A methylation-switchable conformational probe for 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 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 acid 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 tremendous interest in their mechanistic and inhibition studies.

One major class of DNA/RNA demethylase 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 technique for their analysis. Current limited assays rely primarily on indirect approaches, such as the analysis of demethylation co-products (NADPH, FADH, formaldehyde) through coupled assays, or the analysis of demethylated products following their digestion with 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 give poor reproducibility. Although 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, 5-methylcytosine has been shown to induce B- to Z-transition in some DNAs, while N6-methyadenosine (m6A) could induce duplex-hairpin conversion in certain RNA sequences. Inspired by these interesting observations, we envisaged that (de)methylation-induced conformational change could provide a basis for the design of ‘conformational probe’ useful for sensing 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 assay method which is based on methylation-sensitive probe.

Herein, we describe a novel detection strategy for RNA demethylase activity, which combines the discriminatory power of dynamic conformational probe with the sensitivity of Differential Scanning Fluorimetry (DSF) technique (Fig. 1b). In this approach, a structurally dynamic oligonucleotide that changes secondary structure according to its methylation status is employed as conformational probe. By design, when the probe is methylated, it preferentially adopts a hairpin conformation. However, on selective removal of methylation by a demethylase, the probe undergoes spontaneous and rapid transformation to a duplex conformation. Such a major switch from hairpin to duplex structure is accompanied by profound

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The probe is highly responsive to its methylation status. Removal of the methyl group by RNA demethylase triggers a dramatic switch from hairpin to duplex structure. This causes a profound change in the T_m of the probe, which can be sensitively detected via DSF technique. Insert shows the oxidative demethylation of m^A by the m^A-demethylases.

change in a number of biophysical properties, notably the melting temperature (T_m) 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 interaction in ‘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 change. 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 m^A-containing probe with a view of developing a high-throughput assay against m^A-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(CGCGm^AUAUUCGCG) as our model m^A-probe (Table S1, ESI†). It consists of two terminal ‘CGCG segments’ and a middle ‘AAUU segment’ (underlined), where m^A resides (for chemical synthesis, see ESI†). Due to its self-complementary nature, it can inherently adopt a bimolecular duplex, by engaging in intermolecular base pairing, and a monomolecular hairpin, by folding back on itself (Fig. 2a). We reasoned that since m^A impairs Watson-Crick base pairing, the probe likely favours hairpin conformation, where m^A can be stably localised within the ‘loop’ of the hairpin.

To verify our probe design, we performed DSF-based melting analysis of m^A-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 probe exists predominantly as a single conformation under our experimental conditions (Fig. 2b). Moreover, the T_m of m^A-probe is relatively constant over a 100-fold concentration range (Fig. 2c), suggesting that the probe likely adopts a hairpin conformation, as anticipated. In contrast, the melting profile of its demethylated counterpart r(CGCGAAUUUCGCG) shows a concentration-dependent T_m, which is indicative of a duplex structure (Fig. 2b and 2c). The above results were confirmed by circular dichroism (CD)- and UV-melting analyses, which gave melting profiles that are highly consistent with those derived from DSF analysis (Fig. S1 and S2, ESI†). In addition, the CD spectra of m^A- and demethylated probes clearly reveal characteristics that are consistent with B-like hairpin and A-form duplex, respectively (Fig. 2d).

To obtain additional insights into their conformations, we performed ^1H NMR analysis of the probes. The imino spectrum of m^A-probe revealed only four NH peaks at 4 °C, likely suggesting a hairpin structure in which the stem contains four G-C base pairs, and the loop consists of four unpaired m^A AUU nucleotides (Fig. 2e). The demethylated probe, however, showed six imino peaks, implying a fully base-paired duplex structure (Fig. 2f). We are aware that for more vigorous proof of probe conformations, other methods such as 2D NMR analysis 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 m^A-probe may induce a hairpin-duplex

Fig. 1 (a) N-methylation on DNA/RNA that are selectively demethylated by the AlkB subfamilies (* weak activity). (b) The conformational probe-DSF strategy. The conformational probe is highly responsive to its methylation status. Removal of the methyl group by RNA demethylase triggers a dramatic switch from hairpin to duplex structure. This causes a profound change in the T_m of the probe, which can be sensitively detected via DSF technique. Insert shows the oxidative demethylation of m^A by the m^A-demethylases.

Fig. 2 Verification of m^A-probe design. (a) The m^A- and demethylated-probes were found to exist predominantly as hairpin and duplex conformations, respectively, as demonstrated by their (b) DSF-based melting profiles. (c) van’t Hoff plots, (d) CD spectra, and (e) imino ^1H NMR spectra. Assignment of imino peaks for the demethylated-probe was based on literature data. The terminal G-C base pair was not detectable at 25 °C for both probes, likely due to ‘fraying’.
transformation, consistent with our design strategy. Notably, only one set of NMR signals was observed for m^3A- and demethylated-probes, suggesting that they exist almost exclusively as single conformation in solution and do not co-exist as other secondary structures. The absence of the duplex form of m^1A-probe also implies negligible Hoogsteen base pairing between m^1A and U in this RNA sequence context. This result is interesting because Hoogsteen base pairs have been observed between m^1A and T in DNA duplexes.\(^\text{15}\)

The thermodynamic parameters derived from DSF-melting experiments further revealed that the observed hairpin-duplex conversion is primarily an enthalpy-driven process (\(\Delta\Delta H^\circ = -61.9\) kcal/mol), and despite the large entropy cost (\(\Delta\Delta S^\circ = -163.0\) cal/mol/K), the demethylated duplex is significantly more stable than the m^1A hairpin (\(\Delta\Delta G^\circ_{310} = -11.5\) kcal/mol) (Fig. 3, S3 and S4, ESI\(^\text{†}\)). We appreciate that m^1A can potentially undergo base-catalysed Dimroth rearrangement to yield m^A,\(^\text{16}\) nevertheless, in our DSF analyses, the \(T_m\) of m^1A-probe was highly reproducible, suggesting little or no degradation under our experimental conditions. Our combined results, therefore, established m^1A-probe as a stable, methylation-responsive probe suitable for reporting demethylation activity.

We next examine if m^1A-probe could be recognised and demethylated by ALKBH2. In a typical conformational probe-DSF assay, the enzyme (50 nM) was incubated with m^1A-probe (10 \(\mu\)M in the presence of co-substrate 2-oxoglutarate (10 \(\mu\)M), Fe(II) (10 \(\mu\)M), and L-ascorbate (200 \(\mu\)M) in 50 mM HEPES buffer (pH 7.0) at 4 °C for various time intervals, after which the reaction was quenched and product formation was analysed using DSF-melting analysis. The negative first derivative plots are shown in Fig. 4a-e. In the absence of enzyme, only one melting peak \(S\) corresponding to the demethylated m^1A-probe substrate was detected. However, 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-based analysis (Fig. S5, ESI\(^\text{†}\)). Consistent with our detection strategy, there was a large difference in \(T_m\) (\(\Delta T_m = 9.9^\circ\)C) between substrate and product despite only a single methyl group change, presumably due to demethylation-induced hairpin-duplex conversion. This allows a resolution of their melting peaks. By comparison, a methyl mark difference between otherwise identical nucleotide sequences usually produces insignificant \(T_m\) shifts.\(^\text{13}\)

As the reaction progressed, the product showed a time-dependent increase in \(T_m\). Detailed analysis revealed an excellent linear relationship between product \(T_m\) and ln(product concentration), hence, the \(T_m\) of product provides a direct indication of demethylase activity (Fig. 4h-i). Analytical sensitivity tests further showed that DSF has the sensitivity to reliably detect serially diluted product as little as 100 nM (Fig. S6, ESI\(^\text{†}\)). In addition, enzyme titration studies with substrate at 10 \(\mu\)M indicated that activity can be measured at ALKBH2 concentrations as low as 10 nM (Fig. S7, ESI\(^\text{†}\)).

To evaluate the affinity and specificity of m^1A-probe towards the m^1A-demethylases, we determined the kinetic parameters for demethylation of m^1A-probe, m^1A-ssRNA and m^1A-dsRNA by ALKBH2, ALKBH3 and AlkB (Table 1). A comparison of the \(K_{cat}/K_m\) values reveals that m^1A-probe is an excellent substrate for ALKBH2, with efficiency that is comparable to that of m^1A-ssRNA and only slightly lower than m^1A-dsRNA (Table 1, Fig. S8 and S9, ESI\(^\text{†}\)). This result suggests that ALKBH2 does not exhibit strict conformational requirement for substrate recognition. Intriguingly, m^1A-probe can also be efficiently demethylated by ALKBH3 and AlkB, despite their strong preference for single-
stranded substrates (Fig. 4f and Table 1). In fact, ALKBH3 and ALKB exhibit ~5-fold greater activities for m^1A-probe than for m^1A-dsDNA. To our knowledge, this is the first report demonstrating that m^1A-demethylases, in general, are able to recognise and accept substrates with hairpin structures. Importantly, m^1A-probe also displayed remarkable selectivity for m^1A demethylases, as demonstrated by the lack of product formation when incubated with AlkB subfamily members ALKBH5 and FTO, both of which are specific for m^5C (14, 15). Furthermore, our assay displayed remarkable selectivity for m^5C, demonstrating that m^5C exhibit ~5-fold greater activities for m^5C-AlkB than for m^5C-Alf (a) E. N. Nikolova, E. Kim, A. A. Wise, P. J. O'Brien, I. J. Wilkinson, C. M. Rodriokus Lopez, B. Guzman Asenjo, A. J. Lloyd and M. Fischer, L. Koch, C. Emmerling, J. Vierkotten, T. Peters, J.C. Bruning, U. Ruther, Nature, 2009, 458, 894; (c) M. Camp, B.F. Eichmann, Mol Cell, 2011, 44, 343.


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