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Short communication

A facile one-step fluorescence method for the quantitation of low-content single base deamination impurity in synthetic oligonucleotides

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ABSTRACT

Oligonucleotides are being researched and developed as potential drug candidates for the treatment of a broad spectrum of diseases. The characterization of antisense oligonucleotide (ASO) impurities caused by base mutations (e.g. deamination) which are closely related to the target ASO is a significant analytical challenge. Herein, we describe a novel one-step method, utilizing a strategy that combines fluorescence-ON detection with competitive hybridization, to achieve single base mutation quantitation in extensively modified synthetic ASOs. Given that this method is highly specific and sensitive (LoQ = 4 nM), we envision that it will find utility for screening other impurities as well as sequencing modified oligonucleotides.

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1. Introduction

Synthetic antisense oligonucleotides (ASOs) that target precursor mRNA and mRNA, genetic information carriers that encode cellular proteins, are being explored as therapeutic agents [1,2]. The underlying mechanism of this technology is to induce down-regulation or upregulation of gene expression by forming a stable duplex between the ASO and its complementary RNA sequence via Watson-Crick base pair hybridization [3]. Numerous chemical modifications have been introduced to ASOs in the past decades in order to increase the efficacy and stability of ASO drugs in a biological environment. This makes their analytical characterization challenging since traditional DNA/RNA analytical methods may not be suitable for highly modified, nuclease resistant ASOs. During drug development, the characterization of impurities in the product is of fundamental importance to ensure quality, strength, and safety of a drug [4]. Among various ASO impurities, a base mutation impurity that may be generated by deamination of 5-methylcytosine (MeC) to thymine (T) [5], (see Fig. 1A, green circle) is one of the most challenging impurities to quantitate because of its similarity in structure (net addition of oxygen and loss of nitrogen) and mass ($\Delta m = 1$ amu) to the pure product. Despite the low rate of deamination (9.5×10^{-10} s^{-1} at 37 °C) [6], its detection (if present) and quantitation is of importance for dug characterization and process understanding. Any method used to analyze and quantitate deamination also has the potential to be applied to other single base mutations in ASOs.

A variety of analytical methods have been developed to detect mutations in DNA and RNA sequences in academic disciplines [7–14]. However, single-base mutation/deamination quantitation remains a formidable challenge because the existing nuclease- and polymerase-based methods (including PCR amplification [7], enzymatic digestion coupled to LC–MS/MS [8–11], next generation sequencing [12], polymerase-based bioelectronics sensor [13], and hybridization-protection assay followed by LC–MS [14]) involve multiple sample treatment steps and are not effective on chemically modified ASO structures. In fact, literature on resolving deamination impurities in ASO drug development is scarce. Only one method based on high resolution liquid chromatography mass spectroscopy enables deamination quantitation in one step [15]. This method measures the change in the isotope distribution pattern between the sample and standard with software-enhanced mass accuracy (MassWorks by Cerno Bioscience) to quantitate total deamination, i.e. not site-specific, at the 5% level (Fig. 1B). Furthermore, the available methods used to detect other low-content impurities fail to resolve the deamination impurity. For example, Marco and Tony developed LC–MS and NMR methods to quan-

Abbreviations: ASO, antisense oligonucleotide; 2′-MOE, 2′-*O*-methoxyethyl; MB, molecular beacon; PS, pure sequence; DA, deamination marker; cPS, complementary pure sequence.

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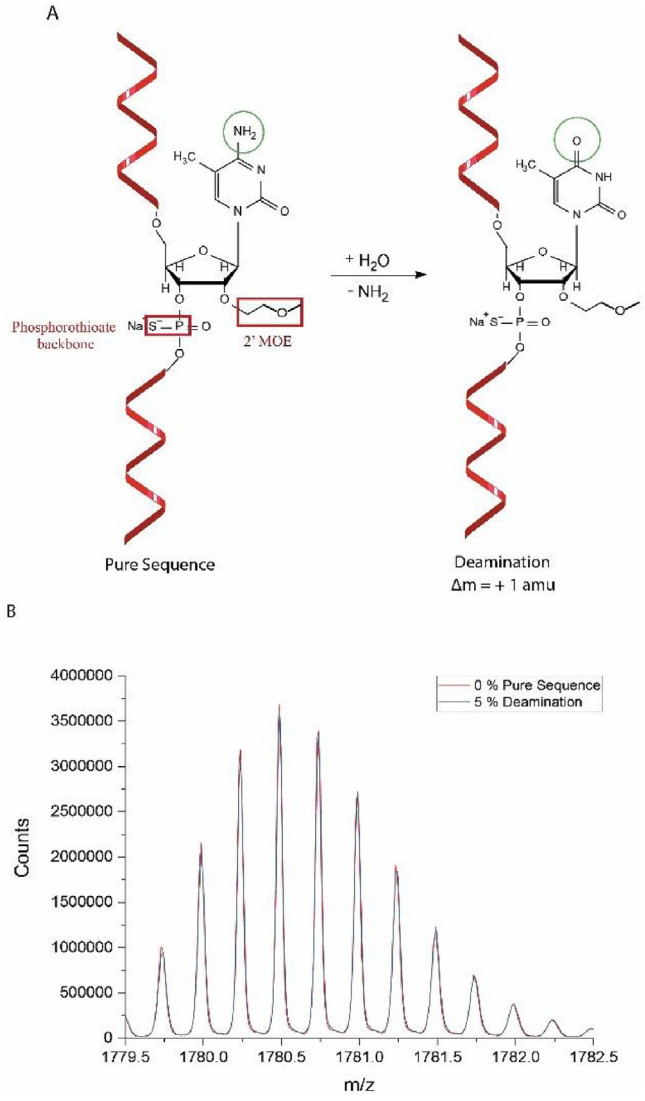

Fig. 1. (A) Schematic of ASO and impurity caused by deamination. The conversion of 5-methylcytosine to thymine is highlighted with green circles. Modifications including phosphorothioate internucleotide linkages and 2′-MOE are highlighted in red boxes. (B) Example of overlaid high resolution mass spectra of pure sequence (red trace) and 5% deamination spiked mixture (blue trace) at the −4 charge state show a slight shift in isotope distribution. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tify a low-level mono phosphate substituted impurity (P = O) [16]. The large mass difference (Δm = 16 amu) from the pure product and distinct chemical shift of P = O in ^{31}P NMR spectrum make its quantitation possible. In contrast, deamination impurity has a mass difference of 1 amu and no distinct NMR signal from the product. In addition to the challenges that the existing methods for deamination quantitation face, to the best of our knowledge, there has been no report on one step site-specific deamination quantitation for ASOs.

In this study, a facile one-step method was developed utilizing a strategy which combines competitive hybridization and fluorescence detection. This is the first time a site-specific one-step quantitation method has been shown for analysis of ASO drugs with significant chemical modifications. In this method, a molecular beacon (MB) designed to complement the deamination impurity is used for specific hybridization which induces fluorescence for low level deamination quantitation. The salt concentration and buffer pH of this method was optimized by monitoring the fluorescence response. Then, competitive hybridization was used to enhance the signal to noise ratio. These optimizations resulted in a sensitive method, with a LoQ of 4 nM, equivalent to 0.24% of the pure sequence, for deamination detection.

2. Material and methods

All experiments were carried out at room temperature.

2.1. Material and reagents

Pure sequence (***PS***) and deamination marker (***DA***) were synthesized and purified by Ionis Pharmaceuticals (Carlsbad, CA). Due the low rate of deamination ($9.5 \times 10^{-10}\,s^{-1}$ at 37 °C) [7] and appropriate storage condition (5 °C), ***PS*** is assumed to contain no deamination impurities. Other sequences used in this work were synthesized and HPLC-purified by Bioneer, Inc. (Alameda, CA). Exonuclease I reaction buffer (10X) was purchased from New England Biolabs (Ipswich, MA). Tris solution (2 M, pH 7.5) was obtained from AmericanBio, Inc. (Natick, MA).

2.2. Instrumentation

Fluorescence and UV spectra were obtained using an EnVision multilabel plate reader (PerkinElmer, Waltham, MA). For all fluorescence intensity measurements, the excitation wavelength was set to 485 nm, and the emission was monitored from 495 to 600 nm for full spectra and at 520 nm for kinetics. The pH of the buffer was determined using an Orio Versa Star Pro pH meter (Thermo Scientific, Waltham, MA). High Resolution mass spectra were collected using a Waters Xevo QTof mass spectrometer.

2.3. Molecular beacon specificity

Aqueous ***PS*** and ***DA*** stock solutions were prepared to a final concentration of 10 μM. Aqueous ***MB*** stock solution was prepared at a concentration of 5 μM. For the ***MB*** specificity study, 10 μL of ***MB*** stock solution was added to a solution containing 10 μL of ***DA*** stock solution and 80 μL of 1X EXO-I reaction buffer. In a control experiment, 10 μL of ***MB*** stock solution was added to a solution containing 10 μL of ***PS*** stock sotluon and 80 μL of 1X EXO-I reaction buffer. Both working solutions were allowed to incubate for 30 min before fluorescence measurement. The fluorescence intensity (FI) at 520 nm of ***DA***:***MB*** (molar ratio, 2:1) working solution was compared with that of the ***PS***:***MB*** (2:1) working solution and the ratio was calculated.

2.4. Salt concentration

A series of 1X EXO-I reaction buffers with sodium chloride concentrations ranging from 200 mM to 1.8 M were prepared. The salt buffers were used to prepare working solutions for fluorescence measurements of both ***DA*** and ***PS*** samples as in the **Molecular beacon specificity** section. The FI ratios of ***DA***:***MB*** (2:1) and ***PS***:***MB*** (2:1) in different buffers were used to determine the optimal buffer salt concentration.

2.5. pH

After the salt concentration was optimized for the method, buffer pH optimization was performed for a pH range from 4 to 11 using Tris buffer. The FI ratios of ***DA***:***MB*** (2:1) and ***PS***:***MB*** (2:1) in Tris buffer at different pH values were calculated to determine the optimal pH condition.

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