Electronic Supplementary Information (ESI)

Dual signal amplification strategy for high-sensitivity detection of copper species in bio-samples with tunable dynamic range

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Experimental Section

1. Materials and reagents.

Cu-Enzy and Cu-Sub (Table S1) of high performance liquid chromatography grade were synthesized by TaKaRa Bio Inc., and the oligonucleotides stock solutions (100 μM) were prepared with TE buffer. Streptavidin-coated magnetic nanoparticles (MNPs-SA) were purchased from BioMag Biotech. Co. Ltd. 4-(2-Hydroxyethyl)- piperazine-1-ethanesulfonic acid (HEPES), 4-phenylphenol (BIP), ascorbic acid and KOH (EG, 99.999%) were bought from Aladdin. HNO_3 (BV-Ⅲ, 70%) were obtained from Beijing Institute of Chemical Reagents. Luminol was obtained from Sigma. Standard solutions of Cu^{2+}, Mn^{2+}, Te^{2+}, Bi^{3+}, Ni^{2+}, Hg^{2+}, Pb^{2+}, Se^{2+}, Cd^{2+}, In^{3+}, Ca^{2+}, Mg^{2+}, Fe^{3+}, and Co^{2+} were bought from the National Standard Research Center of China. All other reagents were of analytical grade and were used without further purification. The glassware used in this experiment was thoroughly soaked in 2 M HNO_3 and rinsed thoroughly with ultrapure water prior to use. Ultrapure water obtained from a Milli-Q water purification system (resistivity < 18.2 MΩ•cm, Millipore) was used throughout the study.

The buffers used in this study were as follows: (1) buffer A: TE buffer; (2) buffer B: 0.01 M Tris-HCl, pH 8.0, 0.3 M NaCl; (3) buffer C: 0.05 M HEPES, pH 7.0, 0.05~1.5 M NaCl; (4) hybridization buffer: 0.05 M HEPES, pH 7.0, 1.5 M NaCl; (5) PBST: 0.01 M PBS, pH 7.4, 0.5% (v/v) Tween-20.

2. Synthesis of the MNPs@Cu-Sub@Cu-Enzy probe

Magnetic nanoparticles (MNPs) were functionalized with oligonucleotides by adding Cu-Sub to a suspension of MNPs (2 mg/mL) to a final concentration of 0.4 nmol/mg. The mixture was incubated at room temperature for 30 min with gentle stirring. MNPs@Cu-Sub was obtained by removing the supernatant through magnetic separation and washing with buffer A. Then, 20 μL of 100 μM Cu-Enzy and 480 μL of hybridization buffer were added to MNPs@Cu-Sub for hybridization with Cu-Sub at 37°C for 60 min. After hybridization, the probe (MNPs@Cu-Sub@Cu-Enzy) was washed with buffer B to remove the unbound Cu-Enzy, then resuspended in 500 μL of buffer B and kept at 4°C. The characterization and the CL catalytic activity of the probe are shown in Figure S1 and Figure S2.

3. Cu^{2+} determination by Cu-DNAzyme-based probe

Cu^{2+} determination was performed in 96-well plates, and 100 μL of the appropriate diluted probe in buffer B was used in each well. The supernatants were discarded through magnetic separation, and then a series of concentrations of Cu^{2+} in buffer C containing 50 μM ascorbate acid were added into the wells. After allowing the reaction to equilibrate for 40 min at ambient temperature, the supernatant was removed, and the probe was washed 5 times with PBST. RLU was recorded by a Victor X light chemiluminescence Reader (PerkinElmer). The concentrations of CL substrates were optimized by response surface optimization experiment, the matrix design and the results are shown in Table S2-S4 and Fig. S3.

4. The accuracy and precision of this method

Pretreated serum diluted 1000-fold (CuT is 1.09 μM, as determined by AAS) was spiked with Cu^{2+} standard solution to obtain the desired concentrations of 0.5, 2.0, 10, 50, and 100 nM. The

spiked samples were tested 6 times to determine the recoveries of Cu^{2+} in human serum. Moreover, the abovementioned samples were measured 6 times in the same day and on 3 different days to obtain the intra- and inter-assays precision. The results are displayed in Table S5.

5. Analysis of copper species in bio-samples

The serum and urine samples of patients with WD receiving treatment and healthy subjects are the remaining samples from clinical testing, which were obtained from Beijing Chao-yang Hospital. This experiment has been approved by the Medical Ethics Committee of Capital Medical University (NO. 2014SY47). Cu-DNAzyme-based probes can respond to only ionic Cu^{2+}. Thus, Copper in bio-samples should be digested to obtain ionic Cu^{2+} before detection. For CuT and urinary copper (CuU) determination, the serum and urine samples were directly digested by using a mixture of H_2SO_4, HNO_3 and H_2O_2 (2:1:1) as a digestion reagent. Serum or urine (100 μL) was mixed with 400 μL of digestion reagent and heated at 70°C for 60 min followed by 150°C until the samples were approximately dry. The residue was dissolved in 800 μL of H_2O, and the pH was adjusted to about neutral using KOH. Next, the digestion solutions were diluted with buffer C and measured by the proposed method and AAS, and the results were further analyzed by SPSS 19.0 software.

For CuEXC determination, EDTA, a high-copper-affinity chelator, was used to chelate the CuEXC loosely bound to serum albumin and amino acids. Briefly, 100 μL EDTA was added to 100 μL serum, incubated with vortex mixing, and then centrifuged for 30 min at 15000 rpm and 4°C by using an ultrafiltration device, the ultrafiltrate was collected for further digestion and detection as mentioned above. The concentration of EDTA and the capture time were optimized (Fig. S4). Additionally, before each experiment, the ultrafiltration device was soaked in 100 mM EDTA for 30 min and rinsed with ultrapure water thoroughly before use to avoid contamination of the filters.

Figures

Dynamic laser scattering (DLS) was used to characterize the change in size distribution and zeta potential in the process of probe synthesis (Figure S1). Compared with MNPs, the average particle size of MNPs@Cu-Sub and MNPs@Cu-Sub @Cu-Enzy is slightly increased, which may be due to the increased of water solubility after modification with Cu-Sub and Cu-DNAzyme. The absolute value of zeta potential increased with the functionalization with Cu-Sub and Cu-Enzy, both of which are negatively charged may be due to the negative ionization of DNA strands in water, thereby confirming the successful assembly of the DNAzyme probe. The zeta potential of MNPs@Cu-Sub@Cu-Enzy is -31.8 mV, indicating the good dispersibility of the probe in aqueous solution.

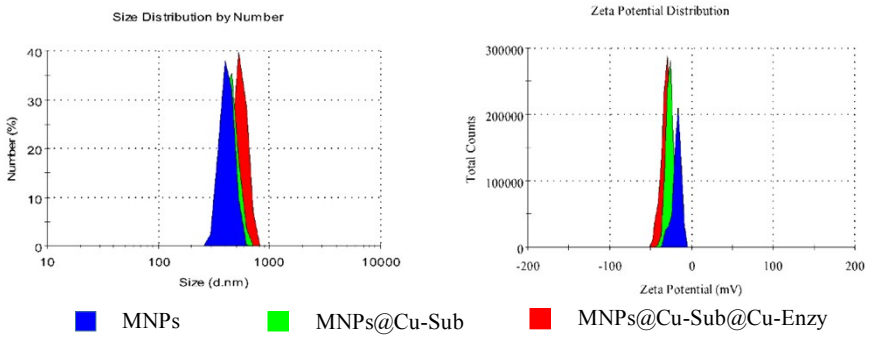

Figure S1. The characterization of probe size distribution and zeta potential.

MNPs with peroxidase-mimicking activity have been reported by various studies [1]. The peroxidase-mimicking activity of MNPs can lead to an increase in background signal and reduction of sensitivity. Therefore, we explored the CL catalytic activity of the MNPs, MNPs@Cu-Sub and probe (MNPs@Cu-Sub@Cu-Enzy). As shown in Fig. S1, the RLU produced by the MNPs was similar to that of buffer C, while after the Cu-Sub was immobilized on the surface of the MNPs, the RLU produced by MNPs@Cu-Sub was much higher than those of buffer C and the MNPs, and the RLU of probe was similar to that of MNPs@Cu-Sub. This demonstrated that the MNPs used in this study do not have peroxidase-mimicking activity because only certain size MNPs exhibit peroxidase-mimicking activity. Therefore, the MNPs used in this study did not affect the background signal and method sensitivity.

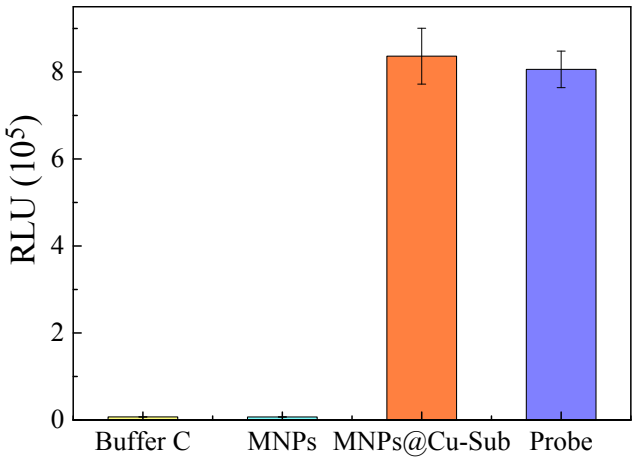

Figure S2. CL catalytic activity of MNPs, MNPs@Cu-Sub and probe. (MNPs, MNPs@Cu-Sub and probe were tested at the same concentration, 2.0 mg/L)

To maximize the RLU values from CL, response surface methodology (RSM) was selected to optimized the CL substrates concentration. RSM, a method for efficient statistical experimental design, can find the combination of parameter levels to achieve the optimal response and avoid conducting a large number of "one-factor-at-a-time" experiments. In this work, a three-factor and three-level Box-Behnken design was adopted to determine the optimum concentrations of luminol, BIP and H_2O_2, the corresponding levels of which are presented in Table S3. The experimental layout was carried out by Design Expert V8.0.5 software, and 17 sets of experimental runs at random were used to determine 9 coefficients of the model, including 12 factorial points and 5 zero-points, zero-point was repeated 5 times to estimate the experimental error.

Regression models of the relationship between these 3 variables and RLU were illustrated graphically by three-dimensional response surfaces (Figure S3), and the regression equation of RLU and each variable is: $Y=3.362\times10^6+8.993\times10^5A- 1.588\times10^5B+3.460\times10^5C+2864.67AB+1.88\times10^5AC-5625.08BC-8.850\times10^5A^2-92188.13B^2-2.398\times10^5C^2$ ($R^2=0.9924$). The detail matrix design of RSM and RLU results are displayed in Table S4.

Analysis of variance (ANOVA) was used to evaluate the adequacy and fitness of the developed regression model, and the results of variance analysis are shown in Table S5. The data demonstrated that the regression model was statistically significant ($P < 0.0001$), and the nonsignificant lack of fit ($P > 0.05$) indicates the reliability of the quadratic model. Therefore, the regression model can be used to make predictions about the response for given levels of each factor. According to the model, the optimum substrate concentrations to obtain the maximum RLU of the CL system were 1.60 mM luminol, 0.03 mM BIP and 10.0 mM H_2O_2.

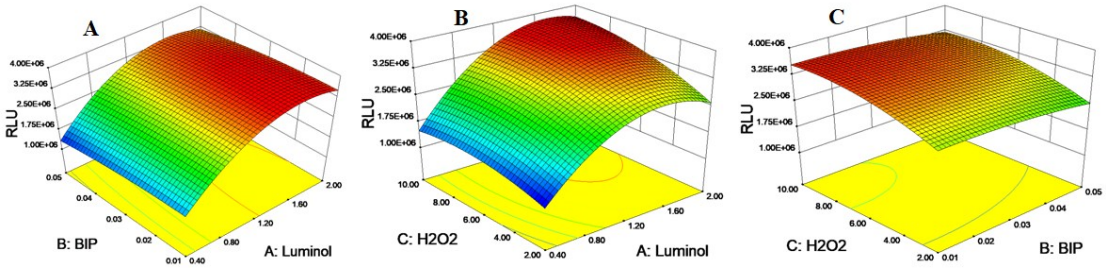

Figure S3. (A) The effect of luminol and BIP on RLU; (B) The effect of luminol and H_2O_2 on RLU; (C) The effect of H_2O_2 and BIP on RLU.

The concentration of CuEXC in the filtrate increased and then reached to a plateau at approximately 1.0 μM, which corresponded to 4 mM EDTA (Figure S4A). However, a linear increase can be observed after the plateau, which may be due to the partial degradation of Cp. Additionally, Cu exchanges between albumin or amino acids and EDTA reached equilibrium at 60 min (Figure S4B).

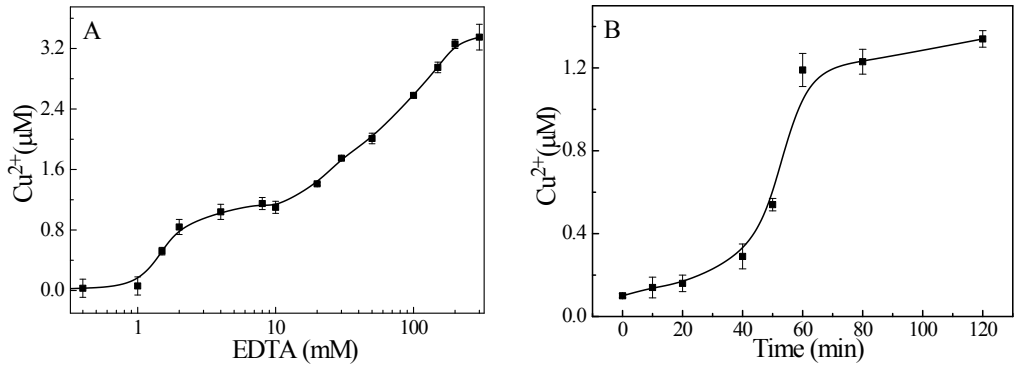

Figure S4. Optimization of the detection conditions for CuEXC in serum. (A) The effect of increasing EDTA concentration on CuEXC; (B) The effect of the incubation time of serum with EDTA before ultrafiltration on CuEXC. Error bars represent standard deviation (n=3)

Tables

Cu-Sub and Cu-Enzy were modified on the basis of their traditional feature structures [2]. The substrate was labeled with biotin and extended by 20 thymine (T) nucleobases at 5' end to reduce the steric hindrance between the magnetic nanoparticles (MNPs) and oligonucleotide, while the 3' end was contained HRP, which is also sensitive to Cu^{2+} in the presence of ascorbic acid. The sequences of Cu-Sub and Cu-Enzy used in this experiment are shown in Table S1.

Table S1. Sequences of Cu-Sub and Cu-Enzy used in this experiment

Oligonucleotides	Sequence (5'to 3')
Cu-Sub	Biotin-T_{20}AGCTTCTTTCTAATACGGCTTACC-HRP
Cu-Enzy	GGTAAGCCTGGGCCTCTTTCTTTTTAAGAAAGAAC

Table S2. Factors and levels of the RSM design

Symbols	Parameters (μM)	Levels and ranges		
		-1	0	+1
A	luminol	0.4	1.2	2.0
B	BIP	0.01	0.03	0.05
C	H_2O_2	2.0	6.0	10.0

Table S3. Matrix design and experimental results in terms of RLU

Experimental	Factors (μM)			Factors coded			Results
Runs	Luminol	BIP	H_2O_2	A	B	C	RLU
1	2.0	0.03	2.0	+1	0	-1	2515584
2	0.4	0.03	10.0	-1	0	+1	1583453
3	1.2	0.05	2.0	0	+1	-1	2514875
4	1.2	0.03	6.0	0	0	0	3296944
5	2.0	0.01	6.0	+1	-1	0	3467124
6	1.2	0.01	2.0	0	-1	-1	2898067
7	1.2	0.03	6.0	0	0	0	3390733
8	0.4	0.01	6.0	-1	-1	0	1544090
9	0.4	0.03	2.0	-1	0	-1	1223293
10	1.2	0.03	6.0	0	0	0	3422354
11	1.2	0.05	10.0	0	+1	+1	3151312
12	2.0	0.05	6.0	+1	+1	0	3231900
13	1.2	0.01	10.0	0	-1	+1	3557004
14	0.4	0.05	6.0	-1	+1	0	1297407
15	1.2	0.03	6.0	0	0	0	3472435
16	1.2	0.03	6.0	0	0	0	3228893
17	2.0	0.03	10.0	+1	0	+1	3627868

Table S4. ANOVA results for the designed response surface

Source	Sum of Square	Degrees of Freedom	Mean Square	*F*	*P*
Model	1.152×10^{13}	9	1.280×10^{12}	101.53	<0.0001
A-Luminol	6.470×10^{12}	1	6.470×10^{12}	513.35	<0.0001
B-BIP	2.019×10^{11}	1	2.019×10^{11}	16.02	0.0052
C-H_2O_2	9.576×10^{11}	1	9.576×10^{11}	75.98	<0.0001
AB	3.283×10^{7}	1	3.283×10^{7}	2.605×10^{-3}	0.9607
AC	1.414×10^{11}	1	1.414×10^{11}	11.22	0.0123
BC	1.266×10^{8}	1	1.266×10^{8}	0.01	0.9230
A^2	3.297×10^{12}	1	3.297×10^{12}	261.65	<0.0001
B^2	3.578×10^{10}	1	3.578×10^{10}	2.84	0.1358
C^2	2.421×10^{11}	1	2.421×10^{11}	19.21	0.0032
Residual error	8.822×10^{10}	7	1.260×10^{10}		
Lack of fit	4.961×10^{10}	3	1.654×10^{10}	1.71	0.3015
Total error	1.160×10^{13}	16			

Table S5. The recoveries and precisions of Cu^{2+} spiked in human serum (n=6)

C_{Cu2+} (nM)	Intra-day			Inter-day		
	$\bar{X}$±SD	Recovery (%)	*RSD* (%)	$\bar{X}$±SD	Recovery (%)	*RSD* (%)
0	10.45±0.84	95.8	7.70	10.17±1.02	93.3	10.0
0.5	11.35±0.98	90.0	8.63	11.48±1.21	116.0	10.54
2.0	13.13±1.25	111.5	9.52	12.64±1.59	87.0	12.57
10	19.96±1.57	90.6	7.86	20.26±1.73	93.6	8.54
50	62.75±4.28	103.7	6.82	65.06±3.68	108.3	5.65
100	107.23±2.95	96.3	2.75	111.23±4.64	100.3	4.17

Table S6. The comparison of different methods for Cu^{2+} detection [a]

Method	Detection strategy	LOD	Linear range	Sample	Ref
FL	The cleavage of Cu-DNAzyme	35 nM	Not available	None	2
CL	Label-free immunoassay	5.16 nM	15.6 nM-15.6 μM	traditional Chinese medicine and water	3
SERS	Dual Hot-Spot Model	0.18 nM	0.5-1000 nM	water samples	4
MPA	Ag MNPs transform to Ag PNPs	5 nM	30 nM-0.7 μM	human serum	5
FL	Fluorescence quenching of Carbon-Dots	1.0 μM	5.0 μM-200 μM	Cell	6
CL	Dual signal amplification	1.0 pM	Tunable	serum and urine	This work

[a] LOD: Limit of detection; FL: Fluorometry; CL: Chemiluminescence; SERS: surface-enhanced Raman scattering assay; MPA: Multimodal plasmonic assay.

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