A methylation-switchable conformational probe for the sensitive and selective detection of RNA demethylase activity†‡

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We describe a novel methylation-sensitive nucleic acid (RNA) probe which switches conformation according to its methylation status. When combined with a differential scanning fluorimetry technique, it enables highly sensitive and selective detection of demethylase activity at a single methylated-base level. The approach is highly versatile and may be adapted to a broad range of RNA demethylases.

N-Methylation of nucleic acid bases has been a subject of intense chemical and biological interest because of its critical roles in several key cellular processes, such as epigenetic gene regulation, RNA metabolism and cellular reprogramming. In recent years, it has become apparent that the methylation status of nucleic acids is dynamically regulated by a complex interplay between DNA/RNA methyltransferases and demethylases, which add and remove ‘methyl marks’ from nucleic acid bases, respectively. It is also increasingly clear that dysregulation of these enzymes may underlie the pathogenesis of a range of human diseases, hence there is currently strong interest being shown in their mechanistic and inhibition studies.

One major class of DNA/RNA demethylases is the AlkB family of iron- and 2-oxoglutarate (2OG)-dependent dioxygenases, which includes Escherichia coli AlkB, and nine human homologues, ALKBH1-8 and FTO (fat mass and obesity-associated protein) (Fig. 1a). The activity of AlkB demethylases is extremely challenging to assay, in part due to the small size and chemical inertness of the methyl group they remove. Consequently, despite significant efforts by various groups, there is, at present, a lack of sensitive and high-throughput techniques for their analysis. Current limited assays rely primarily on indirect approaches, such as the analysis of demethylation co-products (NADPH, FADH, and formaldehyde) through coupled assays, or the analysis of demethylated products following their digestion with the restriction enzyme. However, these methods suffer from false positives/negatives that are typically associated with coupled reactions. They also require multiple steps which are laborious, time-consuming, and generally show poor reproducibility. Although the direct detection of DNA/RNA demethylation has been achieved using radio-labelled substrates, MALDI mass spectrometry, and capillary electrophoresis, these techniques lack throughput and/or sensitivity, which severely hinders the study of this important class of enzymes.

Recent studies have demonstrated that nucleic acid base methylation can induce a major overall conformational change in certain sequence contexts. For instance, 3-methylcytosine has been shown to induce B- to Z-transition in some DNAs, while N6-methyladenosine (m6A) could induce a duplex-hairpin conversion in certain RNA sequences. Inspired by these interesting observations, we envisaged that the (de)methylation-induced conformational change could provide a basis for the design of a ‘conformational probe’ which is useful for sensing the methylation change. To the best of our knowledge, this concept has not previously been applied to the analysis of DNA/RNA methylation, and, to date, there is no report of an assay method which is based on a methylation-sensitive probe.

Herein, we describe a novel detection strategy for RNA demethylase activity, which combines the discriminatory power of a dynamic conformational probe with the sensitivity of a Differential Scanning Fluorimetry (DSF) technique (Fig. 1b). In this approach, a structurally dynamic RNA that changes its secondary structure according to its methylation status is employed as a conformational probe. By design, when the probe is methylated, it preferentially adopts a hairpin conformation. However, upon selective removal of methylation by a demethylase, the probe undergoes a spontaneous and rapid transformation to a duplex conformation. Such a major switch from the hairpin to duplex structure is accompanied by a profound change in a number of biophysical properties, notably the melting temperature (Tm) of the probe, which can be accurately measured using DSF-based melting analysis. DSF is a simple and inexpensive technique that has been
widely used for the detection of SNPs and protein–ligand interactions in the ‘thermal shift assay’. However, to date, DSF (and the related high-resolution melt analysis) has not been applied to the analysis of RNA secondary structure. As we shall demonstrate, DSF is a powerful technique for monitoring methylation-induced RNA conformational changes. When used in combination with a conformational probe, it has the sensitivity to detect changes in RNA down to a single methylated base.

To implement the proposed strategy, we design a ‘conformational probe’ that is structurally responsive to methylation changes. In this study, we used an m1A-containing probe with a view of developing a high-throughput assay against m1A-demethylases ALKBH2 and ALKBH3, which are potential therapeutic targets for cancer.

Preliminary studies of a small library of palindromic RNAs led us to identify r(CGCGm1AUCGCG) as our model m1A-probe (Table S1, ESI†). It consists of two terminal ‘CGCG’ segments and a middle ‘AAUU’ segment (underlined), where m1A resides (for chemical synthesis, see the ESI†). Due to its self-complementary nature, it can inherently adopt a bimolecular duplex through intermolecular base pairing, and a monomolecular hairpin by folding back on itself (Fig. 2a). We reasoned that since m1A impairs Watson–Crick base pairing, the probe likely favours the hairpin conformation, where m1A can be stably localised within the ‘loop’ of the hairpin.

To verify our probe design, we performed a DSF-based melting analysis of the m1A-probe. In this method, the melting profile was obtained by monitoring the fluorescence intensity of an RNA intercalating dye (SYBR Green I) at 520 nm as a function of temperature. The fluorescence spectrum showed a monophasic, sigmoid transition, implying that the m1A-probe exists predominantly as a single conformation under our experimental conditions (Fig. 2b). Moreover, the probe exhibits a relatively constant $T_m$ over a 100-fold concentration range (Fig. 2c), suggesting that it likely adopts a hairpin conformation, as anticipated. In contrast, its demethylated counterpart r(CGCGAAUUCGCG) showed a concentration-dependent $T_m$ which implies a duplex structure (Fig. 2c). The above results are consistent with melting profiles obtained from circular dichroism (CD)- and UV-melting analyses (Fig. S1 and S2, ESI†). The CD spectra of m1A- and demethylated-probes further revealed characteristics which are indicative of a B-like hairpin and a A-form duplex, respectively (Fig. 2d).

To obtain additional insights into the conformations of the probes, we performed 1H NMR analysis. The imino spectrum of the m1A-probe revealed four NH peaks at 4°C, likely suggesting a hairpin structure in which the stem contains four C-G base pairs, and the loop consists of four unpaired m1A nucleotides (Fig. 2e). The demethylated probe, however, showed six imino peaks, implying a fully base-paired duplex (Fig. 2f). We are aware that for more rigorous proof of probe conformations, other analytical methods such as 2D NMR are required. Nevertheless, our combined data clearly demonstrate that the conformation of the probe is highly dependent on its methylation status. It further suggests that demethylation of the m1A-probe may...
induce a hairpin–duplex transformation, consistent with our design strategy. Notably, only one set of NMR signals was observed for m1A- and demethylated-probes, suggesting that they exist almost exclusively as a single conformation in solution and do not co-exist as other secondary structures. The absence of the duplex form of the m1A-probe also implies negligible Hoogsteen base pairing between m1A and U in the context of this RNA sequence. This result is interesting because Hoogsteen base pairs have been observed between m1A and T in DNA duplexes.  

The thermodynamic parameters derived from DSF-melting experiments further revealed that the observed hairpin–duplex conversion is primarily an enthalpy-driven process (ΔΔH° = −61.9 kcal mol⁻¹), and despite the large entropy cost (ΔΔS° = −163.0 cal mol⁻¹ K⁻¹), the demethylated duplex is significantly more stable than the m1A hairpin (ΔΔG° = −11.5 kcal mol⁻¹) (Fig. 3 and Fig. S3, S4, ESI†). We appreciate that m1A can potentially undergo base-catalysed Dimroth rearrangement to yield m1A; nevertheless, in our DSF analyses, the Tm of the m1A-probe was highly reproducible, suggesting little or no degradation under our experimental conditions.

We next examined if the m1A-probe could be recognised and demethylated by ALKBH2. In a typical conformational probe-DSF assay, the enzyme (50 nM) was incubated with the m1A-probe (10 μM) in the presence of co-substrate 2-oxoglutarate (10 μM), Fe(n) (10 μM), and L-ascorbate (200 μM) in 50 mM HEPES buffer (pH 7.0) at 4 °C for various time intervals, after which the reaction was quenched and the product formation was analysed using DSF-melting analysis. The negative first derivative plots are shown in Fig. 4a-e. In the absence of enzymes, only one melting peak S corresponding to the denaturation of the m1A-probe substrate was detected. After a 5 minute incubation with ALKBH2, a distinct biphasic melt transition was observed. The new melting peak P corresponds to the dissociation of the demethylated probe, as verified by HPLC analysis (Fig. S5, ESI†). Consistent with our detection strategy, there is a large difference in Tm (ΔTm = 9.9 °C) between the substrate and the product despite only a single methyl group change, presumably due to demethylation-induced hairpin–duplex conversion. This allows a clear resolution of their melting peaks. By comparison, a methyl mark difference between otherwise identical nucleotide sequences usually produces insignificant Tm shifts.13

As the reaction progressed, the product showed a time-dependent increase in Tm. Detailed analysis revealed an excellent linear relationship between the product Tm and ln(product concentration), hence the Tm of the product provides a direct indication of the demethylase activity (Fig. 4h-i). Analytical sensitivity tests showed that DSF can reliably detect a serially diluted product of concentration as small as 100 nM (Fig. S6, ESI†). Enzyme titration studies with the substrate at 10 μM concentration further indicated that activity can be measured at ALKBH2 concentrations as low as 10 nM (Fig. S7, ESI†).

To evaluate the specificity of the m1A-probe, we compared the kinetic parameters for the demethylation of m1A-probe, m1A-ssRNA and m1A-dsRNA by m1A-demethylases (Table 1). The kcat/Km values indicate that the m1A-probe is an excellent substrate for ALKBH2, with efficiency that is comparable to m1A-ssRNA and only slightly lower than m1A-dsRNA (Table 1, Fig. S8 and S9, ESI†). Thus ALKBH2

Fig. 3 Conformational probes investigated in this study. (a) The Tm values were measured at 5 μM strand concentration using DSF-melting analysis. (b) ΔH° and ΔS° were derived from 1/T versus ln(concentration) plots (for duplexes) and from s versus T plots by curve fitting (for hairpins), assuming a two-state process. (c) Probes containing m1A, m3U, m1G and m5dC likely adopt a hairpin structure, as their Tm values are independent of the strand concentration (Fig. S11–S13, ESI†).

Fig. 4 Conformational probe-DSF assay of ALKB demethylase activity. The negative first derivative plots show melting profiles of reaction mixtures (a) in the absence of enzymes, and after incubation with (b–e) ALKBH2, (f) ALKBH3 and (g) AlkB. (h) There is an excellent linear relationship between the product Tm and ln(product concentration). (i) Time course analyses of the m1A-probe (10 μM) incubated with various m1A demethylases (50 nM). (j) Enzyme inhibition curves of 1 against ALKBH2/5 and AlkB, as determined by the DSF-based assay. The IC50 values are comparable to those derived from the HPLC-based assay (in parentheses). Errors represent S.D. of three replicates.
FTO, both of which are specific for m6A with negligible activity when incubated with AlkB subfamily members ALKBH5 and demethylases, as demonstrated by the lack of product formation for m3dC (Fig. 3). Remarkably, the introduction of these modified bases triggered a similar duplex-hairpin conversion, as determined by DSF-melting analyses (Fig. S11–S13, ESI†). This work was funded by the Singapore Ministry of Health’s National Medical Research Council (NMRC/BNIG/2008/2013) and the Singapore Ministry of Education (Start-Up Grant R148-000-168-133).

Notes and references


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