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Target-triggered transcription machinery for ultra-selective and sensitive fluorescence detection of nucleoside triphosphates in one minute

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ABSTRACT

Nucleoside triphosphates (NTPs) play important roles in living organisms. However, no fluorescent assays are currently available to simply and rapidly detect multiple NTPs with satisfactory selectivity, sensitivity and low cost. Here we demonstrate for the first time a target-triggered *in-vitro* transcription machinery for ultra-selective, sensitive and instant fluorescence detection of multiple NTPs. The machinery assembles RNA polymerase, DNA template and non-target NTPs to convert the target NTP into equivalent RNA signal sequences which are monitored by the fluorescence enhancement of molecular beacon. The machinery offers excellent selectivity for the target NTP against NDP, NMP and dNTP. Notably, to accelerate the kinetics of the machinery while maintain its high specificity, we investigated the sequence of DNA templates systematically and established a set of guidelines for the design of the optimum DNA templates, which allowed for instant detection of the target NTP at fmol level in less than 1 min. Furthermore, the machinery could be transformed into logic gates to study the coeffects of two NTPs in biosynthesis and real-time monitoring systems to reflect the distribution of NTP in nucleotide pools. These results provide very useful and low-cost tools for both biochemical tests and point-of-care analysis.

1. Introduction

Nucleoside triphosphates (NTPs) are essential and vital functional molecules in all living organisms. They are not only the building blocks of RNA, but also critical for cellular structure, function, metabolism and regulation (Zhou et al., 2011). Adenosine triphosphate (ATP) is known as the major energy currency and involved in numerous signal transduction processes such as protein phosphorylation and modulation of MgtE for Mg^{2+} homeostasis (Dong and Zhao, 2016; Acin-Perez et al., 2011; Tomita et al., 2017). Guanosine triphosphate (GTP) serves as an activator of substrates such as GTPase proteins and also an energy source in specific metabolic reactions (Kerr et al., 2017; O'Donnell et al., 2017). Cytidine triphosphate (CTP) is vital for dolichol phosphorylation as well as allosteric regulation of protease (Fernandez et al., 2002; Nm et al., 1989). Uridine triphosphate (UTP) is a fundamental intermediate in *de novo* pyrimidine biosynthesis and controls a lot of signaling pathways (Lecca and Ceruti, 2008; Linan-Rico et al., 2017). Any changes or imbalanced amounts of NTPs may cause abnormal responses, metabolic disorders, apoptosis and necrosis (Bradbury et al., 2000), and are relevant to the pathogenesis of many diseases such as inflammation, neuropathic pain and Parkinson's disease (Karmakar et al., 2016; Masuda et al., 2016; Wang et al., 2007; Ramirez et al., 2017). Besides, modulation of intracellular NTP pools can regulate tumor cell invasion (Bianchi-Smiraglia et al., 2017) and some anticancer or antiviral drugs, as nucleotide derivatives, compete with NTPs in RNA synthesis or metabolic activities and disturb normal NTP levels to achieve pharmacological effects (Van Moorsel et al., 2003). Therefore, increased attention has been paid in recent years to the quantification of NTP pools in cells with high accuracy, high specificity and high speed.

Various methods have been developed for this purpose. Separation-based assays such as high performance liquid chromatography (HPLC) coupled with UV detection or mass spectrometry (MS) have the ability to detect multiple NTPs, but they have the limitations of long sample pre-treatment time, dependence of sophisticated instrument, time-consuming analytical processes (> 2 h) and relatively low sensitivity (≈ 10 pmol) (Huang et al., 2003; Cohen et al., 2009). By comparison, fluorescent sensors based on molecular recognition or NTP-dependent reactions have shown great advantages in simplicity, high sensitivity and high throughput (Dong and Zhao, 2016). However, no fluorescent assays are currently available to rapidly detect all the four different NTPs with satisfactory selectivity. One of the main difficulties is the

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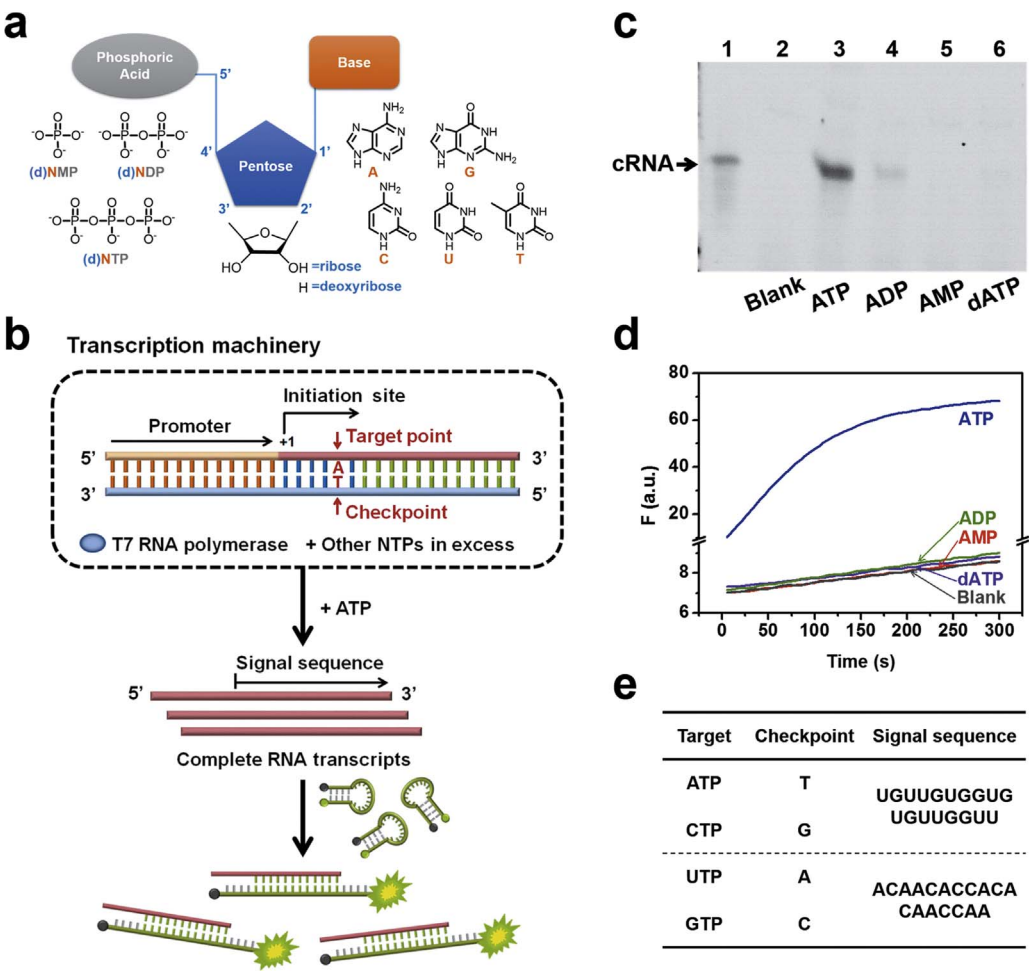

Target	Checkpoint	Signal sequence
ATP	T	UGUUGUGGUG
CTP	G	UGUUGGUU
UTP	A	ACAACACCACA
GTP	C	CAACCAA

Fig. 1. a) Molecular structures of NTPs and their nucleotide analogues. b) Illustration of target NTP-triggered *in-vitro* transcription machinery by using ATP as an example. c) Confirmation of the RNA products of transcription triggered by 250 pmol of ATP and its analogues by using denaturing urea polyacrylamide gel electrophoresis. Lane 1: Standard sample of complete RNA transcripts (cRNA-ATP-1, 30 pmol). Lane 2–6: Transcription products in the presence of 0 nmol (blank), 250 pmol of ATP, ADP, AMP and dATP, respectively. All the reaction products were treated with DNase I to remove the DNA sequences before loaded into the gel. d) Real-time fluorescence curves of the ATP-triggered transcription machinery by using dsDNA-ATP-2 upon the input of 0 pmol of ATP (blank), 8 pmol of ATP, ADP, AMP and dATP, respectively. F: fluorescence intensity. e) Summary of the checkpoints and signal sequences for their corresponding target NTPs.

interference from nucleotide analogues such as nucleoside diphosphates (NDPs), nucleoside monophosphates (NMPs) and deoxyribonucleoside triphosphates (dNTPs) which have very similar molecular structures and chemical properties to NTPs (Fig. 1a). Organic fluorescent chemosensors including metal-organic frameworks (Zhou et al., 2011; Xiao et al., 2014; Srivastava et al., 2015; Deng et al., 2017) and genetically encoded fluorescent reporters (Yoshida et al., 2016) require tedious synthesis and purification processes for each NTP with a defective selectivity and a moderate sensitivity from micromolar to millimolar. DNA or RNA aptamers have been developed into numerous sensors for ATP and GTP (Carothers et al., 2006; Lu et al., 2010; Feng et al., 2014; Wolter et al., 2017), yet few were reported for CTP or UTP. Besides, ATP aptamer usually needs a long time for incubation (30–60 min) and it mainly recognizes the nucleoside moiety, lacking specificity against ADP and AMP (Liu et al., 2009). ATP or GTP-dependent deoxyribozymes based on ligation or self-phosphorylation reaction have also been developed as biosensors (Cho et al., 2005; McManus and Li, 2013; Wang et al., 2014). But even coupled with amplification, their limits of detection (LOD) are only 1 μM. Recently, a novel guanine-vacancy-bearing G-quadruplex (GVBQ) was reported for sensing guanine and its derivatives by structural complementation (Li et al., 2016); nevertheless, its selectivity and sensitivity need to be further improved. Particularly, both luciferase-based (Wood et al., 1989; Sato et al., 2000; Kim et al., 2010) and T4 DNA ligase-based detection (Lu et al., 2011; Lin et al., 2015) have a better selectivity and sensitivity than the afore-mentioned approaches. Deficiently, they can only detect ATP. In addition, the chemiluminescence of luciferin-luciferase reaction is not as stable as fluorophores while the reaction time for ligase is more than 30 min. Thus, it is very urgent to break through the limitations of current methods and develop a novel strategy for fluorescence detection of NTPs with improved selectivity, high sensitivity, short time and simplified operation.

DNA-dependent RNA polymerase (RNAP) catalyzes DNA-directed *de novo* synthesis of RNA by using NTP substrates with high fidelity (error rate of 10^{-4}–10^{-5}) and efficiency (> 200 nucleotides/s) (Vassylyev et al., 2007; Sydow and Cramer, 2009; Anand and Patel, 2006). Although previous studies have reported radioactive determination of NTPs by using *Eschericlzia coli* RNA polymerase (Maxson and Wu, 1976; Sasvariszekely et al., 1975), the use of radioisotopes greatly increased the operational complexity and experimental cost. Besides, the long reaction time and low sensitivity (≈ 25 pmol or 1.2 μM) made it less favored for sensitive point-of-care analysis. Molecular machinery is an autonomous device which is driven by fuel substrates and performs mechanical operations (Li et al., 2008; Wang et al., 2012; Song et al., 2014; Wu et al., 2015). Herein, we report for the first time a target-triggered *in-vitro* transcription machinery that achieves ultra-fast, highly selective and sensitive fluorescence detection of four NTPs with low cost and simplicity. By using the isomerization of RNAP active center to recognize the ribose moiety, strict Watson-Crick base-pairing to discriminate the nitrogenous base and driving force from the cleavage of two phosphoric anhydride bonds to ensure triphosphates, the machinery provides an extremely high degree of selectivity for NTPs over NDPs, NMPs and dNTPs. Moreover, the machinery has the ability to quantify NTPs in less than 1 min with a limit of detection as low as 20 fmol (1 nM), indicating a great application potential in routine biochemical tests and point-of-care analysis.