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A label-free electrochemiluminescent sensor for ATP detection based on ATP-dependent ligation

Tingting Zhao [a,1], Chunshui Lin [b,1], Qiuhong Yao [a], Xi Chen [b,*]

[a] Xiamen Huaxia University, Xiamen 361024, China
[b] State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen 361005, China

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ABSTRACT

In this work, we describe a new label-free, sensitive and highly selective strategy for the electrochemiluminescent (ECL) detection of ATP at the picomolar level via ATP-induced ligation. The molecular-beacon like DNA probes (P12 complex) are self-assembled on a gold electrode. The presence of ATP leads to the ligation of P12 complex which blocks the digestion by Exonuclease III (Exo III). The protected P12 complex causes the intercalation of numerous ECL indicators ($Ru(phen)_3^{2+}$) into the duplex DNA grooves, resulting in significantly amplified ECL signal output. Since the ligating site of T4 DNA ligase and the nicking site of Exo III are the same, it involves no long time of incubation for conformation change. The proposed strategy combines the amplification power of enzyme and the inherent high sensitivity of the ECL technique and enables picomolar detection of ATP. The developed strategy also shows high selectivity against ATP analogs, which makes our new label-free and highly sensitive ligation-based method a useful addition to the amplified ATP detection arena.

1. Introduction

Adenosine triphosphate (ATP) is composed of an adenine ring, a ribose sugar and three phosphate groups, and works as cellular energy currency in living cells through breaking the phosphoanhydride bond [1–3]. Because of its ubiquitous presence in living matter, ATP has been widely used as an indicator in food quality control, environmental analysis and cell viability [4,5]. In addition, aberrant ATP levels are highly connected to many diseases such as Parkinson's disease and angiocardiopathy [6,7]. Therefore, it is of great importance to develop sensitive and selective methods to detect ATP.

Luciferase-mediated bioluminescence is a traditional method in ATP assay [8]. Liposomes are applied to enhance firefly bioluminescence assay for ATP in the presence of cationic surfactants [9]. However, costly and unstable bioluminescence agents are used in this method. Although several methods are reported to detect ATP with good performance, such as synthetic host–guest receptors [10], conjugated polymers [11], mass spectrometry [12], high-performance liquid chromatography [13] and peptides [14], most of these methods suffer from poor sensitivity, multi-step chemical reactions and poor selectivity. Aptamer-based methods for ATP detection are considered to be promising recognition elements for biosensing applications and are widely used in analytical assays because of their simple synthesis, excellent selectivity and stability [15,16]. An ATP modular aptameric sensor transduces recognition events into fluorescence changes through the allosteric regulation of noncovalent interactions with a fluorophore [17]. A signal-on sensor based on an ATP-responsive electrochemical aptamer switch is reported [18]. However, a major disadvantage of aptamer-based methods is their relatively low association constant with ATP, which leads to a rather poor detection limit. As a consequence, in the case of most of aptamer-based biosensors, the detection limit of ATP is usually in the micromolar range, and demonstrates only moderate sensitivity. To improve the sensitivity, many amplification techniques are reported using nanoparticles [19], enzymes [20] and graphene oxide [19], but these methods are time-consuming and complex.

DNA ligases seal 5′-PO_4 and 3′-OH polynucleotide ends via three nucleotidyl transfer steps involving ligase-adenylate and DNA-adenylate intermediates [21]. For the ATP-dependent ligase, it is inactive until it binds a molecule of ATP, which leads to the loss of the pyrophosphate moiety from ATP and the formation of a covalent enzyme [22]. Thus, the enzymatic ligation reaction shows specific dependence on its cofactor ATP, which, in turn, provides an efficient platform for constructing highly selective biosensing systems for ATP [23]. An innovative fluorescence approach to detect ATP is reported based on the ATP-dependent DNA kinase reaction using molecular beacons (MB) [24–26]. Although it is

* Corresponding author.
E-mail address: xichen@xmu.edu.cn (X. Chen).
[1] Equal contribution to this work.

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convenient, quick, sensitive and selective, the fluorophore label causes higher cost and complexity in the assay. In addition to fluorescence methods, a biotin-tagged MB-like DNA probe and a ligase-based signal-on electrochemical sensing method for ATP is reported [27]. However, false opening of the MB probe makes it vulnerable to a false positive signal. Furthermore, it needs a relatively long incubation time to induce the conformation change.

Exonuclease III (Exo III) catalyzes the stepwise removal of mononucleotides from the 3-hydroxyl ends of DNA duplexes, but its activity on single-stranded DNA and the 3′-protruding termini of double-stranded DNA is limited [28]. The combination of T4 DNA ligase and Exo III for ATP detection has been reported [29,30]. The underlying principle relies on the Exo III showing different cleavage capacity for a DNA substrate in the absence and presence of ATP. Graphene oxide increases the sensitivity, but, unfortunately, the fluorescence signal of ATP is reduced [29]. No ATP-dependent enzyme reaction (ATP-DER)-based electrochemiluminescence (ECL) assay has been reported before. Thus, the ability to integrate the ATP-DER with ECL signal detection for developing ATP assay is very encouraging.

Herein, based on ligase-based signal-on, we developed a sensitive ECL assay for ATP using a novel MB-like DNA probe without any electrochemical label modification. The MB-like DNA probe was prepared by hybridizing the DNA probe P1 with the 3′-protruding terminus and DNA probe P2 with thiol modification, forming a P12 complex. P1 and P2 alone were resistant to digestion by Exo III, while the hybridization of P1 and P2 caused the P12 complex to become the substrate of the Exo III digestion reaction. The P12 complex was immobilized on a gold electrode through the interaction between Au and SH of P2. In the presence of ATP, ligation between P1 and P2 blocked the digestion of Exo III. Numerous ECL molecules, $Ru(phen)_3^{2+}$, could thus be efficiently intercalated into the grooves of the P12 complex to give an ECL signal. In contrast, in the absence of ATP, ligation could not occur, thus exposing the digestion of P2 by Exo III, which accompanied the release of P1 from the electrode. As a result, a weak ECL background could be achieved. The MB-like DNA probe designed served as a direct substrate of efficient digestion and ligation without a long incubation time for conformation change. The integration of enzyme reactions (ligation and digestion) with the high sensitivity of the ECL technique was shown here to lead to the sensitive detection of ATP.

2. Materials and methods

2.1. Materials

T4 DNA ligase and Exo III were purchased from the Takara Biotechnology Co., Ltd. (Dalian, China). ATP, adenosine (A), adenosine diphosphate (ADP), adenosine 5′-monophosphate (AMP), uridine triphosphate (UTP), cytidine triphosphate (CTP) and guanosine triphosphate (GTP) were obtained from the Sangon Biotechnology Co., Ltd. (Shanghai, China). Tris (2-carbox-yethyl) phosphine hydrochloride (TCEP) was purchased from Alfa Aesar (Tianjing, China); Dichlorotris (1, 10-phenanthroline) ruthenium hydrate ($Ru(phen)_3Cl_2 \cdot H_2O$) and 6-mercapto-1-hexanol (MCH) from Sigma-Aldrich (Shanghai, China). All other reagents were of analytical grade and used without additional purification. All solutions were prepared with ultra-pure water ($\geq$ 18 MΩ, Milli-Q, Millipore, USA).

The oligonucleotide probes P1 and P2 were 'synthesized' and purified by the Sangon Biotechnology Co., Ltd. (Shanghai, China) and their detailed base sequences are listed as follows:

P1: 5′-PO4-GGA AGG TTG CGG GTG GGG GGT GGG GGC ACC CGC AAC CTT CCT GCA GCT CTC AGG AAG GTG GTT TTT-3′
P2: 5′-SH-TTT TTC CAC CTT CCT GAG AGC TGC A-3′

Buffer solutions employed in this work were prepared as follows: pH 8.0 oligonucleotide stock solution was prepared with 20 mM Tris–HCl, 100 mM NaCl, 5 mM $MgCl_2$, and kept in a refrigerator. pH 8.0 Tris–HCl containing 20 mM NaCl was used as an electrolyte and washing solution. 20 mM Tris–HCl (pH 7.6) buffer solution, 100 mM NaCl, 5 mM $MgCl_2$ and 1 mM dithiothreitol (DTT) were used for Exo III-based digestion and the ATP-based ligation reaction.

2.2. Instruments

ECL experiments were carried out on a set-up consisting of a CHI 660B workstation (Co. CHI, USA) and an IFFM-D FIA Luminescence Analyzer (Ruimai Co., China). A homemade electrochemical cell comprising a traditional three-electrode system with a 3 mm gold electrode as the working electrode, a platinum wire as the counter electrode, and an Ag/AgCl as the reference electrode, was used. All potentials in this work refer to the Ag/AgCl reference electrode. In ECL detection, the ECL cell was placed directly in front of a photomultiplier (PMT, applied potential at −600 V). The PMT window was only opened toward the working electrode in order to eliminate the blank chemiluminescence intensity in the solution, and the ECL signals from the counter electrode. CV experiments were performed using the CHI 660B workstation, and EIS measurements were carried out with a VSP multipotentiostat from Bio-Logic Science Instruments equipped with five channels (Claix, France). UV–vis absorption spectra were recorded using a NanoDrop 1000 Spectrophotometer (Themo Scientific, USA). Native polyacryamide gel electrophoresis was carried out with a Bio-rad vertical electrophoresis system (Bio-Rad Laboratories, Inc., USA) under appropriate conditions.

2.3. Polyacrylamide gel electrophoresis (PAGE)

A 10% native polyacrylamide gel was prepared using 1 × TBE (89 mM Tris–HCl, pH 8.0, 89 mM boric acid, 2.0 mM Na_2 EDTA). 50 μL mixtures of (1) 4 μM P1+P2+Exo III + T4 DNA ligase +100 nM ATP; (2) 4 μM P1+P2+Exo III + T4 DNA ligase; (3) 4 μM P1+P2; (4) 4 μM P1; (5) 4 μM P2 were used for PAGE. The gel was run at 100 V for 60 min in 1 × TBE buffer. Then, it was stained in the stains-all solution (0.1 g stains-all, 450 mL formamide, 550 mL H_2O) for 30 min. After this process, the gel was illuminated under sunlight for 5–10 min to obtain the stained bands. Finally, the PAGE results were photographed using a digital camera.

2.4. Fabrication of ECL ATP sensors

The gold electrode was cleaned by immersion in a freshly prepared piranha solution (a mixture of concentrated H_2SO_4 and 30% H_2O_2, 3:1 v/v) for 10 min, followed by a thorough rinse with ultrapure water. Then, the electrode was polished with 50 nm alumina slurry and sonicated in absolute ethanol and ultrapure water for 5 min, respectively, to remove the residual alumina powder. The electrode was then electrochemically cleaned in 0.5 M H_2SO_4 by cycling the potential between −0.2 and 1.5 V until a reproducible cyclic voltammogram could be obtained. Subsequently, the electrode was washed thoroughly with ultrapure water and dried in a nitrogen stream for use.

The P12 complex was first prepared by mixing the same concentration (0.5 μM) of the auxiliary probe (P1) and the signaling probe (P2) in the oligonucleotide stock solutions. The DNA probe was heated to 90 °C for 5 min and cooled to room temperature for