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Molecular structure and thermodynamic predictions to create highly sensitive microRNA biosensors

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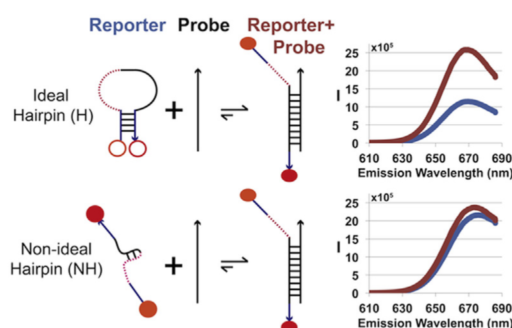
HIGHLIGHTS

- Challenges facing highly sensitive miRNA biosensor designs are addressed.
- Thermodynamic and molecular structure design metrics for reporter+probe biosensors are proposed.
- The influence of ideal and non-ideal reporter hairpin structures on reporter+probe formation and signal change are discussed.
- 5–9 nM limits of detection were observed with no interference from off-analytes.

GRAPHICAL ABSTRACT



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ABSTRACT

Many studies have established microRNAs (miRNAs) as post-transcriptional regulators in a variety of intracellular molecular processes. Abnormal changes in miRNA have been associated with several diseases. However, these changes are sometimes subtle and occur at nanomolar levels or lower. Several biosensing hurdles for *in situ* cellular/tissue analysis of miRNA limit detection of small amounts of miRNA. Of these limitations the most challenging are selectivity and sensor degradation creating high background signals and false signals. Recently we developed a reporter+probe biosensor for let-7a that showed potential to mitigate false signal from sensor degradation. Here we designed reporter+probe biosensors for miR-26a-2-3p and miR-27a-5p to better understand the effect of thermodynamics and molecular structures of the biosensor constituents on the analytical performance. Signal changes from interactions between Cy3 and Cy5 on the reporters were used to understand structural aspects of the reporter designs. Theoretical thermodynamic values, single stranded conformations, hetero- and homodimerization structures, and equilibrium concentrations of the reporters and probes were used to interpret the experimental observations. Studies of the sensitivity and selectivity revealed 5–9 nM detection limits in the presence and absence of interfering off-analyte miRNAs. These studies will aid in determining how to rationally design reporter+probe biosensors to overcome hurdles associated with highly sensitive miRNA biosensing.

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1. Introduction

MicroRNAs (miRNAs) are an emerging class of biomarkers that

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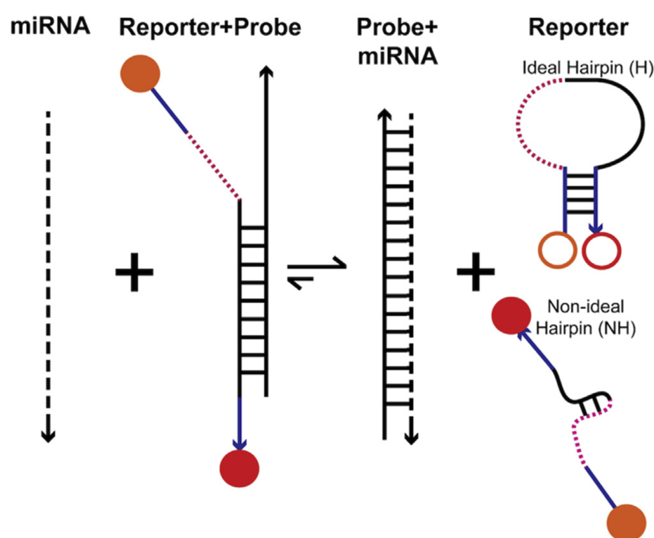
may aid in understanding, diagnosing, and tracking disease progression. One role of miRNAs in biology is to regulate protein expression by interacting with messenger RNA (mRNA) to disrupt translation. In many diseases, the expression of miRNA is altered leading to changes in protein expression, sometimes resulting in an altered phenotype. *In situ* miRNA analysis is needed to fully understand how miRNA regulate gene expression as miRNA often target different messenger RNA in a cell- and tissue-specific manner [1]. To achieve this goal miRNA biosensors are needed that can detect low levels and small changes in miRNA concentration, particularly in the femtomolar to nanomolar range [2–6].

In general, *in situ* miRNA detection is qualitative or semi-quantitative [1,5,7,8]. Recent reports [9,10] indicate that *in situ* detection of miRNA profiles in cells and tissues as a means for diagnostics and the study of disease progression have been hampered due to insufficient sensitivity, selectivity, and robustness of miRNA biosensors [6]. Standard fluorescence *in situ* hybridization (FISH) techniques have several limitations for low abundance miRNA detection: (1) loss of miRNA from copious rinsing steps, (2) often require secondary sensing with horseradish peroxidase or other enzymatic treatment, (3) long time-to-result, and (4) can only be used on fixed cells and tissues [5,11]. Current biosensors capable of *in situ* nucleic acid analysis, such as molecular beacons or ratiometric bimolecular beacons are limited by intracellular nucleases that often degrade the biosensors, leading to an increase in false signals [12,13].

To overcome the issues relating to false signals, our group recently developed a competitive binding biosensor [14,15]. In the presence of a specific miRNA the biosensor releases a self-complementary reporter capable of Förster Resonance Energy Transfer (FRET). We designed these biosensors for *in situ* analyses [10] with a faster time-to-result than quantitative reverse transcription polymerase chain reaction (RT-qPCR) [16,17], microarrays [18], and FISH [19]. Our sensor, referred to as a 'reporter+probe biosensor', has a partially complementary double-stranded design containing a probe sequence and a reporter sequence with dyes on the 3' (3-prime) and 5' (5-prime) ends. The probe strand is completely complementary to the analyte of interest, and the reporter strand is only partially complementary to the probe to permit competitive binding of the miRNA analyte to the probe. The competitive reaction, referred to by some as a toehold-mediated strand displacement/exchange [20], releases the self-complementary reporter from the probe, allowing the reporter to fold onto itself (Scheme 1). Reporters are designed to form an ideal hairpin structure that brings the 3′ and 5′ ends (containing the reporting dyes) into the FRET distance. However, some reporter designs can form a non-ideal internal hairpin that raises the baseline signal due to increased dye-to-dye distance. Designing these reporter sequences to work in an ideal manner takes into account thermodynamics [21–23], kinetics [24,25], and conformation of the reporter strand.

In our previous work, we developed a reporter+probe biosensor that was selective for let-7a [14,15]. Let-7a is one of the thousands of miRNAs that have been discovered and among those that have been found to regulate disease progression [1,26–31]. Here we investigated multiple reporters to be used in reporter+probe biosensors for two different mouse-derived miRNAs associated with cancer progression: miR-26a-2-3p and miR-27a-5p [32–36]. In this work we will build upon our previous research [14,15] to further examine how the predicted thermodynamic and structural parameters of the reporters, probes, and analytes influence the analytical performance of reporter+probe biosensors. Similar to work by others [24,37,38], the relationship between the Cy3 and Cy5 dye emission was used to understand the conformational states of the reporters in solution with and without probe, and was



Scheme 1. Response of reporter+probe biosensor to miRNA analyte. The miRNA (down arrow with dashed line) reacts with reporter+probe complex to form the probe+miRNA complex and free reporter. Reporter (Down arrow with solid and dashed regions) is only partially complementary to probe (Up arrow with solid line) in the region indicated in black. The dotted red region of the reporter is not complementary to probe, and the blue region represents the reporters stems. When in the hairpin conformation, the reporter forms a stem-loop that is either ideal with 5′ and 3′ ends directly adjacent to each other, or a non-ideal hairpin that separates the dyes at different distances (Colored circles, orange for Cy3 and red for Cy5). Empty colored circles indicate quenched emission, and filled circles indicate unquenched emission.

related to the predicted conformations. Specifically, we will discuss the importance of changes in thermodynamic values, molecular structures, and base pairing before and after reporter+probe formation and subsequent probe+analyte formation. To the best of our knowledge, we were the first group to describe a competitive nucleic acid biosensor that uses a self-complementary reporting molecule to reduce false signals [14,15]. This design has since grown in popularity for use in other technologies like Nanoflares for messenger RNA (commercially known as SmartFlares®) [39] and molecular sentinels [40]. The results of the work presented here will enable the community to rationally design reporter+probe biosensors to achieve a robust, selective, and sensitive biosensor with minimal false signals.

2. Experimental

2.1. Reporter design procedure

The process for designing the biosensor sequences is outlined in Scheme 2. DNA analogs of each miRNA strand were used as a substitute because RNA is more susceptible to degradation than DNA. A MATLAB program developed in-house was used to design the nucleic acid sequences for the reporter+probe biosensors based on a particular miRNA analytes, in this case they were miR-26a-2-3p and miR-27a-5p. The computer program starts by designing a probe strand that is fully complementary to a given miRNA analyte. Several reporter strands were then made to be partially complementary to the probe. For consistency each of the reporters in this study contained the same stem sequence, 5′-CGATG——CATCG-3′. The computer program allows control over the length of both the complementary and non-complementary regions on the reporter. The non-complementary regions of the reporters were made of random nucleic acid sequences. The reporters were then filtered to remove those reporters whose non-complementary region bind to

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