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LETTERS TO THE EDITOR

Preparation and Application of a BODIPY-Labeled Probe for a Real-Time Polymerase Chain Reaction

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Abstract—A BODIPY-based (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene, TMB) green fluorescent probe for quantitative real-time polymerase chain reaction (qPCR) was synthesized by azide-alkyne cycloaddition. Comparative studies with the analogous fluorescein-based probe were carried out. We demonstrated that fluorescent probes with the TMB fluorophore can be used in qPCR experiments.

Keywords: qPCR, fluorescent probes, chromophore, BODIPY, click, TaqMan

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INTRODUCTION

Oligonucleotide probes for quantitative PCR (qPCR) based on the fluorescent dye–Taqman-type quencher pair allow high-accuracy and high-specificity quantitative determination of the target region of DNA under analysis [1]. However, the choice of fluorescent labels for certain probes is usually limited to a commercially available set of fluorophores. In the present work, the fluorescent probes for qPCR were obtained by the scheme where an alkyne-containing fluorophore is attached to an oligonucleotide with a fluorescence quencher at one end and the azide group at another end by the click reaction. This approach expands the choice of fluorophores by a set of alkyne derivatives of dyes, which are commercially available or can be synthesized easily [2–4].

RESULTS AND DISCUSSION

We chose 4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene (tetramethyl-BODIPY, TMB) as the fluorescent label, since this dye has a high extinction coefficient and quantum efficiency, which must provide a high probe sensitivity. However, TMB possesses pronounced hydrophobic properties, which restricts its application for labeling of biological objects. In the present work, TMB containing the Taqman-type probe was prepared and the promising outlook for application of such probes for qPCR was assessed. At the first step, the carboxyl derivative of TMB (Fig. 1a, compound (1)) was synthesized according to the procedure described earlier in [5]. For subsequent conjugation with the azido derivative of oligonucleotide, compound (1) was esterified with propargyl alcohol (Fig. 1a, compound (2)). The fluorescent dye was attached to the oligonucleotide by the 1,3-dipolar cycloaddition "click" reaction between azide and alkyne [6] (Fig. 1b) to yield the TaqMan-oligonucleotide probe with the fluorophore at the 5' end and the fluorescence quencher at the 3' end. As noted above, TMB is a hydrophobic compound, which potentially can influence the results of qPCR analysis performed in aqueous solutions. Therefore, a probe with the commonly used fluorescein (FAM) instead of TMB was prepared to serve as the control (Fig. 1). To check the probes obtained, the expression analysis of the β-actin gene isolated from the rat airway epithelium was performed using qRT-PCR. Usually, the beta-actin gene is constitutively expressed and serves as the reference point for determination of relative expression of other genes [7]. In our case, it is sufficient that the gene is expressed reliably in the tissue under analysis. The first DNA chain was obtained by reverse transcription of the total RNA of the tissue to be analyzed. Then, dilutions of the resulted DNA of 1 : 10, 1 : 100, and 1 : 1000 were used as the matrix in qPCR experiments using the synthesized TMB- and

Abbreviations: FAM, fluorescein; BODIPY, 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene; TMB, 1,3,5,7-tetramethyl-BODIPY; TBTA, tris(N-benzyltriazolylmethyl)amine; FRET, Förster resonance energy transfer; qPCR, quantitative PCR; and qRT-PCR, quantitative reverse transcription PCR.

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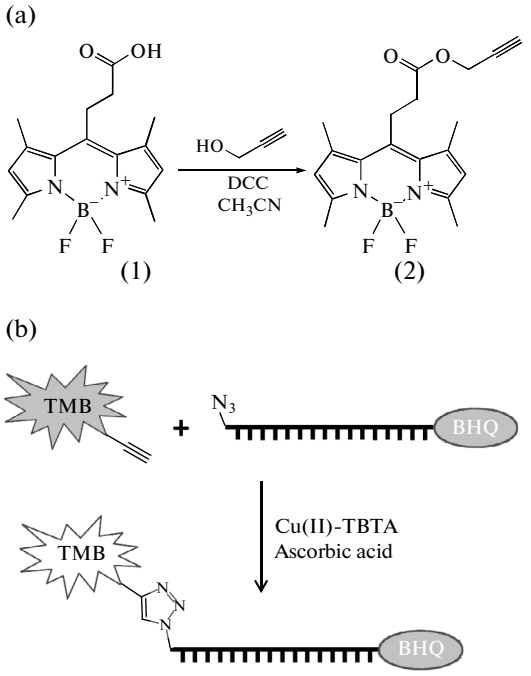

Fig. 1. Scheme for the synthesis of fluorescent BODIPY probe. (a) preparation of the TMB alkyne derivative; (b) "click" addition of TMB to oligonucleotide.

FAM-containing probes. Both the TMB- and FAM-containing probes showed similar results (Fig. 2). The number of threshold cycles in both cases changed from 20 to 27 for the minimum and maximum dilutions, respectively. The equation parameters of the calibration plots obtained were also similar.

$$\text{Cycle number} = A + B \times \log(\text{dilution})$$

The coefficients A and B were 31.3 ± 0.2 and -3.9 ± 0.1 for the FAM probe and $A = 30.5 \pm 0.1$ and $B = -3.53 \pm 0.07$ for the TMB probe, respectively.

In addition, we performed qPCR adding 5% DMSO to the reaction mixture to prevent possible aggregation of TMB in water. The resulted calibration plots (data are not shown) were close to those without DMSO. Probably, this is explained by the solubilizing effect of highly polar oligonucleotide with the attached fluorophore. Thus, the hydrophobic properties of TMB had no considerable effect on the efficiency of qPCR.

In conclusion, in the present work we demonstrated that the TaqMan-type qPCR probes with a necessary fluorophore can be obtained by the azide-alkyne cycloaddition "click" reaction. The alkyne-containing TMB fluorophore was synthesized and then conjugated with the azide-containing oligonucleotide. The results of qPCR showed that the oligonucleotide probes with TMB can be applied in qPCR experiments on a par with hydrophilic fluorescent labels, such as FAM.

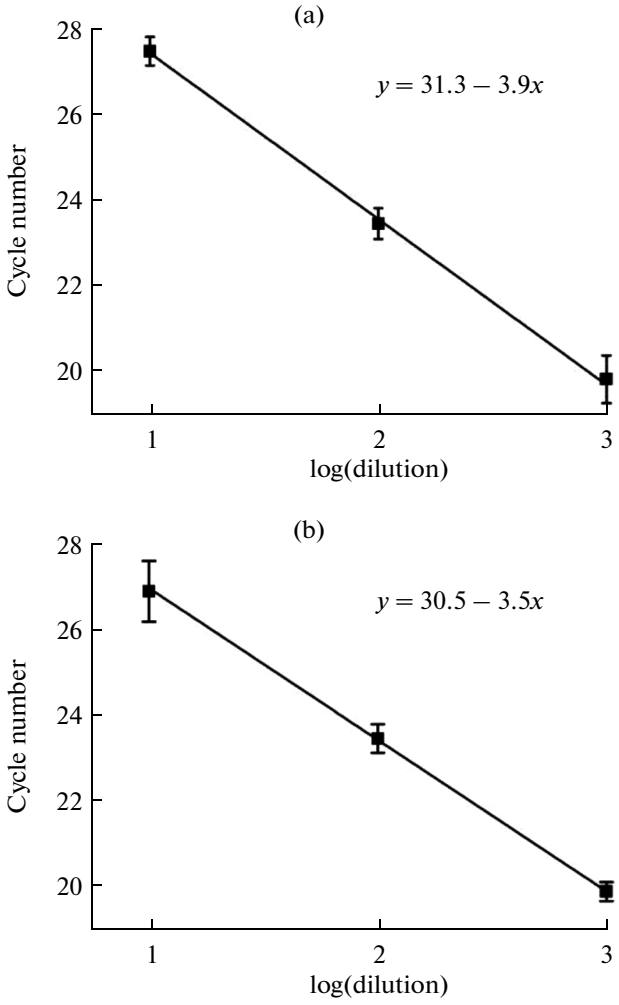

Fig. 2. qPCR calibration plots for the FAM probe (a) and the BODIPY probe (b).

EXPERIMENTAL

Propargyl 3-(4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacen-8-yl)propionate (2). To a solution of 3-(4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacen-8-yl)propionate (1) (38 mg, 0.12 mmol) in dry acetonitrile (20 mL), DCC (30 mg, 0.145 mmol) and propargyl alcohol (14 μL, 0.24 mmol) were added in an argon atmosphere. The mixture was left with stirring overnight and then filtered. The solvent was evaporated on a rotary evaporator and the resulted solid was purified by preparative silica gel column chromatography (the eluent was toluene, Rf = 0.4). The yield was 25 mg (61%), a crystalline dark-red substance, ESI-MS, *m/z*: 359.15 $[M + H]^+$, 339.15 $[M - F]^+$. ^{1}H NMR ($CDCl_3$): 2.44 (6H, s, CCH_3), 2.49–2.51 (1H, t, J 2.35 Hz, CCH), 2.52 (6H, s, CCH_3), 2.62–2.67 (2H, m, J_1 8.8 Hz, J_2 2.58 Hz, $COCH_2$-C$\underline{H}_2$), 3.31–3.35 (2H, m, J_1 8.8, J_2 2.8, CO$\underline{CH}_2$-CH_2), 4.74 (2H, d, J 2.35, O$\underline{CH}_2$), 6.07 (2H, br. s, ArH). ^{13}C NMR ($CDCl_3$): 14.44, 16.36,

23.44, 35.09, 52.45, 75.19, 77.23, 122.01, 131.21, 140.38, 142.82, 154.8, 170.84.

TaqMan-type fluorescent probes were prepared by conjugation of the alkyne-substituted dye (compound (2) or FAM alkyne (N-(prop-2-yn-1-yl)-5-carboxamide-fluorescein, Lumiprobe, United States)) with the azide-containing oligonucleotide (Syntol, Russia) using the Cu(I)-TBTA catalyst (Lumiprobe, United States) according to manufacturer's procedure. Probes were purified by HPLC on a C4 reversed-phase column (Phenomenex) equilibrated with 100 mM ammonium acetate in the acetonitrile gradient (from 8 to 60% of acetonitrile per 40 min); the flow rate was 0.7 mL/min. Labeled oligonucleotides were detected by two wavelengths at 260 and 495 nm. The retention times of fluorescent probes with the FAM and TMB dyes were 29 and 34 min (38 and 44% of acetonitrile), respectively.

The total RNA for qRT-PCR was isolated from frozen liquid-nitrogen-milled samples of the rat airway epithelium. The stable fraction of the total RNA isolated using RNeasy Mini kit (Qiagen, United States) was treated with RQ1 Rnase-Free DNase (Promega, United States). The quality of resulted RNA was assessed by the data from agarose gel electrophoresis. The purity of the RNA sample was determined by spectrophotometry. For the synthesis of the first cDNA chain, the total RNA (1 μg) was sampled and a MINT-Universal kit (Evrogen, Russia) was used according to manufacturer's protocol.

The gene expression analysis of rat β-Act (NM_031144.3, GenBank) was performed by quantitative real-time polymerase chain reaction (qPCR) using a Taq DNA Polymerase kit (Syntol, Russia) following manufacturer's recommendations. The primers used were as follows: rβActFor: 5'-AGCCATGTACGTAGCCATCCA-3'; and rβActRev: 5'-TCTCCGGAGTCCATCACAATG-3'. For specific detection of amplification products, TaqMan probes with the 5'-(F)TGTCCCTGTATGCCTCTGGTCGTACCAC(BHQ1)-3' sequence, where F is the FAM or TMB fluorophore and BHQ1 is the fluorescence quencher. To achieve certainty, each qPCR was performed in triplicate using the negative control on a ANK-32 (DNA-Technology, Russia); the temperature conditions were as follows: 1 cycle: 95°C for 300 s and 50 cycles: 62°C for 50 s and 95°C for 15 s.

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