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Isothermal amplified detection of ATP using Au nanocages capped with a DNA molecular gate and its application in cell lysates†

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A novel controlled-release biosensor for isothermal amplified detection of ATP using Au nanocages (AuNCs) capped with a DNA molecular gate is reported for the first time, and has been successfully tested in intracellular ATP detection. Two kinds of SH-modified short strand DNAs S1 and S2 were assembled on the surface of the AuNCs by means of Au–thiolate bonding. The hybridization of a long-strand DNA S3 with the two immobilized SH-DNAs leads to the formation of molecular gates. The molecular gates were designed to inhibit the release of the fluorescent molecules such as Rhodamine-B (RhB), which were filled in the hollow interiors of AuNCs. The primer S4 was employed to play the role of a recognition moiety. The specificity recognition reaction between ATP and ATP aptamer gave rise to the primer S4 released from a double-stranded hybrid formed with the ATP aptamer. The released S4 will initiate the autonomous replication–scission–displacement process with the assistance of DNA polymerase and nicking endonuclease. As a result, the DNA synthesis and the DNA cycle achieved the opening of the DNA-based molecular gates and the significant amplification of the release of the guest molecules from AuNCs. In order to realize the cyclic enzymatic amplification of the release of the guest molecules from AuNCs, the long-strand S3 is ingeniously designed in such a way that it contains a Nb.Bpu10I nicking endonuclease recognition sequence and a sequence complementary to the primer S4. The fabricated system was demonstrated to be an efficient biosensor for target molecule detection qualitatively and quantitatively.

Introduction

Among a variety of Au nanoparticles, Au nanocages (AuNCs) have received increasing attention in biomedical applications in recent years owing to their excellent biocompatibility and bioinert nature, as well as their spectacular physical and chemical properties.[1–3] These versatile nanostructures, characterized by hollow interiors and ultrathin porous walls, have shown enormous potential and have a promising future in the fields of cancer theranostics including imaging,[4,5] diagnosis,[6,7] drug delivery,[8] and therapy.[9,10] The localized surface plasmon resonance (LSPR) peaks of AuNCs can be readily and precisely tuned to the so-called transparent window of blood and soft tissues in the near-infrared (NIR) region ranging from 700 to 900 nm[11,12] to meet the requirements for *in vivo* application by controlling their size, wall thickness, or both.[13] In addition, AuNCs possess many other significant features such as a compact size, extraordinarily large scattering and absorption cross-sections (almost five orders of magnitude greater than those of conventional organic dyes), low cytotoxicity, as well as an easy and straightforward procedure for surface modification using the well-established thiolate–Au chemistry.[14–16]

To fully realize their potential in cancer theranostics, AuNCs as promising nanoscale carriers must have the capability of controlled release of the loaded guest. Recent reports on the design of capped and gated AuNCs have shown promise in the generation of controlled-release systems. For example, thermally responsive polymers,[17,18] calcium phosphate coated magnetic nanoparticles,[19] and phase-change material (PCM) have been applied to block the pores of AuNCs.[20] To control opening of the pore entrances of AuNCs and to achieve the controlled release, various stimuli including pH, NIR laser irradiation, high-intensity focused ultrasound, and temperature, have been used as "triggers" for uncapping the pores and releasing the guest molecules from AuNCs.[17–20]

Despite these achievements, it is still a challenge to design more practical controlled-release AuNC systems not requiring

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external stimuli. Driven by the need, our group has developed a novel bioresponsive controlled-release biosensor using Au nanocages capped with an aptamer-based molecular gate.[21] Aptamers have proven to be effective pore blockers to make AuNCs a controllable release system. Inspired by this strategy, we combined the advantages of a DNA amplification technique with a bioresponsive molecular gate to develop a novel controlled-release biosensor based on AuNCs for isothermal amplified detection of trace amounts of target molecules. To the best of our knowledge, an AuNC-based isothermal amplified detection strategy triggered by the primer and its application in cancer cells have not yet been reported.

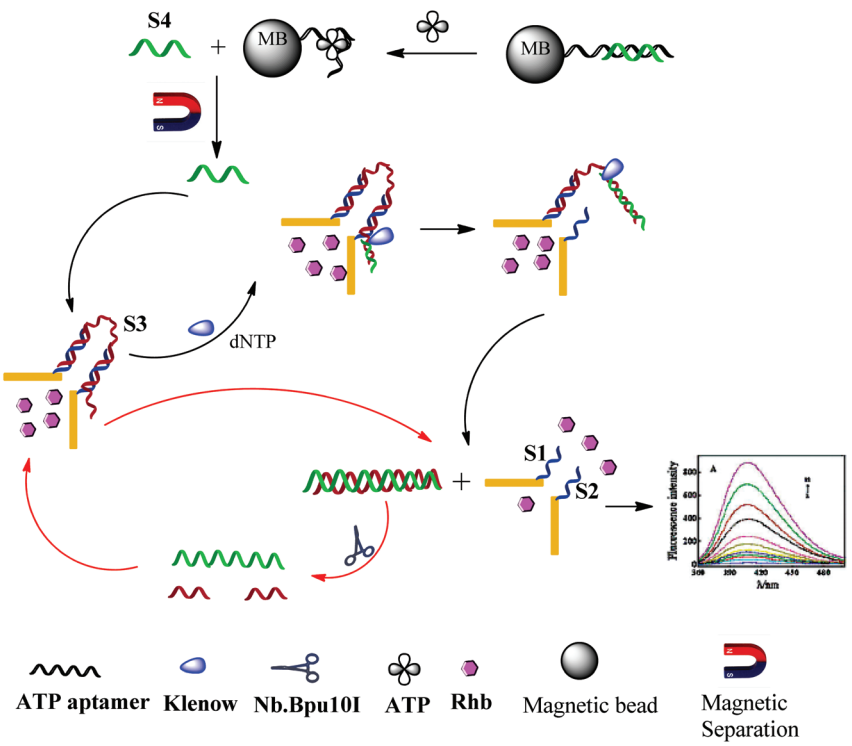
Herein, we describe the development of a novel controlled-release biosensor for isothermal amplified detection of ATP with a combination of AuNCs capped with a DNA molecular gate and DNA machine amplification. Two kinds of SH-modified short strand DNAs S1 and S2 were assembled on the surface of the AuNCs by means of Au–thiolate bonding. The hybridization of a long-strand DNA S3 with the two immobilized SH-DNAs leads to the formation of molecular gates. The molecular gates were designed to inhibit the release of the fluorescent molecules such as Rhodamine-B (RhB), which were filled in the hollow interiors of AuNCs. The primer S4 was employed to play the role of a recognition moiety. The specificity recognition reaction between ATP and ATP aptamer gave rise to the primer S4 released from a double-stranded hybrid formed with an ATP aptamer. The released S4 will initiate the autonomous replication–scission–displacement process with the assistance of DNA polymerase and nicking endonuclease. The DNA synthesis and the DNA cycle achieved the opening of the DNA-based molecular gates and the significant amplification of the release of the guest molecules from AuNCs. As a result, highly sensitive and selective detection of ATP is achieved.

Results and discussion

Principle of the assay

The working principle of the bioresponsive controlled-release system is illustrated in Scheme 1. In this work, we chose ATP as a target molecule for proof-of-concept experiments. ATP is important for regulating cellular metabolism and biochemical pathways, and as such, the intracellular ATP level is used as an indicator of living organisms and their biological activity.

AuNCs as supports were functionalized with two kinds of SH-modified short strand DNAs S1 and S2 by means of Au–thiolate bonding on the surface of the AuNCs. S1 and S2 were complementary to the two ends of a long-strand DNA S3, respectively. The hybridization of S3 with the two immobilized SH-DNAs leads to the formation of the molecular gate. In order to realize the cyclic enzymatic amplification of the release of the guest molecules from AuNCs, the long-strand S3 is ingeniously designed in such a way that it contains a Nb. Bpu10I nicking endonuclease recognition sequence and a sequence complementary to the primer S4. Once the primer S4, complementary to the 3′ end of S3, was mixed with the capped AuNCs, it will hybridize with S3, which was assembled



Scheme 1 Schematic illustration of the bioresponsive controlled-release biosensor system for isothermal amplified detection of ATP.

on the surface of the AuNCs. Subsequently, with the assistance of DNA polymerase and nicking endonuclease, the autonomous replication–scission–displacement process was initiated. In the process, S4 as a primer initiates the polymerization and the DNA synthesis of the complementary strand of S3 (S3′), which will result in the displacements of S1 and S2, respectively, and eventually leading to S3 to be uncapped from the AuNCs and opening of the DNA-based molecular gate. The released S3 in the formed duplex was sheared into two single-stranded DNAs, resulting in the dissociation of the duplex and the release of S3′. Then, S3′ is free to bind to another S3. As a result, the DNA cycle achieved the significant amplification of the release of the guest molecules from AuNCs.

Based on the design, we constructed a novel bioresponsive controlled-release fluorescent biosensor by filling the hollow interiors of AuNCs with fluorescent molecules such as Rhodamine-B (RhB). First of all, target ATP was allowed to react with the ATP recognition probe, which was a double-stranded hybrid formed by ATP aptamer and the primer S4. Because of the specificity recognition reaction of ATP and ATP aptamer, the double-stranded DNA dissociated through the competitive displacement reaction, thus resulting in the release of the primer S4. After magnetic separation, the divorced primer S4 was added to the proposed biosensor system, in which the autonomous replication–scission–displacement process was initiated with the cooperation of nicking endonuclease and polymerase. That is to say, the cyclic process that ultimately leads to the release of the guest molecules from AuNCs can be repeated as long as there is ATP available, which caused the release of the primer S4 through the specific binding of ATP with aptamer, and then the free primer S4 initiated the autonomous process. As a result, the release of a lot of fluorescent molecules achieved the significant amplification of the fluorescence signal.

Fabrication of the DNA-based controlled-release biosensor

AuNCs were synthesized by means of the galvanic replacement reaction between Ag nanocubes and chloroauric acid ($HAuCl_4$).[22] The morphology of the synthesized AuNCs is shown in Fig. 1. As shown in Fig. 1, AuNCs with a clear hollow interior and thin wall were observed. The morphology and particle size of the synthesized AuNCs were consistent with the literature reports.[21] The UV-visible-near-IR absorbance spectrum of AuNCs (Fig. S1, ESI†) is provided to show its corresponding surface plasmon resonance peak of the AuNCs. The spectrum proved that the synthesized AuNCs had a near-infrared LSPR peak at 782.94 nm, indicating the hollow structure of AuNCs. To improve the separation process, the magnetic nanoparticles (MNPs) modified with sulfhydryl groups (SH-MNPs) were used to combine with the as-prepared AuNCs. Then, S1 and S2 were immobilized on the MNP-combined AuNC surface through Au–S binding. After modification with S1 and S2, the MNP-combined AuNCs were soaked in PBS solution (pH 7.4) of RhB for loading cargo molecules RhB. To encapsulate the loaded RhB inside the AuNCs, the long-strand DNA S3 was used to hybridize with the two SH-modified short-strand DNAs S1 and S2, respectively, resulting in the formation of the controlled-release biosensor capped with the DNA-based molecular gate.

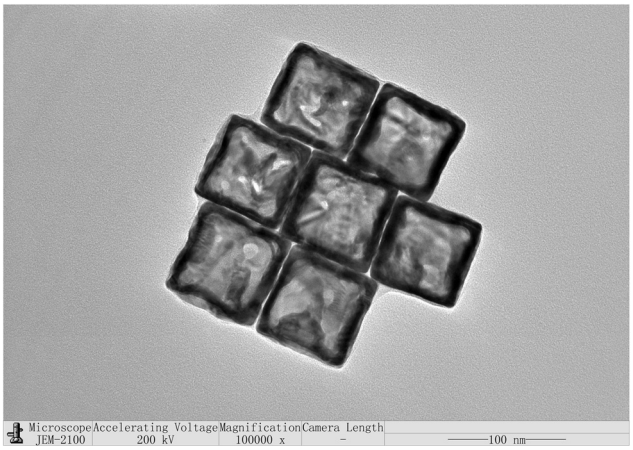


Fig. 1 Transmission electron microscopy (TEM) image of the synthesized AuNCs.

To test the feasibility of our proposed biosensor system, control experiments were performed. Fig. S2 (ESI†) shows the fluorescence signals of RhB released from the hollow interiors of AuNCs, in which curve b shows the fluorescence signal of released RhB without ATP. In contrast to curve b, significantly enhanced fluorescence signal was indeed observed in the presence of ATP (curve a, Fig. S2, ESI†). The results confirmed that the pores of AuNCs really could be blocked with DNAs and opened by the autonomous replication–scission–displacement process initiated by S4, which was released by ATP. Therefore, it was expected that the release of RhB would be sensitive to ATP.

Fig. S3 (ESI†) shows the relationship between the incubation time and the fluorescence signal in the presence of 10.0 μM ATP with the proposed biosensor. It could be seen that the fluorescence signal increases with the incubation time ranging from 0 to 2 h and then levels off at an incubation time of more than 2 h. That is to say, 2 h was essential for the accomplishment of the cyclic enzymatic signal amplification process. Therefore, 2 h was chosen as the optimal incubation time.

Detection of ATP

To test the ability of *in vitro* probing of the proposed biosensor, we performed the detection of ATP by employing the fabricated biosensor based on the proposed platform. Fig. 2 shows the fluorescence intensity of RhB released from the hollow interiors of AuNCs toward different concentrations of ATP. As shown in Fig. 3 and S4 (ESI†), there was a good linearity between the fluorescence intensity and the concentrations of ATP ranging from 5.0×10^{-8} to 1.0×10^{-6} M, and the linear regression equation could be expressed as: FL = 254.87145 + 46.59514 × C_{ATP} (10^{-7} M) (γ = 0.9925) with the detection limit of 1.0×10^{-8} M (3σ, n = 11). As shown in Fig. S4,† the fluorescence signal increased with the increase of the concentration of ATP, and then reached an equilibrium at 2.0 μM ATP

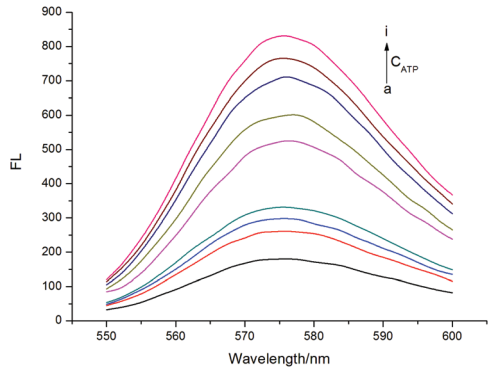


Fig. 2 Fluorescence intensity corresponding to different concentrations of ATP (a → i: 0, 5.0×10^{-8}, 1.0×10^{-7}, 2.0×10^{-7}, 5.0×10^{-7}, 8.0×10^{-7}, 1.0×10^{-6}, 5.0×10^{-6}, 1.0×10^{-5} M).

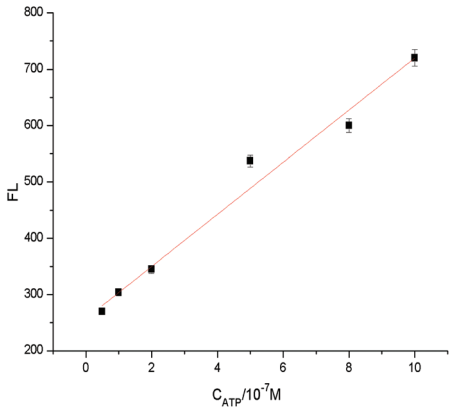


Fig. 3 The linear relationship of ATP concentration and the fluorescence intensity.

or more. The results demonstrate that DNAs can play the role of the molecular gate effectively, and the fabricated bioresponsive controlled-release system is an efficient biosensor for target molecule detection qualitatively and quantitatively through aptamer-target binding and the cyclic enzymatic signal amplification process.

Specificity

The ATP analogues including guanosine triphosphate (GTP), uridine triphosphate (UTP) and cytosine triphosphate (CTP) were utilized to investigate the specificity of the proposed biosensor under the same experimental conditions, respectively. As shown in Fig. 4, only ATP could result in the maximum fluorescence enhancement, which demonstrated the high specificity of the sensing platform.

Real sample assay

Finally, Ramos cells (a B-cell lymphoma cell line) were applied to evaluate the capability of the biosensor system presented here in biological samples. Fig. S5 (ESI†) shows the fluorescence intensity of RhB released from the hollow interiors of AuNCs toward the Ramos cell lysate (curve a) and PBS (curve b). The experimental results demonstrated that the fluorescence signal was significantly enhanced as compared with PBS control. The concentration of ATP in the Ramos cell lysate is 5.81×10^{-7} M per 1.0×10^{5} cells.

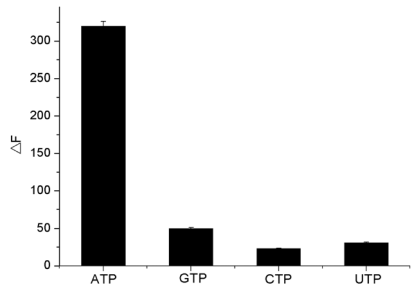


Fig. 4 Specificity of the proposed biosensor toward UTP, GTP, CTP and ATP (3.0×10^{-6} M), respectively. ΔF is the fluorescence intensity after blank deduction.

Conclusions

In conclusion, a novel controlled-release biosensor for isothermal amplified detection of ATP was developed by blocking the pores of AuNCs with DNAs. The results demonstrate that the obtained bioresponsive controlled-release system is an efficient biosensor for target molecule detection with high sensitivity and selectivity through aptamer-target binding and the cyclic enzymatic signal amplification process, allowing it to be successfully applied in intracellular ATP detection. The novel hybrid system described in this work can be further developed into a theranostic system with a diverse array of applications for molecular imaging, as well as for chemo- and photothermal therapy.

Experimental section

Materials

Hydrogen tetrachloroaurate(III) trihydrate ($HAuCl_4 \cdot 3H_2O$) and tris-(2-carboxyethyl)phosphine hydrochloride (TCEP) were purchased from Sigma-Aldrich. Rhodamine B (RhB) was obtained from Shanghai Aladdin Chemistry Corp (China). Adenosine triphosphate (ATP), cytosine triphosphate (CTP), guanosine triphosphate (GTP), and uridine triphosphate (UTP) were obtained from Sigma, and their stock solutions (1.0 mM) were prepared with doubly distilled water. The resulting solution was further consecutively diluted with doubly distilled water in order to obtain a suitable solution for fluorescence detection. Unless stated otherwise, all reagents were purchased from commercial sources and used without additional purification. The 0.01 M PBS buffer (pH 7.4) was prepared by standard methods. Deionized and doubly distilled water was used throughout the experiments. The synthetic oligonucleotides were purchased from SBS Genetech. Co. Ltd, China. Their sequences are as follows: the ATP-aptamer sequences, 5′-ACC

TGG GGG AGT ATT GCG GAG GAA GGT-3′; 5′-GGT CCA GCT GGC TTT TTT--SH-3′ (S1); 5′-SH--TTT TTT CCG TCG CAC GCT TTT-3′ (S2); 5′-GCC AGC TGG ACC TG *GCT AAG G*CA GCG TGC GA*C GGT GGG GGA*-3′ (S3); the primer strand sequences, 5′-AAT ACT CCC CCA CCG-3′ (S4). Klenow DNA Polymerase (Thermo Scientific, USA); Nb.Bpu10I nicking endonuclease (Thermo Scientific, USA); and dNTPs (Bioteke Corporation, Beijing, China) were also obtained. Silver nanocubes were prepared according to literature procedures.[23,24] Ramos cells were obtained from Chinese Academy of Medical Sciences.

Cell culture

Ramos cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 100 IU mL^{-1} of penicillin–streptomycin. The cells were maintained at 37 °C under a humidified atmosphere (95% air and 5% CO_2). The amount of cells was determined by using a hemocytometer prior to each experiment. The cells were collected and separated from RPMI cell medium buffer by centrifugation at 3200 rpm for 2 min, followed by washing twice with a sterile phosphate buffer solution (10 mM, pH 7.4 PBS). The sediment was resuspended in PBS (1.0 mL) containing Ca^{II} (0.1 mM) and Mg^{II} (0.1 mM) to obtain a homogeneous cell suspension (approximately 1.0×10^5 cells mL^{-1}).

Apparatus

Magnetic nanoparticles (MNPs) modified with sulfhydryl groups (3.0–4.0 μm, 10 mg mL^{-1}) (SH-MNPs) and a magnetic rack were obtained from BaseLine Chrom Tech Research Centre (Tianjin, China). Fluorescence spectra were recorded using a F-4600 fluorescence spectrophotometer (Hitachi, Japan); UV-visible spectra were recorded with a Cary 50 UV-vis-NIR spectrophotometer (Varian, Agilent). Transmission electron microscopy (TEM) image was taken with a JEM-2000EX/ASID2 instrument (Hitachi, Japan).

Synthesis of AuNCs

AuNCs were synthesized by the galvanic replacement reaction between Ag nanocubes and chloroauric acid ($HAuCl_4$) with some modifications.[25,26] Briefly, 400 μL of silver nanocubes was dispersed in 10 mL water containing 1 mg mL^{-1} PVP in a 50 mL flask under magnetic stirring. The dispersion was then refluxed for 10 min before 6.0 mL of 0.2 mM $HAuCl_4$ aqueous solution was added to the flask at a rate of 0.8 ml min^{-1}. The mixture was refluxed for another 10 min until its LSPR peak had reached about 780 nm. After cooling down to room temperature, the sample was centrifuged and washed. The obtained AuNCs were finally redispersed in deionized water for further use.

Assembly of the AuNCs onto SH-MNPs

After washing 10 μL SH-MNPs (10 mg mL^{-1}) with 100 μL PBS buffer, magnetic separation was performed. 600 μL of the as-prepared AuNC suspension was added to the washed MNPs, and the mixture was agitated overnight at room temperature.

Modification of the AuNCs with DNAs

Magnetic separation was performed and the obtained MNPs-combined AuNCs were washed thrice with 100 μL PBS. 100 μL of each 1.0 μM DTT activated thiol-modified short-strand DNAs S1 and S2 were added, respectively, and incubated in a constant temperature oscillator at 37 °C for 12 h. The DNA-AuNC-MNP conjugates were "aged" for 24 h by adding 20 μL of 1.0 M NaCl solution in the system.

Loading the DNA-modified AuNCs with RhB

To load RhB into the hollow interiors of the AuNCs modified with DNAs, magnetic separation was performed and the obtained complexes DNA-AuNC-MNPs were washed with 100 μL PBS. The washed complexes were re-suspended in PBS, and then 2 μL of 5.0×10^{-4} M RhB solution was added. The complexes were incubated in a constant temperature oscillator at 37 °C for 12 h.

Fabrication of the DNA-based controlled-release biosensor

In order to encapsulate the loaded RhB inside the AuNCs, the long-strand DNA S3 was used to hybridize with the two thiol-modified short-strand DNAs S1 and S2, respectively, which results in the formation of the DNA-based molecular gate. 100 μL of 1.0×10^{-6} M S3 was added into the above solution. The solution was incubated at room temperature for 1 h. After the hybridization procedure, the obtained conjugates were washed three times with PBS buffer solution on a magnetic rack and finally were resuspended in 100 μL PBS and stored at 4 °C for future use. The fabrication of the DNA-based controlled-release biosensor was thus completed.

Preparation of the ATP recognition probe

The ATP recognition probe was a duplex probe constructed by the hybridization of ATP aptamer with the primer S4. 100 μL of a PBS solution containing 10 μL of the activated carboxyl-modified MNPs (10 mg mL^{-1}) and 10 μL of 1.0×10^{-5} M amino-modified ATP aptamer was agitated overnight at 37 °C, and followed by magnetic separation. 10 μL of 1.0×10^{-5} M S4 was added to the obtained complexes, and the mixture was then diluted to a final volume of 100 μL with 0.01 M PBS (pH 7.4). After incubation for 1 h at 37 °C, magnetic separation was performed. The as-prepared recognition probe 1 was a hybrid of the ATP aptamer with the primer S4.

Optimization of the incubation time

The incubation time for the cyclic enzymatic signal amplification process has an important influence on the fluorescence signal. The tested time was varied from 0 to 3 h while keeping the incubation temperature constant at 37 °C.

Detection of ATP by the controlled-release biosensor

To test the ability of *in vitro* probing of the controlled-release biosensor, we performed the detection of ATP by employing the fabricated biosensor based on the proposed platform. First of all, 50 μL of ATP solution was allowed to react with the ATP

recognition probe for 1 h at 37 °C. The double-stranded DNA dissociated through the competitive displacement reaction, thus resulting in the release of the primer S4 into the solution. After magnetic separation, the divorced primer S4 was added to 100 μL of the proposed biosensor dispersed in PBS, followed by incubation for 1 h at 37 °C. After that, 1 μL of DNA Polymerase and 20 μL buffer, 1 μL of nicking endonuclease and 20 μL buffer, 5 μL of dNTPs were added, and the mixture was then diluted to a final volume of 200 μL with 0.01 M PBS (pH 7.4). After incubation for 2 h at 37 °C, magnetic separation was performed and the supernatant was collected for fluorescence detection with an excitation wavelength of 530 nm and an emission wavelength of 573 nm.

Determination of ATP in a cultured cell extract

Finally, Ramos cells (a B-cell lymphoma cell line) were applied to evaluate the capability of the proposed biosensor in biological samples. A suspension of 1.0×10^5 Ramos cells (1.0 mL) dispersed in RPMI cell media buffer was centrifuged at 3000 rpm for 5 min and washed with PBS (0.01 M, pH 7.4) for sometime and resuspended in 200 μL of PBS. 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 18 000 rpm for 20 min at 4 °C. The cell lysate was added into the ATP recognition probe, and the mixture was subjected to incubation at 37 °C for 1 h to ensure the full reaction between the aptamer and ATP. After magnetic separation, the supernatant containing the released primer S4 was introduced into the proposed biosensor system for the cyclic enzymatic amplification process, and PBS was used as a control in our experiments. At the end of the process, magnetic separation was performed and the supernatant was collected for fluorescence detection.

Acknowledgements

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