Polymerization-sensitive switch-on monomer for terminal transferase activity assay

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ABSTRACT
We herein describe a simple but efficient method for the determination of terminal transferase (TdT) activity, which relies on our finding that Fe(III)-quenched boron-dipyromethene (BODIPY)-ATP is utilized as a switch-on monomer for polymerization and enables the facile synthesis of fluorescence oligonucleotides without additional, post-processing steps. As TdT carries out the synthesis of DNA by adding the monomers into growing chains, Fe(III) is displaced from BODIPY with the release of pyrophosphate group, which consequently leads to the generation of highly fluorescent long oligonucleotides. With this strategy, we selectively detected the TdT activity with high sensitivity. In addition, its practical applicability was successfully demonstrated by determining TdT activities in human serum.

Introduction
DNA molecules labelled with a fluorescent moiety, which is termed “fluorescence oligonucleotides”, have been utilized in various fields such as biotechnology, biomedicine, and molecular and cellular biology [1–3]. In general, the synthesis of fluorescence oligonucleotides is broadly classified into two types: (i) chemical DNA synthesis based on phosphoramidite method [4] and (ii) enzymatic DNA synthesis using modified mononucleotides [5,6]. In particular, the second one has gained momentum due to its simplicity, cost-effectiveness and especially, no limitation to form long DNA strands (up to thousands of bases) [5,6]. Many enzymes from DNA polymerase family that are capable of incorporating the modified dNTPs, have been exploited to synthesize the fluorescence oligonucleotides [5–7]. However, some methods require extra, post-processing steps such as separation of unreacted dNTPs or secondary labelling with fluorophore, which makes procedures tedious, time-consuming and complicated, consequently limiting the widespread application.

In this study, we devised a new, facile method for in situ formation of the fluorescence oligonucleotide based on the enzymatic DNA synthesis. As a key component, we employed ATP conjugated with fluorescent boron-dipyromethene (BODIPY) analogues (BODIPY-ATP), which have been known to possess unique fluorescence properties including high quantum yield (almost 1.0 in water), outstanding photostability, high extinction coefficient (more than 80,000 cm⁻¹M⁻¹), and pH-insensitivity [8–12]. In addition, on the basis of the effective fluorescence quenching effect of Fe(III) on BODIPY-ATP via its coordination with the triphosphate group and N7 nitrogen of BODIPY-ATP, the quenched form of BODIPY-ATP complexed with Fe(III) (BODIPY-ATP/Fe(III)) was prepared (Figure 1(a)) [13]. We expect that as DNA polymerase catalyses the synthesis of DNA by adding the specially designed monomers into the growing chains, Fe(III) would be liberated from BODIPY with the concomitant release of pyrophosphate group, which would lead to the high fluorescence signal from the produced, long oligonucleotides (Figure 1(b)).

As a model DNA polymerase, we selected terminal transferase (TdT), a template-independent DNA polymerase that catalyses the addition of mononucleotides to the 3’ terminus of DNA [14–17]. It is known that TdT serves as an indicator for acute leukaemia and thus there is high demand for a simple and novel strategy for the determination of TdT activity [18,19]. In the effort described below, we successfully demonstrated the proposed concept with TdT, which was applied for the simple determination of TdT activity with high sensitivity and selectivity.

Material and methods

Materials
TdT-primer (5’-AATACAAACCTCTCA-3’) used in the study was synthesized by Genotech Co. (Daejeon, South Korea). TdT, phi29 DNA polymerase, exonuclease I (Exo I), Klenow

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fragment (exo’), Vent (exo’) DNA polymerase, T4 DNA ligase
and T4 DNA polymerase were purchased from New England
Biolabs Inc. (Beverly, MA), and i-pfu DNA polymerase was pur-
chased from iNtRON Biotechnology (Seongnam, Korea).
BODIPY-ATP and FeCl₃·6H₂O were purchased from Invitrogen
(Carlsbad, CA) and Sigma-Aldrich (St. Louis, MO), respectively.
All other chemicals were of analytical grade and used with-
out further purification. Ultrapure DNase/RNase-free distilled
water purchased from Bioneer (Daejeon, Korea) was used in
all experiments.

Procedure to determine TdT activity

TdT-primer (1 μM), BODIPY-ATP/Fe(III) (5 μM), and TdT at vary-
ing concentrations were incubated in 1X TdT reaction buffer (20
mM Tris-acetate, 50 mM potassium acetate, 10 mM magnesium
acetate, 0.25 mM CoCl₂, pH 7.9) at 37 °C for 1.5 h (Total vol-
ume: 20 μL). Then the mixture was heated to 75 °C for 20 min
to inactivate the enzyme and the resulting fluorescence emis-
sion spectra were measured in the range of 510–600 nm at an
excitation wavelength of 480 nm by using Tecan Infinite M200
pro-microplate reader (Mnndorf, Switzerland).

Gel electrophoresis analysis for TdT-catalysed
polymerization products

The reaction products were resolved on a 16% polyacryl-
amide gel using 1X TBE as a running buffer at a constant
voltage of 100 V for 40 min. After staining with SYBR Green II
(Invitrogen), a gel image was taken with Gel Doc EZ Imager
(Bio-rad, Hercules, CA).

Determination of TdT activity in human serum (10%)

The known concentrations of TdT were first spiked into the
human serum to prepare a set of standards and then a cali-
bration curve was created according to the procedures
explained above. Based on this calibration curve, the concen-
trations of TdT of unknown samples were determined from
the resulting fluorescence intensities.

Results and discussion

Detection feasibility

First, we investigated the quenching effect of Fe(III) on
BODIPY-ATP by measuring the fluorescence signals at 516
nm, an emission maximum of BODIPY, in the presence of
varying concentrations of Fe(III). As shown in Figure S1(a),
Fe(III) quenches the fluorescence signals of BODIPY-ATP in a
dose-dependent manner through a process of the photo-
induced electron transfer [13]. It was also confirmed that
Fe(III)-induced fluorescence quenching of BODIPY-ATP is sta-
bile at temperatures up to 90 °C (Figure S1(b)).

Next, the detection feasibility of this strategy was verified
by employing BODIPY-ATP/Fe(III) as the monomer for

Figure 1. (a) Fe(III)-induced fluorescence quenching of BODIPY-ATP. (b) Schematic illustration of the TdT activity assay utilizing BODIPY-ATP/Fe(III).

Figure 2. Feasibility of the TdT activity assay. (a) Fluorescence spectra from
BODIPY and (b) polyacrylamide gel electrophoresis images under different con-
ditions (1: TdT-primer + BODIPY-ATP/Fe(III)); 2: TdT-primer + BODIPY-ATP/Fe(III)
+ TdT). The insets in (a) show the corresponding photographs under UV light.
The final concentrations of Fe(III) and TdT are 1 mM and 200 U/mL, respectively.
TdT-catalysed polymerization. As envisioned, the presence of TdT produced the significantly increased fluorescence signal compared to that in the absence of TdT, which was supported by the formation of long oligonucleotides (Figure 2).

In addition, TdT-catalysed polymerization was monitored at different time points. The results in Figure S2 show that fluorescence signal increases by TdT as the reaction time increases until 90 min, over which it reaches a plateau. Overall, these observations confirm that TdT transforms BODIPY-ATP/Fe(III) into BODIPY-adenosine of the long oligonucleotides, resulting in the significantly increased fluorescence signal, which can be used for the simple, fluorescence turn-on determination of enzyme activities.

Detection sensitivity and selectivity

To assess the sensitivity of the proposed strategy, the fluorescence intensities at 516 nm were measured as a function of TdT concentration. As shown in Figure 3, the linear relationship is observed in the range from 0 to 50 U/mL ($R^2=0.9942$, $F_{516} = 1141.4 \times C_{TdT}/\text{U mL}^{-1} + 3416.9$, where $C_{TdT}$ is the concentration of TdT) with the detection limit of 0.64 U/mL (3σ/slope), a value that is comparable or superior to those associated with other fluorescence-based TdT assays [20–24].

The selectivity was also determined by employing other enzymes such as exonucleases, ligases and template-dependent DNA polymerases. The results in Figure 4 show that the fluorescence of BODIPY is efficiently switched on by the action of TdT, while other enzymes induce the negligible fluorescence enhancement even though they are present at 10 times higher concentration than that of TdT, confirming that it has high selectivity toward TdT.

Practical applicability

Finally, the ability of this strategy to determine TdT activity in human serum was evaluated to check its practical applicability. Following that the known amounts of TdT were spiked into diluted human serum (10%), the recovery experiments were carried out. As evidenced by good recovery ratio between 98% and 102% in Table 1, it worked well even in the serum containing various interfering agents, which indicates that it can be applied for the determination of TdT in real samples.

Conclusion

In summary, we found that BODIPY-ATP/Fe(III) serves as the effective monomer for the in situ synthesis of fluorescence oligonucleotides, which was utilized for the sensitive and selective determination of TdT activity. Importantly, the use of BODIPY-ATP/Fe(III) enables the cheap and convenient synthesis of fluorescence oligonucleotides while overcoming the drawbacks of previous methods. To the best of our
knowledge, this is the first report that exploits BODIPY-ATP/Fe(III) in DNA polymerization to develop biosensing platforms. We believe this strategy has a great potential to be applied for the simple and sensitive detection of other biological targets such as small molecules, nucleic acids, and proteins, and even for the formation of versatile nanostructures.

Disclosure statement
No potential conflict of interest was reported by the authors.

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