows: The template was a mixture of λDNA and Pet28a-EGFP plasmid. The primers were mixed primers of λDNA and Pet28a-EGFP. The PCR products contained target bands of 283 bp and 662 bp. The sequence of Pet28a-EGFP primers was as follows: FP: 5′-GGCACCTGTCCTACGAGTTG-3′, RP: 5′-GTCTGGCTGGCTGGCATAA-3′; 25 μL of PCR components included 0.28 ng of λDNA, 0.12 ng of Pet28a-EGFP plasmid, λDNA primers (0.2 μM each), Pet28a-EGFP primers (0.2 μM each), 12.5 μL of premix Taq (Ex Taq Version 2.0 plus dye), copper salts, and the remaining volume was filled with deionized water. The PCR program was exactly the same as those used in an error-prone two-round PCR system.

4.3. Data Analysis. PCR products were loaded into the 2% agarose gel stained with an ultrasensitive fluorescent nucleic acid chromophore (GelRed) and electrophoresed at 110 V for 30 min. Results of gel electrophoresis containing PCR products were observed with a gel imager (GelDoc-It 310 Imaging System). ImageJ software was used to quantify the band intensity in gels, and Origin software was used to present graphs and data analysis. Error bars showed the average value and standard deviation of three repeated measurements. The PCR efficiency was defined as the ratio of the intensity of target bands in experimental groups (with Cu^{2+}) and the control group (without Cu^{2+}). The intensity of target bands in the control group was normalized to 1.0. If the PCR efficiency was higher than 1.0 after adding Cu^{2+}, the PCR efficiency was improved. PCR specificity was defined as the ratio of target bands to the total bands in each group.[11] If the intensity of target bands increased and the nonspecific band decreased, the PCR specificity was improved.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c04507.

> Copper ions improving PCR specificity in different polymerase systems; construction of an error-prone two-round PCR system; EDTA alleviating the effect of copper ions; sequencing results of PCR products containing copper ions; compared copper ions with gold nanoparticles; effect of annealing temperature on PCR specificity; effect of magnesium ions on PCR specificity with or without copper ions; nontarget DNA reversed the effect of copper ions; effect of copper ions under different template amounts; effects of other divalent metal ions on PCR specificity; effect of copper ions on nonspecific amplification in the absence of targets; and effect of copper ions on the amplification of different amplicons (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Feng Zhang − *Key Laboratory of Optical Technology and Instrument for Medicine, Ministry of Education, School of Optical-Electrical Computer Engineering, University of Shanghai for Science and Technology, Shanghai 200093, China; Quantum Biophotonic Lab, Wenzhou Institute, University of Chinese Academy of Sciences, Wenzhou 325001, China; School of Biomedical Engineering, Guangzhou Medical University, Guangzhou 511436, China;* orcid.org/0000-0001-6035-4829; Email: fzhang@usst.edu.cn

Authors

Xianjing Zhang − *Key Laboratory of Optical Technology and Instrument for Medicine, Ministry of Education, School of Optical-Electrical Computer Engineering, University of Shanghai for Science and Technology, Shanghai 200093, China*

Jun Guo − *Key Laboratory of Optical Technology and Instrument for Medicine, Ministry of Education, School of Optical-Electrical Computer Engineering, University of Shanghai for Science and Technology, Shanghai 200093, China;* orcid.org/0000-0001-6944-0731

Bo Song − *Key Laboratory of Optical Technology and Instrument for Medicine, Ministry of Education, School of Optical-Electrical Computer Engineering, University of Shanghai for Science and Technology, Shanghai 200093, China;* orcid.org/0000-0001-5600-106X

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.2c04507

Notes

The authors declare no competing financial interest.

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