Journal of Photochemistry and Photobiology A: Chemistry xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology A: Chemistry

journal homepage: www.elsevier.com/locate/jphotochem

Invited feature article

An aptamer-based ligation-triggered rolling circle amplification strategy for ATP detection and imaging *in situ*

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ARTICLE INFO

Article history:
Received 26 July 2017
Received in revised form 12 September 2017
Accepted 30 September 2017
Available online xxx

Keywords:
Fluorescent detection
ATP
Rolling circle amplification (RCA)
Fluorescence *in situ* hybridization (FISH)

ABSTRACT

Adenosine 5′-triphosphate (ATP), one of the most widely known small natural biological molecules, is a multifunctional molecule existing in living organisms and plays an important role in many biological processes. In this work, we have developed an aptamer-based ligation-triggered rolling circle amplification strategy for ATP detection. Based on ATP aptamer, phosphorylated single oligonucleotide could be specifically ligated and circularized in the presence of ATP. Then branched rolling circle amplification (BRCA) was used as a signal amplification method for the analysis of ATP. Due to the high binding affinity and specificity of ATP aptamer, the proposed method showed significant advantages in high sensitivity and excellent selectivity. In addition, the selectivity was further improved in the ligation step by T4 ligase, it can also distinguish ATP from other adenosine analogues. Furthermore, this simple and facile method could be used to detect ATP in complex biological matrix, cell lysates, and the detection limit was as low as five cells. Moreover, the strategy realized the visualization of ATP level *in situ* and providing the location of ATP at single-cell level.

1. Introduction

The well-being of living organisms is highly dependent on the integrity and stability of microenvironment [1], yet microenvironment is under constant assault by exogenous and endogenous agents. In the past decades, scientists endeavor to develop chemical and bio-logical labels to detect or monitor the crucial impacts, such as pH [2,3], level of damaged nucleobase [4–6] and amount of adenosine 5′-triphosphate (ATP) [7,8] in the microenvironment. However, most of the molecular probe are chemical dyes (Rhodamine, Cy5 and BODIPY etc.) based [4,9,10]. The mechanism of chemicals probing the substrates limited their sensitivity and selectivity. Especially for ATP, some reported probes cannot perfectly differentiate ATP and UTP [11].

ATP, the ubiquitous energy currency of all living organisms, the abundance changes of which threatens proper cell function such as synthesis and degradation of biological molecules, active transport, and muscle contraction. In addition, it has been suggested that ATP performs as an indicator for cell viability, cell injury, cell motility and other cellular process [12]. Therefore, exploring a reliable and sensitive method to detect ATP levels in vitro and visualize ATP distribution in cells is crucial for precise understanding these bio-logical processes.

Other than chemical labeling method that suffer from multistep reactions or the synthesis of complex compounds, new strategies have been developed for the measurement of ATP level based on liquid chromatography [13] capillary electrophoresis [14], chemilumi-nescence [15] and aptamer-based strategy [8]. The aptamer-based strate-gy has come to the forefront due to its high selectivity and low toxicity during the past decade.

Aptamers are a class of synthetic DNA or RNA oligonucleotides that can bind specific targets ranging from proteins, peptides, small organic or inorganic molecules, and fold into specific tertiary structures. These oligonucleotides are selected through the SELEX (systematic evolution of ligands by exponential enrichment) and pos-sess many advantages, such as thermal stability, low-cost, target versatility [16]. Till now, lots of aptamer-based biosensors have been developed for the determination of ATP [17–20]. Among them, fluorescence-based optical detection is the most widely used approach owing to its ease of operation in homogeneous solution [11]. Furthermore, in order to improve the sensitivity, many amplification techniques have been introduced in aptamer-based strategy includ-ing the use of nanoparticles, enzymes, and graphene oxide [21–23]. These methods improve the sensitivity

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https://doi.org/10.1016/j.jphotochem.2017.09.076
1010-6030/

of ATP analysis but also increase the complexity of the system but few of them can be applied in visualizing ATP level *in situ* in cells. And another critical problem in the most of aptamer-based ATP detection strategy is that other adenosine analogues are hardly distinguished because present aptamer cannot perfectly recognize ATP in the present of AMP, ADP and adenosine. Therefore, efforts are still need to be made to develop general strategies to transduce aptamer recognition events to detectable signals, which can be used *in situ* and promote the selectivity for ATP.

In order to obtain a vastly sensitive aptamer-based probe, we chose rolling circle amplification (RCA) strategy among various kinds of signal amplification techniques, such as amplification molecular beacons used in strand displacement amplification [24], polymerase chain reaction DNA amplification techniques [25]. Due to its robustness and simplicity, the RCA holds a distinct position in DNA diagnostics among other isothermal methods [26]. It is an isothermal process which can convert a short DNA primer into a long single-stranded DNA containing a large number of repeats that are the complementary sequences to the circular template in the pres-ence of polymerase and deoxyribonucleotides (dNTPs [27].

Herein, we designed a simple and highly sensitive method for analyzing the ATP levels in vitro and visualization of ATP distribution in cells on the basis of branched rolling circle amplification (BRCA) [28]. Based on ATP aptamer, phosphorylated single oligonucleotide could be specifically ligated and circularized in the presence of ATP. Then BRCA was used as a signal amplification method for the analysis of ATP. Due to the high binding affinity and specificity of ATP aptamer, the proposed method showed sig-nificant advantages in high sensitivity and excellent selectivity. In addition, the selectivity was further improved in the ligation step with the help of T4 ligase. Because ATP is the cofactor for activa-tion of T4 DNA ligase, the ligation step can provide an additional selection and distinguish ATP from AMP and other adenosine analogues [29]. Furthermore, our strategy successfully realized the visualization of ATP distribution in cells *in situ* by using fluores-cence *in situ* hybridization (FISH).

2. Material and methods

2.1. Materials and equipment

ATP, TTP, GTP, CTP, UTP, AMP, ADP, A, dATP and apyrase were bought from Sigma-Aldrich. T4 ligase, phi29 polymerase, Exo I and Exo III were bought from New England Biolabs Inc. (Beijing, China). Oligonucleotides were synthesized and purified with HPLC by Sangon Biochemistry Co., Ltd. (Shanghai, China). All other chemicals used in this work were of analytical grade and obtained from standard reagent suppliers. All the solutions were prepared with ultrapure water from Millipore Co., Ltd. (18 MΩ cm). DNA concentration was quantified by NanoDrop 2000c (Thermo scientific, USA). Fluorescent emission spectra were collected on PerkinElmer LS 55 (PerkinElmer, USA).

2.2. Fluorescent measurement of ATP

Ligation reaction was conducted in NEB buffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9). To detect ATP, a 10 μL of mixture containing 0.2 μM recognition probe and 0.5 μM padlock probe was denatured at 65 °C for 5 min and then cooled down to 16 °C gradually. Then varying concentrations of ATP was added into the mixture and incubated at room temperature for 20 min. After adding 40 U T4 ligase, the mixture was incubated at room temperature for another 50 min to complete the ligation process. 10 U Exo I and Exo III were added into the mixture solutions and were incubated at 37 °C for 40 min. After this procedure, RCA reaction was conducted in a volume of 10 μL containing 5 μL prepared ligation product, 1 μL 10 × phi29 DNA polymerase reaction buffer (500 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM $(NH_4)_2SO_4$, 40 mM DTT), 2 μL 2.5 mM dNTP, 1 μL primer 1 and primer 2 and 5 U phi29 DNA polymerase. All the samples were incubated at 37 °C for 30 min. Then, SYBR Green I was added for 10 min incubation. Finally, the mixture was diluted into 200 μL, and its fluorescence intensity with different concentrations of ATP was measured. The excitation wavelength was set at 497 nm, and the emission spectra were collected from 500 to 700 nm with both excitation and emission slits of 10 nm.

2.3. Cell culture and sample preparetion for ATP assay from cell extracts

HeLa human cervical carcinoma cells (CCTCC, China) were cultured in DMEM (Hyclone, China) supplemented with 10% FBS (Hangzhou Sijiqing Biological Engineering Materials Corporation, China). Cells were maintained in a humidified atmosphere of 5/95 (V/V) CO_2/air at 37 °C.

First, HeLa cells were treated with 0.5% trypsin to digest from the culture flask, then they were split into 6 well cell culture clusters and cultured overnight. After attaching to the plate, the cells were transferred into a 1.5 mL tube after treatment with trypsin. The cell numbers were counted for accurate quantitation. Then the cells were washed with 1.0 mL of PBS buffer and spun at 3000 rpm on a centrifuge for 5 min, after which the supernatant was removed. After that, 200 μL PBS was added into the tube, mixing the suspension with gentle shake. Then the cell suspension was placed at −80 °C refrigerator for 15 min before dissolving at 37 °C and mixing. After 3 times, the cell lysates were obtained. According to cell number quantitation, an accurate volume of lysate containing 6.0×10^3 cells was picked up for the ATP assay.

2.4. Detection of ATP level in situ

HeLa cells seeded on 35-mm glass-bottomed dishes (Nest) were washed with 2 mL PBS (10 mM) buffer for 3 times, followed by a postfixation step in 4% (W/V) paraformaldehyde in PBS for 20 min at room temperature. After postfixation, the samples were washed 3 times in PBS. Then 1 mL 0.5% Triton X-100 was added and incubated at 4 °C for 20 min, followed by 3 times wash by PBS. Then the dishes were dehydrated using a series of 70%, 85% and 99.5% ethanol for 3 min each. The preincubated samples, which contained 10 μL 10 × NEB bufffer 2, 10 μL 2 μM recognition probe, 10 μL 5 μM padlock probe and 70 μL H_2O and annealed from 65 °C gradually, were added into dried dishes and incubated for 1 h at 37 °C. Then T4 ligase was added and incubated for another 2 h at 37 °C, followed by removing the mixture and wash once in PBS gently. For FISH, ATP already existed in HeLa cells, and after fixing, it can be immobilized so the complex structure consisting of circular padlock probe/ATP aptamer can recognize ATP *in situ*, in the result, they can be retained in the washing, but excess complex structure of circular padlock probe/ATP aptamer can be removed by washing because of no ATP *in situ* to combine. Then RCA reaction mixture was added for 4 h, containing 10 μL 10 × phi29 buffer, 20 μL 2.5 mM dNTP, 2 μL phi29 polymerase and 68 μL H_2O. ATP was visualized using 100 nM of primer 2-FAM in 2 × SSC and 20% formamide at 37 °C for 30 min. Dishes were then washed in PBS 3 times. Finally, the dishes were stained by DAPI and MitoTracker Deep Red FM (MDRF) to counterstain the cell nuclei and mitochondria.

The cells were mounted on the microscope stage after washing with PBS (10 mM) buffer again. White-light laser was used as the light source. The excitation wavelength was 405 nm (DAPI), 488 nm

Please cite this article in press as: F. Wu, et al., An aptamer-based ligation-triggered rolling circle amplification strategy for ATP detection and imaging *in situ*, J. Photochem. Photobiol. A: Chem. (2017), https://doi.org/10.1016/j.jphotochem.2017.09.076