Effect of 5-hydroxymethylcytosine Modifications on Stability of VEGF i-Motifs

by
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A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

Oxford
May 2016

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ACKNOWLEDGEMENTS

I would like to thank the Sally McDonnell Barksdale Honors College for encouraging undergraduate research and giving me the opportunity to publish this thesis.

Thank you to my advisor, Dr. Randy Wadkins, for allowing me to work in your laboratory for the past two years and for all of your help along the way.

Much thanks to Samantha Reilly for showing me the ropes in the laboratory and answering my numerous questions.

Lastly, thank you to my second and third readers, Dr. Tracy Brooks and Dr. Bradley Jones, for putting in the time and effort to help me complete my thesis.
Abstract

DNA i-motifs are a secondary structure of DNA formed at low pH by internal folding of strands rich in cytosine. This study focuses on trying to determine if the DNA from the vascular endothelial growth factor (VEGF) promoter, which can form an i-motif, can be stabilized with the epigenetic modification to 5-hydroxymethylcytosine in four separate locations on the DNA strand, and to what extent the degree of stabilization depends on the location of the modification. Stability of the i-motif was determined by measuring pK$_a$ and melting point ($T_m$) of each VEGF modification using circular dichroism spectroscopy. The modified samples showed an insignificant change in pK$_a$ values but a significant increase in $T_m$ when compared to the wild type, indicating stabilization by epigenetic modification. The ability to stabilize i-motifs has potential for using i-motif DNA in new drug delivery methods.
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Introduction:

DNA can exist as secondary structures aside from the normally depicted Watson and Crick double helix. In single stranded cytosine rich strands, DNA can internally fold to form i-motifs (iMs). iMs are four stranded 3D structures that form at low pH due to the stabilization of the molecule by hydrogen bonding of two hemi-protonated cytosine bases (1) (Figure 1). Because of the need for a proton, iMs usually form at acidic pH. At high and neutral pH, the iM will exist in an unfolded state. The single stranded DNA found to form intramolecular iMs is the result of negative supercoiling of typical Watson and Crick DNA, which unwinds the double stranded helix (2). While this study deals with single stranded sequences, iMs can also form intermolecularly; a study by Li et al. demonstrates the possibility of bimolecular iM formation due to bonds betweens two C-rich strands (3). With the discovery of iMs, there have been ongoing studies of their possible biological role.

Recent studies have shown that the addition of molecular crowding agents and epigenetic modifications stabilizes iMs, evident by an increase in pKₐ and melting point, which in turn allows them to form at a pH closer to neutral (4). Because of the potential occurrence of iMs in vivo and their biocompatibility, interest has grown in the role iMs could play in drug delivery, drug targeting, and gene regulation (5-8). For example, a study by Keum et al. demonstrates how important iMs could be to drug delivery; DNA pyramids, composed of four iMs,
were found to form the iM structure under acidic conditions and unfold during alkaline conditions, resulting in pH-responsive cargo holders (8). Keum et al. bound a fluorescent protein to the DNA pyramid, which was then released after placement in an acidic solution (8). Tumors often produce an acidic environment, so drug release directly near the tumor would prove to be groundbreaking. Further studies on pH-dependence and the release of cargo by iMs could allow the structures to be used in a wide variety of environments.

The DNA used in this study is a single-stranded DNA sequence from the promoter of the vascular endothelial growth factor (VEGF) protein. VEGF is often found upregulated in tumors and plays a role in angiogenesis. A 5-hydroxymethylated cytosine (5mhC) modification was applied to the ssDNA. 5mhC occurs at CpG islands in the body due to the oxidation of 5-methylcytosine by TET enzymes (7). While 5mC has been studied more extensively and is known to act as a transcriptional repressor, the function of 5mhC is unknown in iMs.

**FIGURE 1:** A. Cytosine-Cytosine base pairing showing the reliance of the bond on the presence of a proton. B. Molecular model of a VEGF i-motif. Cytosines are shown in yellow.
FIGURE 2: Sequences of all samples, with the placement of the 5hmC modification shown in red.

However, due to its presence in stem cells and the effect it has on pluripotency, 5mhC is thought to play a role in gene regulation and expression (9). Previous studies from our lab have shown that a single 5mhC modification on the c-myc gene promoter can cause a decrease in the stability of iMs (4). However, in the study presented here, the modifications will occur at four different points on a strand, testing the importance of modification placement. Four 5hmC modifications were performed; a wild type VEGF was also studied for comparison (Figure 2). Our hypothesis was that the addition of a 5hmC epigenetic modification would potentially result in a conformational change in the iM. The addition of the –OH group would lead to an effect on transcription and ultimately gene regulation.
Methods:

In order to measure $pK_a$, each modified DNA stock solution was first diluted to 1 mM in pH 8.0, 10 mM TE Buffer. The wild type was diluted to 0.1 mM. A calculated amount of DNA solution was placed in 0.03 M Cacodylate buffer at each of the respective pHs in order to form a sample of 2 µM DNA in each cuvette. The pH was measured in 0.3 unit increments from 5.0 – 8.1. Before absorption was measured on a Cary 100 UV Visible spectrophotometer, all samples were heated to approximately 80 °C on a heating block. All samples displayed absorbance levels falling between 0.5 – 0.6, demonstrated by a peak at 260 nm. Each sample was then scanned at room temperature using circular dichroism (CD) with an Olis DSM-20 spectrophotometer over a range of 225 – 350 nm. An overall CD scan was also performed with all samples at this time to ensure the presence of i-motifs. $pK_a$ was determined by data analysis of the difference in ellipticity at 285 and 267 nm and a curve fit model to determine $pK_a$ with KaleidaGraph software. Baseline runs were performed using only buffer before each sample was scanned.

Samples for thermal melts were prepared in the same manner as described above. However, all samples were placed in only pH 5.4 buffer. Melting temperatures ($T_m$) were measured on the Olis DSM-20 using wavelengths of 320 nm and 298 nm. The temperature range began at 20 °C and ended at 90 °C, with measurements taken at each 1 °C increment. A 30 second
hold at each temperature took place before data was recorded. $T_m$ was determined by data analysis and using a custom equation with KaleidaGraph software. All scans were completed in triplicate.

Results:

**FIGURE 3**: CD scan of unmodified iM, showing transition from folded to unfolded state based on pH.
FIGURE 4: CD scan of Mod 1, showing transition from folded to unfolded state based on pH.
FIGURE 5: CD scan of Mod 2, showing transition from folded to unfolded state based on pH.
FIGURE 6: CD scan of Mod 3, showing transition from folded to unfolded state based on pH.
FIGURE 7: CD scan of Mod 4, showing transition from folded to unfolded state based on pH.
**FIGURE 8**: Graphical representation of all samples, using a curve fit to find $pK_a$. 
Discussion:

In comparison to the wild type VEGF, the modified iMs demonstrated an increase in stability. CD scans confirmed if the DNA was in the folded or denatured state at each of the pHs tested. Based on Figure 3 - Figure 7, a shift from iM to single stranded DNA due to loss of a proton can be seen to occur somewhere between a pH of 6.0 and 6.1 for modified iMs and closer to 6.0 for

**Table 1:** pK$_a$ values for all iM samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>pK$_a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmod</td>
<td>6.05 ± 0.06</td>
</tr>
<tr>
<td>Mod 1</td>
<td>6.07 ± 0.04</td>
</tr>
<tr>
<td>Mod 2</td>
<td>6.13 ± 0.06</td>
</tr>
<tr>
<td>Mod 3</td>
<td>6.13 ± 0.04</td>
</tr>
<tr>
<td>Mod 4</td>
<td>6.09 ± 0.03</td>
</tr>
</tbody>
</table>

**Table 2:** Average T$_m$ values for all iM samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>T$_m$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmod</td>
<td>42.23 ± 3.49</td>
</tr>
<tr>
<td>Mod 1</td>
<td>56.40 ± 1.11</td>
</tr>
<tr>
<td>Mod 2</td>
<td>52.09 ± 10.04</td>
</tr>
<tr>
<td>Mod 3</td>
<td>54.63 ± 11.33</td>
</tr>
<tr>
<td>Mod 4</td>
<td>58.60 ± 8.55</td>
</tr>
</tbody>
</table>
the unmod. A peak around 290 nm as well as a horizontal shift of the curve is indicative of DNA in a folded confirmation.

\( pK_a \) analysis revealed a slight, but insignificant shift toward physiological pH for the modified iMs (Table 1, Figure 8). Mods 2 and 3 demonstrated the largest shift in comparison to the wild type \( pK_a \) of 6.05, with an increase in 0.08 units resulting in a \( pK_a \) of 6.13. These 5hmC modifications are located in the central loop region of the iM, which perhaps contributes more to the pH dependence of the molecule as a whole than the cytosines in the outer loops. Mods 1 and 4, located in the outer loops, also exhibited an increase in \( pK_a \) of 0.02 and 0.04 units, respectively. These results are consistent with what was seen on the CD graphs. The insignificant increase of \( pK_a \) leads us to believe that 5hmC is not a factor in \( pK_a \) dependence of iM formation.

\( T_m \) is a measurement of the temperature at which DNA unfolds from the iM structure to the single stranded structure or vice versa. The \( T_m \) of the wild type sample was found to be \( \sim 42 \, ^\circ C \), meaning that at temperatures above 42°C only 50% of the DNA is in the iM form. An increase in \( T_m \) would imply an increase in stability and temperature/energy needed to melt or unfold the iM and a change in the temperature-dependence of the iM, which was indeed the case. All mods demonstrated notably higher \( T_m \) values (Table 2). Mods positioned on the outer loops (mods 1 & 4) exhibited the highest increase in \( T_m \), with values of 56.4 and 58.6 \( ^\circ C \), respectively. Mods 2 & 3 also confirmed an increased stability, with increased \( T_m \) values of 52.09 and 54.63, respectively. The amount of error could
be attributed to sample contamination or DNA disintegration. However, samples were run upwards of 5 times to ensure accuracy.

The increased stability of iMs with the addition of the 5mhC modification indicates that the addition of 5hmC is a factor in stabilizing the iM in terms of temperature, while there was no evidence of an effect on pK_a. The increase in T_m indicates that 5hmC could play a role in gene expression. Adding additional modifications such as crowding agents could lead to even more stability and a pK_a consistent with physiological pH. Studies from our lab have shown that the addition of PEG-300 to iMs results in higher T_m and pK_a values near physiological pH (4). Future studies pointed in this direction could uncover the means necessary to allow iMs to act as drug delivery vehicles. Whereas previous studies have focused on the formation of iMs at acidic conditions to release proteins (8), the ability of iMs to also release or transport materials at neutral pH would allow for iMs to be used in numerous environments throughout the body. Having control over the pH-dependence of iM folding in the future may be vital to carrying drugs to specific tissue locations.
References:


