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Aptamer-based detection of adenosine triphosphate via qPCR

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ABSTRACT

Sensitive and specific detection and quantification of small molecules often remain challenging. We developed a novel magnetic bead-based aptamer-assisted real-time PCR (Apta-qPCR) assay to provide a versatile platform for quantification of small molecules. The assay has been realized for the detection of ATP as a model system. The assay relies on a combination of qPCR with the target-induced dissociation (TID) of ATP aptamer from an oligonucleotide, complementary to the ATP binding site of the aptamer. The complementary oligonucleotide was immobilized on deoxythymidine (dT)-modified magnetic beads (dT-beads) and hybridized with the aptamer. The presence of ATP resulted in dissociation of the aptamer from the dT-beads and the dissociated aptamer was quantified using qPCR. The Apta-qPCR assay was able to detect 17 nM ATP with a broad dynamic range from 50 nM to 5 mM. The assay is label-free, and real-time PCR-based detection of aptamer facilitates high sensitivity. The presented method is highly versatile and can be applied to various aptamer-target pairs to allow detection of a broad range of target analytes.

1. Introduction

Nucleic acid aptamers have come up with broad applicability in various fields ranging from detection of environmental pollutants to measurement of carcinogens and drug levels in blood [1]. They are the congeners of antibodies in terms of their binding properties to corresponding target molecules. While antibodies bind to the epitopes of the antigen, aptamers bind to the so-called aptatopes of respective target molecules [2]. Aptamers have been developed and studied as capture probes for many targets ranging from whole cells [3–5], proteins [6,7], or small molecules like antibiotics [8,9], dyes [10], metal ions [11], pesticides [12], toxins [13] and others. They can be easily modified and conjugated to solid matrices for affinity separation [7] or to transducer surfaces [8,14,15] to exploit their interaction with the target molecule for precise detection and quantification.

Small molecules are routinely quantified by hyphenated gas chromatographic (GC) techniques and high-performance liquid chromatography (HPLC) with UV and/or fluorescence detection [16,17]. These techniques offer high sensitivity but require additional steps of extraction and clean-up processes which are time-consuming and costly. Simple and label-free methods, e.g. for the detection of small molecules (e.g., metal ions) [18], are scarce and limited to specific target analytes. Alternatively, small molecules can be detected using aptamers due to the conformational changes in aptamers during their binding to the target molecule. These conformational changes can be transduced to colorimetric [19,20], fluorescence [21], mass [22], surface plasmon resonance [8], or electrochemical signals [23,24]. Target-induced dissociation (TID) of oligonucleotides complementary to the target-binding site of the aptamer is another mechanism that can be exploited to convert the binding of a small molecule target into a detectable signal.

In TID, binding of the target induces dissociation of the complementary sequence from the aptamer [25]. Aptamers come with the advantage of consisting of an oligonucleotide sequence; thus they allow the straightforward design of complementary oligonucleotides for competitive assays and easy amplification via PCR to further enhance the sensitivity of the assay [26]. In the early 1990s, Cantor's group was the first to utilize the amplification potential of DNA in so-called immuno-PCR, where specific antibodies were labeled with double-stranded DNA, and the latter served as a template for qPCR following the formation of an immunocomplex [27]. The Apta-PCR represents a further advancement and simplification of immuno-PCR, where an aptamer molecule replaces the cumbersome antibody-DNA complex. Recently, the aptamer specificity has been combined with qPCR sensitivity in various approaches for the ultrasensitive detection of proteins, including a proximity ligation assay [28,29], nuclease protection assay [30], capillary electrophoresis [31], and the use of target-modified magnetic microparticles [32,33]. Up to now the combination

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of aptamers and qPCR was solely applied to the detection of proteins, while the detection of small molecules has not been realized. To overcome this limitation, we used an aptamer directed against ATP as a model system to demonstrate the applicability of Apta-PCR for small molecule detection for the first time.

ATP is acknowledged as the mediator of energy exchanges that occur in all living cells and plays a critical role in the regulation of cellular metabolism and biochemical pathways in cell physiology [34]. The highly sensitive and selective detection of ATP is essential for biochemical studies as well as in clinical diagnosis [35,36]. The DNA aptamer against ATP was originally created by Huizenga and Szostak through *in vitro* selection [37]. The aptamer was reported to bind ATP and adenosine monophosphate (AMP) [38], while no binding of cytidine triphosphate (CTP), guanosine triphosphate (GTP), and uridine triphosphate (UTP) was observed [39]. NMR studies revealed that the aptamer (G5 – A10, and G18 – A23) binds two ATP molecules (Fig. S-1) [40].

So far, the combination of qPCR with aptamers has been limited to the detection of large biomolecules [41–45]. This study is the first to report on the application of Apta-qPCR for the detection of small molecules. We have recently used TID for the development of an aptamer microarray for sensitive detection of ethanolamine [25]. In this study, the TID strategy was applied to the ATP-binding aptamer (Fig. S-2). The complementary sequence was chosen to hybridize to the part of ATP aptamer which is directly involved in binding of the target (Table 1) [40]. In the absence of ATP, the ATP aptamer hybridizes with the complementary sequence, which is immobilized on dT-modified beads (dT-beads). In the presence of ATP, the ATP aptamer is released from the dT-beads and quantified by qPCR. The Apta-qPCR assay is illustrated in Scheme 1. The sensitivity of the optimized Apta-qPCR assay for ATP was found to be 17 nM in cell lysates obtained from HeLa cells, and the dynamic range was 50 nM–5 mM. The approach combines the specificity of aptamers with the sensitivity of qPCR, and by transfer of the method to other aptamers, detection and quantification of a wide range of target analytes could be feasible.

2. Experimental section

2.1. Chemicals and materials

All DNA oligonucleotides including 5′-amino modified DNA were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The sequences utilized in the study are shown in Table 1. The concentrations of oligonucleotides were determined using NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) with the corresponding extinction coefficients. Adenosine triphosphate (ATP), cytidine triphosphate (CTP), guanosine triphosphate (GTP), thymidine triphosphate (TTP), and Dynabeads® MyOne™ Carboxylic acid were all purchased from Life Technologies GmbH (Darmstadt, Germany). 2-(*N*-morpholino) ethane sulfonic acid (MES) was purchased from AppliChem GmbH (Darmstadt, Germany). Adenosine monophosphate (AMP), and 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC) were purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany). SYBR Green Real-Time PCR Master Mix was purchased from Promega GmbH (Mannheim, Germany). HeLa cells were purchased from German Collection of Microorganisms and Cell Cultures (DSMZ) GmbH (Braunschweig, Germany). All chemicals were of analytical grade. All stock solutions and buffers were prepared with deionized water (arium 611, Sartorius AG, Göttingen, DE).

2.2. Preparation of dT-beads

Magnetic beads (7-12×10^9 beads mL^{-1}, Dynabeads® MyOne™ Carboxylic Acid, Invitrogen) of about 1.05 µm diameter with carboxyl groups on the surface were used. 100 µL suspension of magnetic beads (10 mg beads mL^{-1}) was dispensed to a micro-tube and washed three times with 500 µL MES buffer (25 mM MES, pH 4.5). The carboxyl groups on the magnetic beads were activated using 500 µL 50 mM EDC in MES buffer for 30 min. 100 µL 5′-amino modified dT (0.5 µM, 1 µM, 1.5 µM, 2.5 µM, 5 µM, 10 µM, and 20 µM) in MES buffer was added to the micro-tube after removing unreacted EDC. Immobilization was carried out by incubation of dT and magnetic beads at room temperature for 2 h with slow tilt rotation to prevent sedimentation of the magnetic beads. Magnetic beads were washed three times with 200 µL MES buffer to remove non-immobilized dT. In order to quench the non-reacted activated carboxylic acid groups on the magnetic beads, the magnetic beads were incubated with 200 µL 50 mM Tris pH 7.5 for 15 min. The dT-beads were washed twice with 200 µL of the aptamer selection buffer (20 mM Tris-HCl buffer containing 150 mM NaCl, 5 mM $MgCl_2$, pH 8.2) and then stored in 200 µL selection buffer at 4 °C. In order to confirm the immobilization of dT on the magnetic beads, all washing fractions were collected, and dT was quantified using NanoDrop ND 1000.

2.3. Hybridization of aptamer to dT-beads using complementary sequences

6.25 µL 100 nM aptamer was mixed with 6.25 µL 100 nM complementary oligonucleotides (dA-cOligo) (1:1; aptamer:dA-cOligos) in 6.25 µL selection buffer in a micro-tube. Different lengths of dA-cOligos (11, 14, 17, 20, and 25 nt) were investigated to improve the efficiency of TID mechanism. During incubation, the solution was heated up to 95 °C for 5 min and cooled to room temperature for 20 min. 6.25 µL dT-beads suspension (labeled with 2.72 pmol, 6.61 pmol, 10.23 pmol, and 16.96 pmol dT) was added to the reaction mixture at room temperature and incubated for 20 min with slow tilt

Table 1
List of sequences used in the work.

Name of the sequence	Sequence[a]
ATP PCR Aptamer	5′GGAACACTATCCGA*CTGGCACC*A*CCTGGGGGAGTATT*GCGGAGGAAGGTCCTTGGGCATGTCTAGCGATCC3′
dA-cOligo11	3′A_{25}-TT*TGGACCCCCTC*-5′
dA-cOligo14	3′A_{25}-TT*TGGACCCCCTCATA*-5′
dA-cOligo17	3′A_{25}-TT*TGGTGGACCCCCTCATA*-5′
dA-cOligo20	3′A_{25}-TT*CGTGGTGGACCCCCTCATAA*-5′
dA-cOligo25	3′A_{25}-TT*GACCGTGGTGGACCCCCTCATAA*-5′
Random sequence	5′- NH_2-C_{12}-TGGACCCCCTC-3′
Forward primer	5′-GGAACACTATCCGACTGGCACC-3′
Reverse primer	5′-GGAACCCGTACAGATCGCTAGG-3′
dT	5′-NH_2-C_6-TTTTTTTTTTTTTTTTTTTTTTTTT-3′
Cy5-labeled ATP PCR aptamer	5′-Cy5-GGAACACTATCCGA*CTGGCACC*A*CCTGGGGGAGTATT*GCGGAGGAAGGTCCTTGGGCATGTCTAGCGATCC-3′

[a] Underlined bases correspond to primer binding regions. Italics bases indicate complementary sequences within the aptamer and oligonucleotides. dA means $d(A)_{25}$ and dT means $d(T)_{25}$. 2 T nucleotides were used as spacer between $d(A)_{25}$ region and complementary sequence. Justification of the sequences used in the assay is given in supplementary information (Fig. S-2).