Biomaterials 100 (2016) 172–183

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Single-molecule detection and tracking of RNA transcripts in living cells using phosphorothioate-optimized 2′-*O*-methyl RNA molecular beacons

Dan Zhao [a], Yantao Yang [a], Na Qu [a], Mingming Chen [a], Zhao Ma [a], Christopher J. Krueger [a], Mark A. Behlke [b], Antony K. Chen [a, *]

[a] Department of Biomedical Engineering, College of Engineering, Peking University, Beijing, 100871, China
[b] Integrated DNA Technologies Inc., Coralville, IA, 52241, USA

ARTICLE INFO

Article history:
Received 19 March 2016
Received in revised form
10 May 2016
Accepted 17 May 2016
Available online 24 May 2016

Keywords:
Molecular beacons
2′-*O*-methyl RNA
Phosphorothioate
RNA imaging
Single-molecule detection
RNA dynamics

ABSTRACT

Molecular Beacons (MBs) composed of 2′-*O*-methyl RNA (2Me) and phosphorothioate (PS) linkages throughout the backbone (2Me/PS_{FULL} MBs) have enabled long-term imaging of RNA in living cells, but excess PS modification can induce nonspecific binding, causing false-positive signals. In this study, we evaluate the intracellular stability of MBs composed of 2Me with various PS modifications, and found that false-positive signals could be reduced to marginal levels when the MBs possess a fully PS-modified loop domain and a phosphodiester stem (2Me/PS_{LOOP} MB). Additionally, 2Me/PS_{LOOP} MBs exhibited uncompromised hybridization kinetics, prolonged functionality and >88% detection accuracy for single RNA transcripts, and could do so without interfering with gene expression or cell growth. Finally, 2Me/PS_{LOOP} MBs could image the dynamics of single mRNA transcripts in the nucleus and the cytoplasm simultaneously, regardless of whether the MBs targeted the 5'- or the 3′-UTR. Together, these findings demonstrate the effectiveness of loop-domain PS modification in reducing nonspecific signals and the potential for sensitive and accurate imaging of individual RNAs at the single-molecule level. With the growing interest in the role of RNA localization and dynamics in health and disease, 2Me/PS_{LOOP} MBs could enable new discoveries in RNA research.

1. Introduction

In recent years, considerable progress has been made in the discovery of novel RNA molecules and their functions. Fundamental to the success of these findings is the use of gene expression analysis techniques such as northern blotting, RT-qPCR and DNA microarrays. While these methods have provided valuable insights into the expression status of specific RNAs from populations to single cells, the need to lyse the cells limits gathering of sufficient spatiotemporal information necessary to completely characterize RNA activities. Currently, single-molecule fluorescence *in situ* hybridization (smFISH) is the method of choice for mapping the intracellular distribution of RNAs with single-transcript sensitivity [1,2]. In one method, multiple fluorophore-labeled oligonucleotide (ODN) probes are hybridized to individual RNA transcripts in fixed cells. As a result, each transcript appears as a discrete bright spot readily detectable via conventional fluorescence microscopy. While smFISH has increasingly become a valuable tool in RNA research, the need for cell fixation makes the technique impractical for studying RNA dynamics. Therefore, in order to obtain a more in-depth understanding of the role of RNAs in health and disease, there remains a need to visualize individual RNA molecules directly in native live-cell contexts.

One tool that has great potential for imaging RNAs in their native environment is the molecular beacon (MB) [3], a class of oligonucleotide-based probes that are capable of forming a stem-loop structure with a fluorophore at one end and a quencher at the other end. In the unhybridized state, the complementary short-arm sequences flanking the loop domain anneal to form a double-stranded stem. This brings the quencher into close spatial proximity with the fluorophore to significantly quench its fluorescence.

* Corresponding author. Department of Biomedical Engineering College of Engineering, Peking University, No. 5 Yiheyuan Road, Haidian District, Beijing, 100871, China.
E-mail address: chenak@pku.edu.cn (A.K. Chen).

http://dx.doi.org/10.1016/j.biomaterials.2016.05.022

Hybridization of the loop domain to the target RNA disrupts the stem, causing separation of the quencher from the fluorophore, allowing emission of a fluorescent signal upon excitation. The ability to report hybridization events with fluorescence emission has spurred applications of MB technology from test tubes to living cells, enabling direct visualization of RNA molecules, including mRNA [4–13], viral RNA [14–17], microRNA [18–20] and long non-coding RNA [21,22] in various physiological and pathological contexts.

Paralleling the growing use of MBs for intracellular RNA analysis, accumulating evidence has shown that when delivered into cells, MBs synthesized with unmodified DNA or 2′-*O*-methyl RNA (2Me) backbones are quickly sequestered in the nucleus where they generate false-positive signals as a result of nonspecific interactions such as nuclease degradation and/or binding to cellular components [6,9]. In an attempt to overcome this problem, several groups have developed methods to minimize nuclear localization of MBs [9,23,24]. Alternatively, MBs have been synthesized with chemically-modified oligonucleotide (ODN) backbones to enhance biostability [11,17,25]. In one design, the phosphate group of the 2Me MB has been substituted with a phosphorothioate (PS) linkage throughout the backbone (2Me/PS_{FULL} MB), a modification that renders an ODN highly nuclease-resistant [17,25]. In living cells, 2Me/PS_{FULL} MBs have been used to visualize the replication of Coxsackie viral RNA for up to 12 h [17]. Consistent with this, we showed that 2Me/PS_{FULL} MBs have longer intracellular bioactivity, in addition to eliciting lower false-positive signals, compared with MBs without PS modification (2Me MBs) [25]. Despite these reported attributes, it was also shown that within a few hours following cellular entry, 2Me/PS_{FULL} MBs can form discrete, bright spots that may be misinterpreted as, for example, sites of RNA accumulation or even single RNA transcripts. Additionally, by 24 h many of the MBs were found to localize to lysosomes, accompanied by an increase in false-positive signals. Therefore, in order to better take advantage of the nuclease resistance property of the PS modification, it is necessary to eliminate these undesirable signals.

The observed nonspecific fluorescence exhibited by 2Me/PS_{FULL} MBs reflects the problem of nonspecific binding commonly encountered with highly PS-modified ODNs [26–31]. Given that 2Me RNAs are fairly nuclease resistant [32], we hypothesize that partially PS-modified 2Me MBs may avoid nonspecific binding while still maintaining the nuclease stability needed for sensitive intracellular RNA imaging. To investigate this possibility, we synthesized MBs composed of 2Me RNA with different numbers and locations of PS linkages and evaluated their long-term stability in living cells. The optimal configuration that exhibited the least false-positive signal was further investigated for its ability to accurately image single RNA transcripts and measure RNA dynamics in both the nucleus and the cytoplasm without affecting gene expression and cell viability.

2. Materials and methods

2.1. Synthesis and spectral analysis of MBs

The MBs used in this study are listed in Tables 1 and 2. Luciferase (sequence: 5′-GUCAGGACAUCACUUACGCUGAGUUU-3′) and MB repeat (sequence: 5′-CUCGACAGGAGUUGUGUUUGUGGACGAAGAG-3′) target RNA oligonucleotides were also synthesized. All oligonucleotides were synthesized by Integrated DNA Technologies Inc (Coralville, IA, USA).

The emission profile of each MB was acquired on a Lumina fluorescence spectrometer (Thermo Scientific) by setting the excitation wavelength to 647 nm and recording the emission from 655 to 800 nm. These experiments were carried out in 1xPBS, 0.05% Tween-20, pH 7.4 at 25 °C using 50 nM MBs in the presence or absence of 300 nM complementary target.

2.2. Melting profile analysis

The melting profiles of all MBs in the absence and presence of RNA target were acquired using a CFR96 Touch™ Real-Time PCR Detection system (Bio-Rad). Specifically, the fluorescence intensity of a 30 μL solution containing 1 μM MB in the absence or the presence of 2 μM RNA target in 1xPBS was recorded from 5 to 98 °C. Temperature was increased at one degree per five minutes to ensure that the solution reached equilibrium prior to each fluorescence recording. The derivative of the emission recording, with respect to temperature, is plotted to identify the temperature at which the fluorescence exhibits the greatest rate of change. This temperature corresponds to the melting temperature (Tm) of the MB alone or the MB-target duplex. Melting temperatures are listed in Tables 1 and 2.

2.3. Hybridization kinetics

To evaluate the effect of loop-domain PS modification on the rate of MB-target hybridization, a 500 μL sample of 250 nM luciferase RNA target was added to 500 μL of 250 nM 2Me/PS_{LOOP} or 2Me MBs. Fluorescent measurements were taken immediately on a Lumina fluorescence spectrometer (Thermo Scientific). Measurements were performed in 1x PBS, 0.05% Tween-20, pH 7.4 at 25 °C.

2.4. Synthesis of fluorescently-labeled dextrans

The IRDye®800-labeled dextran used as a reference probe was synthesized as previously described [25]. In brief, IRDye®800CW NHS ester (Li-Cor) was reacted with 2.5 mg/mL aminodextran (MW:10 kDa, Life Technologies) at a dye-to-dextran molar ratio of 3:1 in 50 mM sodium borate buffer (pH 8). After overnight reaction at 25 °C, the mixture was purified on NAP-5 gel chromatography columns (GE Healthcare) in phosphate buffer (48 mM K_2HPO4, 4.5 mM KH_2PO4, 14 mM NaH_2PO4), pH 7.2, to remove unbound dye. The IRDye®800-labeled dextrans were then concentrated on a Centricon device (Millipore) and the concentration of the IRDye®800 fluorophore was determined on a BioMate 3S spectrophotometer (Thermo Scientific).

2.5. Plasmid construction

pGEM-64x and pGEM-32x plasmids, which encode transcripts containing 64 and 32 tandem repeats of the 50-base sequence 5′-CAGGAGTTGTGTTTGTGGACGAAGAGCACCAGCCAGCTGATCGACCTCGA-3′ were kind gifts of Sanjay Tyagi, Rutgers University, NJ, USA. The underlined sequence is the unique MB target site. The derivative constructs, pEGFP-N1-32x and pEGFP-N1-64x, were constructed by inserting the respective tandem repeats (total length 1,600bp and 3,200bp) from the parental constructs into pEGFP N1 (Clontech) using EcoRI and BamHI. To construct pEGFP-C1-TAA-32x, a TAA stop codon was first introduced into the EGFP sequence of the pEGFP-C1 vector (Clontech) by PCR using forward primer 5-'ACCTGCACCGGTCGCCACCATGGTGAGCAAGG-3' and reverse primer 5′ACTGCTGAATTCTTACTTGTACAGCTCGTCCATGCCGAGAGTGAG-3'. The PCR product was then inserted into the same vector using AgeI and EcoRI to create pEGFP-C1-TAA. The EcoRI- and BamHI-digested 32x fragment was then cloned into pEGFP-C1-TAA to create pEGFP-C1-TAA-32x.