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An Autonomous Bio-barcode DNA Machine for Exponential DNA Amplification and Its Application to the Electrochemical Determination of Adenosine Triphosphate

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Abstract: A novel autonomous bio-barcode DNA machine that is driven by template-dependent DNA replication is developed to exponentially amplify special DNA sequences. Combined with a DNA aptamer recognition element, the DNA machine can be further applied in the aptamer-based, amplified analysis of small molecules. As a model analyte, adenosine triphosphate (ATP) is determined by using the DNA machine system in combination with a DNA aptamer recognition strategy and differential pulse anodic stripping voltammetry (DPASV). Under the optimum conditions, detection limits as low as 2.8×10^{-17} M (3σ) for target DNA and 4.7×10^{-9} M (3σ) for ATP are achieved. The satisfactory determination of ATP in K562 leukemia cell and Ramos Burkitt's lymphoma cell reveal that this protocol possesses good selectivity and practicality. As a promising biomolecular device, this DNA machine may have an even broader application in the rapidly developing field of nanobiotechnology.

Keywords: adenosine triphosphate · aptamers · DNA · DNA recognition · voltammetry

Introduction

DNA performs important functions in carrying, memorizing, and transcribing genetic information. Its specificity of the A–T and G–C hydrogen-bonded Watson–Crick interaction provides a means to construct diverse DNA constructions of programmed base sequences, which makes DNA a useful construction biomaterial for nanoscale machinery.[1,2] DNA machines, which are themselves machine-assembled by DNA, are capable of performing machine-like functions at the molecular level. Recent research has seen the development of various DNA machines, such as tweezers,[3] gears,[4] walkers,[5] DNA-based logic gates[6] and amplified sensors.[7–12] These DNA-based machines provide new methods in the construction of DNA nanodevices that function as sensitive sensors, transporters, or drug-delivery systems. For example, DNA machines that amplify sensing through enzymes-assisted replication/scission reactions and employing synthetic HRP-mimicking DNAzymes or stem-loop-structured fluorescent molecular beacons as reporters are utilized in the detection of DNA,[7–10] small molecules[11] and metal ions.[12] These kinds of amplified sensors are competitive with usual sensors that amplify signal through polymerase chain reaction,[13] nanopartilces,[14] and enzymes.[15] Compared with sensing approaches known so far, DNA machines can carry out the function of circular amplification autonomously and effectively only under simple operations. More importantly, the signal amplification event is controllable in a DNA machine system, such that the whole reaction system can be controlled more easily according to the needs. However, existing DNA machines are still not efficient enough. DNA machines that possess much higher amplified efficiency, such as exponential circular amplification, may have more important future applications.

Herein, a novel autonomous bio-barcode DNA machine is developed to carry out an exponential DNA amplification. This DNA machine is based on template-dependent DNA replication that is manipulated by the Klenow fragment (KF) of *Escherichia coli* DNA polymerase I. When some primer DNA enters into the DNA machine system, the circular amplification reaction will be triggered. As a result, reporter DNA products, amplified exponentially, will be outputted. Combined with a DNA aptamer recognition

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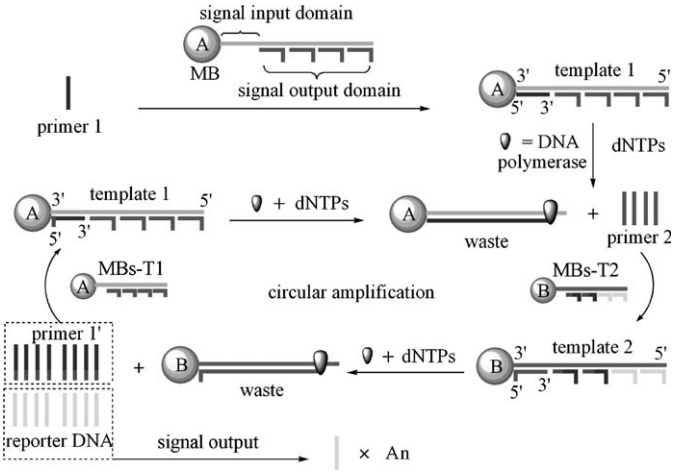
strategy, the DNA machine system can be further used in amplified, aptamer-based detection of small molecules. To be brief, when an aptamer recognizes and combines with its target molecule, competitor DNA will be released from the DNA/aptamer hybrids and then be inputted into the DNA machine system to obtain an amplified signal. As a model analyte, adenosine triphosphate (ATP) is determined by the DNA machine. ATP molecules displace the competitor sequence (primer DNA) from the ATP-binding aptamer, and these displaced competitors enter into the circular, exponential amplification reaction, referred to the DNA machine. Finally, an electrochemical detection strategy using Au nanoparticles (NPs) and CuS NPs with differential pulse anodic stripping voltammetry (DPASV) is used to detect the reporter DNA products outputted from the DNA machine system. Furthermore, ATP in K562 leukemia cell and Ramos Burkitt's lymphoma cell is determined successfully by the proposed method.

Results and Discussion

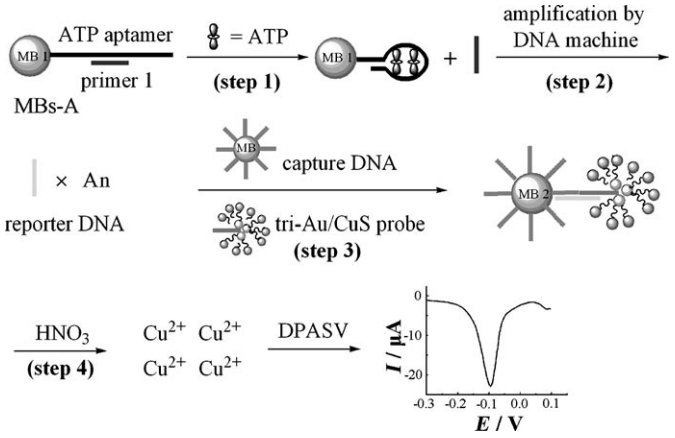
Configuration and operation of autonomous bio-barcode DNA machine: The Klenow fragment (KF), which catalyzes template-dependent DNA replication with great efficiency, is used in our DNA machine system. KF can recognize the double-strand junction of its primer/template substrate to form a binary complex. The incoming deoxynucleoside triphosphates (dNTPs) then bind and grow a new DNA strand from 3′-end of primer. Some DNA strands, which are hybridized to the template in advance, block the growing of new DNA strands. As a result, those DNA strands are displaced from the template. Based on the above principle, the DNA machine for exponential DNA amplification is designed as shown in Scheme 1. Nucleic acid template 1 consists of two domains—a signal input domain containing complementary sequence of primer 1 and a signal output domain hybridizing with four reduplicate units of primer 2.



Scheme 1. Configuration and operation of autonomous bio-barcode DNA machine.

It is noted that primer 2 is terminated at the 3′-end with a domain noncomplementary base to prevent undesired polymerization reaction of the template 1/primer 2 assembly. For convenient separation, template 1, functionalized with amido group on the 3′-end, is bound to carboxyl-modified magnetic beads (MBs, named MBs-T1), which are good solid-phase carriers of biomolecules. Having a similar basic structure, nucleic acid template 2, also immobilized on MBs, consists of a signal input domain containing complementary sequence of primer 2 and a bio-barcode signal output domain that hybridizes with two reduplicate units of primer 1′ and reporter DNA, respectively (named MBs-T2). Primer 1′ is a nucleic acid that has the same sequence of primer 1, but has six more bases. Similar to a fuel substrate, the function of primer 1′ is to undergo two steps of polymerization reaction, so that a chain-type alternant activation of the polymerization reaction (taking place in two templates) is formed. The circular amplification process by the DNA machine can be described as follows. When one primer 1 enters into the DNA machine system, four primer 2 will be displaced from template 1 through a polymerization reaction, which will lead to a increased output of eight primer 1′ and eight reporter DNA from template 2. The eight primer 1′ outputs reactivate the polymerization reaction in template 1, which will cause more and more polymerization reactions to be triggered, so the DNA machine actualizes automatic and continuous running. Meanwhile the exponentially amplified reporter DNA are exported from the DNA machine system. In theory, the relationship between circular reaction times (n) and circular amplified multiples (A_n) will follow the calculated formula of $A_n=8(8^n-1)/7$.

Aptamers are nucleic acid ligands that exhibit affinity and selectivity for their target molecules. Specific molecular recognition occurs through stacking and hydrogen-bonding interactions to the target and is typically accompanied by ligand-induced structural changes in both RNA and DNA aptamers.[16] Recently, various designs tend to develop aptamer-based sensors using generic approaches for coupling small molecule binding events to signal readout.[17–20] Herein, combined with DNA aptamer recognition element, and the DNA machine can be applied in the amplified, aptamer-based detection of small molecules. To demonstrate the generality of the strategy, low-affinity aptamer like ATP-binding DNA aptamer with a dissociation constant of 6 ± 3 μM[21] for ATP is used as model systems in this paper. Scheme 2 shows the whole process of amplified analysis of ATP model molecule. Firstly, the proportional relationship between ATP and primer 1 is constructed on the basis of competition between ATP molecule and primer 1 for ATP binding of the aptamer. Primer 1 is displaced from ATP aptamer immobilized on MBs. Secondly, primer 1 is inputted into the DNA machine system. After a polymerization reaction period of time, exponentially amplified reporter DNA is outputted from this system. Thirdly, a one-to-one recognition triple Au NPs DNA probe labeled with CuS tags (tri-Au/CuS NPs probe) is used in a "sandwich-type" strategy to detect the reporter DNA. Fourthly, CuS tags are dissolved



Scheme 2. The whole process of amplified analysis of ATP.

in strong acidic solution and detected using a sensitive DPASV method.

Optimization of polymerization reaction time: The DNA machine provides a general protocol for exponential DNA amplification. Involved in a circulating signal transformation process, the system is time-dependent and not free of limitations. For the detection procedure of the DNA machine, the polymerization reaction time was optimized, as shown in Figure 1. Electrochemical signals of nucleic acid primer 1

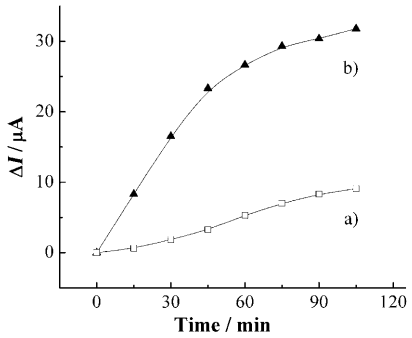


Figure 1. Relative anodic stripping peak current intensity of Cu^{II} in the presence of primer 1 a) 1×10^{-15} M and b) 4×10^{-15} M, recorded at different time intervals of DNA machine operation. See the Experimental Section for details on the procedure.

were determined after different polymerization reaction times ranging from 0 to 105 min. Figure 1 showed that the relative anodic stripping peak current of Cu^{II} generated by the DNA machine changed with different polymerization reaction times in the presence of primer 1. As anticipated, electrochemical signals increased with the prolonging of polymerization reaction time, indicating that the DNA machine could amplify sensing through a circular polymerization reaction. Ultimately, 1 h was regarded as the optimum polymerization reaction time in view of both sensitivity and time consumption.

Sensitivity of DNA analysis by the DNA machine: As the target DNA, the concentration of primer 1 was determined by the DNA machine under the optimum polymerization reaction time of 1 h. The result is shown in Figure 2 (top),

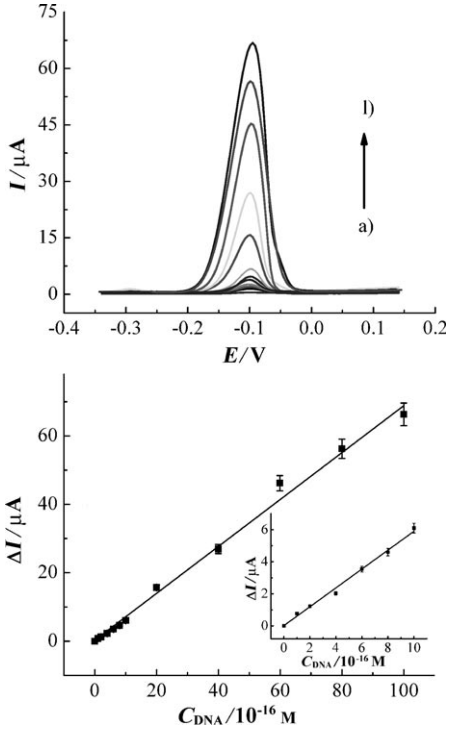


Figure 2. Top: DPASV signals amplified by the DNA machine for primer 1 of different concentrations: a) 0, b) 1.0×10^{-16}, c) 2.0×10^{-16}, d) 4.0×10^{-16}, e) 6.0×10^{-16}, f) 8.0×10^{-16}, g) 1.0×10^{-15}, h) 2.0×10^{-15}, i) 4.0×10^{-15}, j) 6.0×10^{-15}, k) 8.0×10^{-15} and l) 1.0×10^{-14} M. The DNA machine operation is detailed in in the Experimental Section under optimum polymerization reaction time of 1 h. Bottom: Calibration curve; inset: the amplification of the first seven data points.

which indicates that the anodic stripping peak current of Cu^{II} increased with an increasing concentration of primer 1. The concentration of primer 1 could be determined in a linear range from 1.0×10^{-16} M to 1.0×10^{-14} M with a detection limit of 2.8×10^{-17} M at 3σ. The regression equation used was $\Delta I=0.6905\,C-0.0071$ (in which ΔI is the relative anodic stripping peak current in μA; C is the concentration of primer 1, 10^{-16} M; $n=12$, $R=0.9977$; Figure 2, bottom). Compared with a simple "sandwich-type" DNA analysis only using a one-to-one recognition tri-Au/CuS NPs probe (see Supporting Information), the sensitivity of DNA machine system was found to be increased by about two orders of magnitude with an assay time of 1 h. Therefore, remarkable amplification of DNA was obtained by the DNA machine.

Sensitivity and selectivity of ATP analysis by the DNA machine: On the basis of above research, ATP standards of different concentrations were determined by amplification of the proposed DNA machine. As shown in Figure 3 (top), the higher the concentration of the fixed ATP standard, the

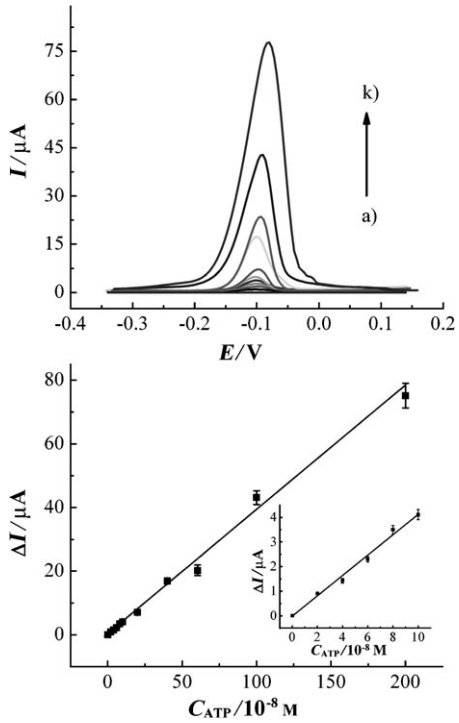


Figure 3. Top: DPASV signals amplified by the DNA machine for ATP standard of different concentrations: a) 0, b) 2.0×10^{-8}, c) 4.0×10^{-8}, d) 6.0×10^{-8}, e) $8.0\times10^{-}$, f) 1.0×10^{-7}, g) 2.0×10^{-7}, h) 4.0×10^{-7}, i) 6.0×10^{-7}, j) 1.0×10^{-6} and k) 2.0×10^{-6} M. Mixture of ATP standard (20 μL) and MBs-A solution (5 μL) was incubated for 60 min to make the aptamer change its structure to bind ATP. The supernatant of primer 1 was obtained by magnetic separation. The ATP analysis was carried out as described in the Experimental Section. Bottom: Calibration curve; inset: the amplification of the first six data points.

higher the anodic peak current. The result indicated that the anodic stripping peak current of Cu^{II} had a good linear relationship with the concentration of ATP standard ranging from 2.0×10^{-8} M to 2.0×10^{-6} M (as shown in Figure 3, bottom). The linear regression equation was calculated as $\Delta I=0.3934\,C+0.2673$ with a correlation coefficient as 0.9965 ($n=11$; ΔI is the relative anodic stripping peak current in μA and C is the concentration of ATP standard: 10^{-8} M). A detection limit as low as 4.7×10^{-9} M at 3σ for ATP was obtained. With respect to sensitivity, this method was better than some established aptamer-based detections of ATP which is listed in Table S2 in the Supporting Information. The proposed ATP analysis amplified with the DNA-based machine was also competitive with electrochemical methods, such as electrochemical sensors with aptamer-complementary DNA oligonucleotides as probe[20] and the target-responsive electrochemical aptamer switch method for reagentless detection of ATP.[18]

The selectivity of the method was investigated by using the proposed method to determinate the same concentration of ATP and its analogues—cytosine triphosphate (CTP), guanosine triphosphate (GTP) and uridine triphosphate (UTP), under the same conditions. The result was shown in Figure 4. High anodic stripping peak current of Cu^{II} was obtained for ATP, whereas electrochemical signal of GTP, UTP and GTP was as low as that of the blank; the results indicate that these small molecules could not interfere with ATP analysis in the proposed protocol based on specific recognition between ATP-binding aptamers and ATP. With good sensitivity and selectivity, the proposed method provides a versatile aptamer-based method to detect minute amounts of small molecules.

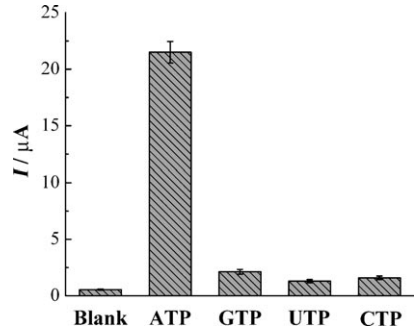


Figure 4. Histogram of relative anodic stripping peak current versus different analytes. The same concentration of ATP, GTP, UTP, and CTP (6.0×10^{-7} M) was detected by the method mentioned in the Experimental Section.

To demonstrate that the proposed method could be used in the detection of practical samples, ATP extracts from K562 leukemia cells and Ramos Burkitt's lymphoma cells were treated with 100-fold dilution to reduce the levels of ATP and then determined by this method. The results are listed in Table 1. The DNA machine-based assay showed that K562 cells released 2.18 ± 0.09 μg (average ±S.D., $n=3$) of ATP per 10^5 cells, whereas Ramos cells released 2.69 ± 0.13 μg of ATP per 10^5 cells. To assess veracity of the results, high-performance liquid chromatography (HPLC) method was also used to determine the same samples for comparison (see Supporting Information). The comparative result indicated that data obtained by HPLC method were consistent with the proposed method.

Table 1. Analysis of ATP in K562 cell and Ramos cell.

	This work [μg 10^5 cells^{-1} mL^{-1}]	RSD [%, n=3]	HPLC [μg 10^5 cells^{-1} mL^{-1}]	RSD [%, n=3]
K562	2.23	3.6	1.93	4.1
cell	2.34	5.2	2.02	4.6
	1.98	4.1	1.85	3.8
Ramos	2.55	4.8	2.32	4.6
cell	2.89	5.2	2.59	4.7
	2.62	3.8	2.41	4.2

Conclusion

In summary, we present a novel autonomous bio-barcode DNA machine that can amplify special DNA exponentially. In the DNA machine system, every sub-assembly is designed and fitted together to perform its circular amplification function induced by polymerization reaction. The result of

target DNA detection shows high sensitivity of the DNA machine. Furthermore, combined with a DNA aptamer recognition strategy, the DNA machine can be applied in the amplified detection of small molecules. As a model analyte, ATP was determined by the DNA machine system assisting with a "sandwich-type" hybridization strategy in which a one-to-one recognition tri-Au/CuS NPs probe is used for electrochemical analysis. The method is sensitive to relevant changes in ATP concentrations and shows a high selectivity for ATP when compared to other nucleoside triphosphate analogues. Furthermore, successful determination of ATP in cancer cells proves the practicability of the DNA machine. In conclusion, the DNA machine provides not only a general protocol for the amplified detection of DNA and small molecule, but also a model for applications of biomolecular devices.

Experimental Section

Reagents: Polymerase KF, dNTPs and all the synthetic oligonucleotides were purchased from SBS Genetech. Co. Ltd. (China). The sequences of oligonucleotides were listed in Table S1 in the Supporting Information. ATP, CTP, GTP, and UTP were obtained from Sigma, and their stock solutions (1.0 mM) were prepared by doubly distilled water. Bovine serum albumin (BSA), hydrogen tetrachloroaurate(III) ($HAuCl_4$), mercaptoacetic acid, tris(2-carboxyethyl) phosphine hydrochloride (TCEP) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were also purchased from Sigma and used as received. The carboxyl-modified MBs (Size: 1.0–2.0 μm) were purchased from Tianjin Baseline ChromTech Research Centre (China). Mercury chloride ($HgCl_2$), copper sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$), trisodium citrate and imidazole were obtained from Yuanhang Chemical Company (China). All other reagents were of analytical grade.

Apparatus: The electrochemical measurement for DPASV was carried out on a CHI 660C electrochemical working station (Texas, USA) using a three-electrode system consisted of a platinum wire as an auxiliary electrode, an Ag/AgCl electrode as reference electrode, and a 3 mm-diameter glassy carbon electrode (GCE) as working electrode. Transmission electron microscopy (TEM) images were recorded on a JEM 1200EX transmission electron microscope (JEOL, Japan). UV/Vis absorption spectra were obtained with a Cary 50 Series Spectrophotometer (Varian, Australia). HPLC analysis was performed in a Hitachi HPLC system (Hitachi, Japan).

Immobilization of capture DNA and template DNA onto MBs:[22] In a typical experiment, carboxyl-modified MBs (50 μg) were firstly washed three times and then activated in imidazole buffer (200 μL, 0.1 M, pH 6.8) containing EDC (0.1 M) with gentle shaking for 30 min. Capture DNA (S1 or S8, 200 pmol) or template DNA (S6 or S7, 200 pmol) was separately added into the activated MBs, and the resultant mixture was incubated for 12 h at 37 °C with gentle shaking. The oligonucleotide-modified MBs were rinsed three times with buffer B (200 μL, 10 mM, pH 7.0 PBS containing 0.15 M NaCl), magnetically separated, and then resuspended in buffer B (200 μL) containing BSA (10 %) for 1 h to minimize nonspecific adsorption effects, and the conjugates were resuspended in buffer B (200 μL) before use.

Primer 1 (S2, 200 pmol) was added to a solution of ATP-binding aptamer (S1)-immobilized MBs, named MBs-A. Primer 2 (S3, 800 pmol) was added to a solution of template 1 (S6)-modified MBs, named MBs-T1. Primer 1′ (S4, 400 pmol) and reporter DNA (S5, 400 pmol) were added to a solution of template 2 (S7)-modified MBs simultaneously, named MBs-T2. After incubating for 1 h at 37 °C with gentle shaking, the MBs were washed three times with buffer B (200 μL) and resuspended in buffer B (200 μL) before use.

DNA amplification by using the DNA machine and electrochemical measurements: Solutions of primer 1 sequence at different concentrations (1.0×10^{-16} M ~ 1.0×10^{-14} M) were prepared before use. Amplification analysis with the DNA machine[23] were performed by mixing MBs-T1 solution (5 μL), MBs-T2 solution (5 μL), polymerase KF (10 U), dNTPs (0.2 mM) and primer 1 (20 μL) solution in buffer A (0.1 mM DTT, 7 mM $MgCl_2$ and 10 mM pH 7.5 Tris-HCl) to a final volume of 100 μL and incubating at 37 °C for 1 h. After separating magnetically, a solution capture DNA (S8)-modified MBs (10 μL) and a solution of tri-Au/CuS NPs probes (10 μL, see Supporting Information) were added simultaneously to the supernatant. The mixture was incubated with continuous shaking at 37 °C for 1 h to complete the "sandwich-type" DNA hybridization. The resultant hybrids were separated by magnetic force and washed three times with buffer B containing BSA (1 %).

CuS NPs anchored on the hybrids were dissolved in an aqueous solution of HNO_3 (10 μL, 0.5 M) for 5 min. After magnetic separation, the supernatant was transferred into an analytical cell containing Hg^{II} (1.8×10^{-4} M) and the final volume was adjusted to 2 mL with acetate buffer (0.01 M, pH 5.3). To detect the dissolved Cu^{II} sensitively, DPASV was used with the in situ preparation of mercury film on the surface of a GCE with a deposition time of 300 s and deposition potential of −1.2 V. After a 20 s rest time, the anodic stripping peak current located at −0.1 V was measured and taken as the analytical response. The conditions selected for DPASV were increment in potential 0.004 V, amplitude 0.05 V, pulse width 0.06 s, sample width 0.02 s, pulse period 0.2 s.

Preparation of ATP extracts from cancer cells and ATP analysis: The K562 leukemia cell and Ramos Burkitt's lymphoma cell were separately cultured in cell flasks according to the instructions from the American Type Culture Collection. The cell line was grown to 90 % confluence in RPMI-1640 Medium supplemented with fetal bovine serum (10 %) and penicillin–streptomycin (100 IU mL^{-1}) at 37 °C, and the cells were harvested by trypsinization. The cell density was determined by a hemocytometer prior to each experiment. Then, the suspension of K562 cell (1.0 mL, 1.02×10^6 cells) and Ramos cell (1.0 mL, 4.42×10^5 cells), dispersed in RPMI cell media buffer, was centrifuged at 3000 rpm for 5 min and washed with phosphate-buffered saline (18.6 mM PBS, 4.2 mM KCl, and 154.0 mM NaCl, pH 7.4) three times and resuspended in deionized water (1.0 mL). Finally, the cells were disrupted by sonication for 20 min at 0 °C. To remove the homogenate of cell debris, the lysate was centrifuged at 10 000 rpm for 10 min at 4 °C.

For the detection procedure, ATP standard solution at different concentrations (20 μL, 2.0×10^{-8} M to 2.0×10^{-6} M) or ATP extracting solution from cancer cells with proper dilution was added into MBs-A solution (5 μL) and incubated for 60 min to make the aptamer change its structure to bind ATP. The supernatant was obtained by magnetic separation. The above-mentioned process was repeated to obtain data of ATP analysis.

Acknowledgements

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