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Nano-graphene oxide as a novel platform for monitoring the effect of LNA modification in nucleic acid interactions

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Abstract

Graphene oxide has gained significant attention due to its exceptional physical properties at biological interfaces. It has extraordinary quenching, fast adsorption and desorption properties that are suitable for detection of molecular interactions in nucleic acids. Here we studied the interaction between locked nucleic acid (LNA) modified oligonucleotides and its complementary miR-10b DNA analog. We demonstrate that LNA modification does not alter the hybridization yield, despite a slight difference in the rate, however it does increase the duplex stability dramatically. The noncovalent nucleic acid-graphene oxide complex maintained stability between 25 and 90 °C in the absence of an oligonucleotide-induced desorption. The melting temperatures of duplexes with or without LNA base modification were determined due to remarkable fluorescence quenching and fast oligonucleotide adsorption with graphene oxide. The difference in melting temperatures was used to control the release of surface adsorbed nucleic acids at 70 °C. Finally, a mutation in the oligonucleotide sequence is detected by the complementary oligonucleotides on graphene oxide surface. Due to its extraordinary physical properties, graphene oxide represents a remarkable platform for studying nucleic acid interactions and serves as a promising material for biomedical applications.

1. Introduction

Bionanotechnology is emerging as the construction of novel nanomaterials with fascinating roles at biological interfaces increases.¹⁻³ Recently graphene oxide (GO) has gained significant attention due to its exceptional physical properties. Graphene oxide is a one-atom-thick, two-dimensional carbon structure with remarkable surface area and extraordinary electronic, mechanical and thermal properties.^{4, 5} It has emerged as an excellent platform for tracing numerous biological events due to its attractive physical properties at the bio-nano/micro interfaces.⁶ Graphene oxide has a remarkable DNA adsorption capacity due to aromatic stacking, hydrogen bonding, hydrophobic interactions, and van der Waals forces.⁷⁻¹⁰ Unlike gold nanoparticles, graphene oxide can release the surface-adsorbed DNA in the presence of complementary oligonucleotides¹¹⁻¹³ or DNA binding target molecules.¹⁴ This reversible interaction has been an attractive tool for designing graphene oxide based biosensors,¹² biomedical imaging tools and gene delivery vectors.¹⁵⁻¹⁷ Moreover, graphene oxide has incredibly fast and efficient fluorescence quenching capability. For example, a fluorophore labeled DNA can be remarkably quenched while forming a GO-DNA noncovalent assembly.⁶ This has been used as an analytical tool for nucleic acid detection or aptasensor design using fluorescence techniques.^{13, 18-30} Recently, exploring the use of graphene oxide for miRNA imaging has gained significant attention due to its advantageous properties *in vivo* environment.³¹

MicroRNAs (miRNA) are endogenously expressed small non-coding RNA molecules (~22 nt) which regulate post-transcriptional gene expression by binding numerous target mRNAs.³² Due to their dysregulation at many stages of cancer and critical role in the disease progression, they are considered as tumor biomarkers or therapeutic targets.³³⁻³⁵ Targeting, silencing or imaging miRNA molecules have been considered promising methods to understand and fight cancer at the molecular level. There have been several synthetically modified oligonucleotides designed for targeting miRNAs, among which Locked Nucleic Acids (LNAs) have shown remarkable results.³⁶⁻³⁸

LNAs are a new class of synthetic oligonucleotides where the 2' oxygen in the modified nucleobase is bridged to 4' carbon, leading to new extraordinary properties. LNAs exhibit excellent binding affinity to target oligonucleotides and have improved duplex stability by increasing melting temperatures dramatically.^{37, 39} Due to these properties, LNAs have been used

in oncomiR – a miRNA linked to cancer - imaging and knock down.^{35,38} miR-10b is one of these oncomiRs which is overexpressed in the metastatic stage of breast, lung, colon and bladder cancer.⁴⁰ Targeting miR-10b with LNA has shown effective therapeutic results for inhibiting breast cancer metastasis.^{38,41}

Here we used graphene oxide to investigate the interaction between an LNA modified antisense oligonucleotide and DNA analog of its target oncomiR (miR-10b). We demonstrate that graphene oxide serves as a firm nano-platform for nucleic acids with or without base modifications while providing kinetic and spectroscopic information about their interaction. Moreover, we were able to validate the stability of the GO-DNA noncovalent assembly at different temperatures. We believe that owing to the extraordinary quenching, fast adsorption and desorption properties; graphene oxide represents a remarkable template for studying nucleic acid interactions and serves as a promising material for biomedical applications.

2. Experimental

Materials and reagents

All DNA sequences were purchased from Integrated DNA Technologies (IDT), USA with the following sequence information and modifications, (1) FITC-labeled miR-10b DNA 5′-/56-FAM/TAC CCT GTA GAA CCG AAT TTG TG-3′, (2) The anti-miR-10b DNA 5′-CAC AAA TTC GGT TCT ACA GGG TA-3′, (3) non-complementary DNA 5′- TAC CCT GTA GAA CCG AAT TTG TG -3′ (4) FITC-labeled mutated DNA with one-base mismatch (M1) 5′-/56-FAM/TAC CCT CTA GAA CCG AAT TTG TG-3′. The kdhsa-miR-10b LNA (anti-miR-10b LNA, unique batch number 600911) was purchased from Exiqon, MA, USA.

Carboxyl graphene water dispersion was purchased from ACS Material, Medford, MA 02155, USA and sonicated 12 hours before use. All other reagents were purchased from Sigma-Aldrich, St. Louis, MO 63103, USA and used without further purification. Double distilled water was used in preparation of all solutions.

Methods

Adsorption and desorption of DNA molecules on graphene oxide

The FITC labeled miR-10b DNA (100 nM) was dissolved in 25 mM HEPES (pH 7.5), 100 mM NaCl and 1 mM MgCl₂. The fluorescence emission value at this concentration was determined to be approximately 3.0×10^7 . The Fluorescence measurements (Ex. 495 nm, Em. 518 nm) were performed using the Fluorolog-3 spectrofluorometer (Horiba Jobin-Yvon, Inc.)

equipped with a temperature controller and its software. The fluorescence readings were recorded over 90 minutes with one reading per three seconds resolution. At 500-seconds time point, 10 $\mu\text{g/mL}$ of GO was added into the DNA solution in order to induce adsorption. At 1000-seconds time point 150 nM of complementary or non-complementary oligonucleotides were added into the graphene oxide-DNA complex in the spectrophotometer reaction cell. The fluorescence recovery was recorded thereafter. The same experimental parameters were used for performing desorption of the mutated oligonucleotides. FITC labeled DNA molecules were prepared by dilution in reaction buffer from 1 mM stock solution immediately before each experiment. The concentration dependent studies were carried out with 200, 100, 50 and 10 nM of anti-miR-10b DNA or LNA. All experiments were performed in triplicate.

Temperature-dependent DNA desorption on graphene oxide

To study desorption of FITC-labeled DNA (20 nM) on GO (5 $\mu\text{g/mL}$) surface with temperature, a typical desorption kinetics study was performed between 25 and 90 $^{\circ}\text{C}$ with 1 $^{\circ}\text{C}$ intervals. In this study, 20 nM FITC-labeled miR-10b DNA was first quenched with 5 $\mu\text{g/mL}$ of GO. The temperature was increased to 90 $^{\circ}\text{C}$ while recording to fluorescence at every 1 $^{\circ}\text{C}$.

Monitoring duplex denaturation with graphene oxide

For melting temperature studies, pre-adsorbed FITC-labeled miR-10b DNA on graphene oxide was incubated with its complementary DNA or LNA oligonucleotide in a 1:1.5 ratio until the hybridization was complete. This sample was used to observe the duplex denaturation by monitoring change in fluorescence with increase in temperature. Briefly, 20 nM FITC-labeled miR-10b DNA was quenched by 5 $\mu\text{g/mL}$ of GO. Then, 30 nM of complementary anti-miR-10b DNA or LNA molecules was added into the reaction cell. Fluorescence was monitored until the hybridization curve plateaued. These samples were then used for monitoring the melting temperature of duplexes between 45 and 90 $^{\circ}\text{C}$. A sharp decrease in fluorescence was observed at corresponding melting temperatures of duplexes.

For determining the possible re-hybridization scenario after denatured strands were adsorbed on graphene oxide, a reverse temperature study from 90 to 45 $^{\circ}\text{C}$ was performed. Here the samples were taken from the melting temperature study where both complementary strands were adsorbed on graphene oxide. The re-hybridization efficiency was determined by monitoring the fluorescence while decreasing temperature 90 to 45 $^{\circ}$ with 1 $^{\circ}\text{C}$ intervals.

LNA modification induced DNA desorption at elevated temperatures

For this study temperature was set to 65, 70 or 75 °C. Similarly, 100 nM FITC labeled miR-10b DNA was first adsorbed on 10 µg/mL GO. 150 nM of complementary anti-miR-10b DNA or LNA was added into the reaction medium at 70 °C. The fluorescence readings were recorded at this temperature for 90 minutes to observe the LNA or DNA induced release at this temperature.

3. Results and Discussion

Here we investigated the adsorption, desorption and denaturation rate of miR-10b DNA analog on graphene oxide (GO) using complementary DNA (anti-miR-10b DNA) or LNA (anti-miR-10b LNA) molecules, Fig. 1a. A mutated oligonucleotide with a single base-mismatch was used to determine the specificity of the LNA or DNA oligonucleotides to target sequence. We have used graphene oxide as a fluorescence quencher and a nano-platform for adsorbing single stranded DNAs. Graphene oxide presented a remarkable template for studying the DNA and LNA interactions at various temperatures.

Briefly, fluorescently labeled miR-10b DNA analog was adsorbed on GO. This complex was used as our starting template for comparing LNA and DNA induced release from GO surface. Initially the adsorption affinity of FITC-labeled miR-10b DNA analog on GO surface was studied using fluorescence spectroscopy. The fluorescence of FITC labeled miR-10b DNA was measured for 500 seconds at room temperature. As seen in Fig. 1b, DNA exhibits a steady fluorescence without any major fluctuation at this condition. Then GO was added into the DNA solution, which resulted in an immediate quenching in fluorescence due to adsorption. The resulting GO-DNA complex exhibited steady fluorescence, which indicates the stability of the complex at this condition. Next, anti-miR-10b DNA analog was added into the complex at 1000-seconds data point in order to study the hybridization with the complementary DNA strand. As seen in Fig 1b, an immediate sharp fluorescence increase (blue curve) was observed, which plateaued over time. We then examined the desorption and hybridization with anti-miR-10b LNA. Similarly, addition of LNA molecules induced an increase in fluorescence, however the rate of the increase was slightly slower with LNA (red curve). Moreover, DNA induced-hybridization saturates faster than LNA which must be due to the faster rate of DNA:DNA duplex formation. We have also used a non-complementary DNA sequence to see nonspecific desorption of miR-10b DNA on the surface. The fluorescence increase with the non-complementary DNA was significantly lower (red curve) than full complementary, which is due

to displacement of the adsorbed molecules.¹¹ The overall recovery of fluorescence with DNA (52%) and LNA (47%) at 5000 seconds suggests that their interaction with complementary miR-10b DNA results in similar duplex yield despite the difference in the hybridization rate (Fig. S1). The ~15% fluorescence recovery is attributed to nonspecific displacement by added oligonucleotides as suggested by the non-complementary control experiment. A concentration-dependent release was performed with anti-miR-10b DNA or LNA to demonstrate the sensitivity of the system. We observed fluorescence recovery in the presence of as low as 10 nM of complementary oligonucleotides with or without LNA modifications, Fig S2.

Next we visualized the denaturation of DNA:LNA and DNA:DNA duplexes using graphene oxide. Graphene oxide is an ideal platform for melting temperature studies because it adsorbs single stranded oligos (1) but not double stranded oligos⁴² and (2) quenches the fluorescence so fast that this process can be monitored in real time with fluorescence measurements. These attractive properties were used for studying the duplex melting with GO. Before performing the melting temperature studies, the temperature-dependent desorption of the single stranded miR-10b DNA was studied in order to confirm that the stability of DNA-GO complex over the projected temperature range. The FITC labeled miR-10b DNA was first adsorbed on GO surface at room temperature as demonstrated by the scheme in Fig 2. The fluorescence is quenched immediately after GO addition at room temperature. The fluorescence was measured at RT for 30 minutes and no change was observed. Then the fluorescence readings were recorded for every 1 °C increase between 25 and 90 °C. During this time course no significant change in the fluorescence intensity was observed, which suggests that the DNA-GO complex is stable between these two temperatures and suitable for performing melting temperature studies, Fig 2.

After determining the stability of the GO-DNA complex with heating we employed the melting study between FITC-labeled miR-10b DNA and complementary anti-miR-10b DNA (DNA:DNA) or LNA (DNA:LNA), respectively as schematically described in Fig. 3a. Heating the duplexes in the presence of GO showed a sharp decrease in the fluorescence around the melting temperatures of both duplexes (Fig. 3b). This decrease was attributed to adsorption of melted stands on GO surface. As expected the DNA:DNA duplex denatured at ~57 °C while the DNA:LNA duplex melted ~72 °C. The results were also compared with the melting curves of duplexes, which were obtained using standard UV-Vis spectroscopy method without GO, Fig S3. We observed ~5 °C decrease in melting temperatures of both duplexes in the presence GO. Our

results agree with the fact that LNA modifications in oligonucleotide strands increase the melting temperature significantly. Overall result suggests graphene oxide can be used to determine the duplex stability between DNA: DNA or LNA:DNA and is an ideal nano-platform for monitoring nucleic acid interactions in real time with fluorescence spectroscopy.

Next the re-hybridization of denatured and GO-adsorbed strands was observed by gradually decreasing the temperature from 90 to 45 °C. The goal was to see if DNA or LNA molecules (1) were stably adsorbed on GO and (2) would desorb with the decrease in temperature and re-hybridize with the FITC labeled complementary DNA strands. Both data (red and blue curves) in Fig. 4 showed no increase in fluorescence with temperature decrease, which suggested that both the DNA and LNAs are stably adsorbed on the GO surface after melting and temperature decrease does not result in any significant re-hybridization.

Later, the difference in melting temperatures was used to control the release of GO-adsorbed FITC labeled miR-10b DNA molecules. These experiments were carried out at 70 °C which is below the melting temperature of DNA:LNA however above DNA:DNA duplexes. This temperature was chosen in order to monitor the hybridization induced desorption with LNA or DNA. Addition of anti-miR-10b LNA (red line) into the GO-DNA complex showed significantly higher fluorescence recovery than anti-miR-10b DNA (blue line), Fig. 5 and S4. This difference in recovery is due to higher stability of the DNA:LNA duplex at 70 °C compared to the DNA:DNA duplex.

The recovered fluorescence in both systems slowly decreased over time. This was observed because at 70 °C, the duplexes are not as stable as they are at room temperature. At this dynamic state, the duplexes tend to hybridize and de-hybridize quickly due to equilibrium between double and single stranded states. However the presence of GO in the system adsorbs denatured single strands immediately and therefore constantly shifts the equilibrium towards single stranded state. This was observed as a decrease of the fluorescence after the maximum recovery was observed, Fig 5. The experiments were also performed at 65 and 75 °C, Fig S5. Similar response was observed at 65 °C, however at 75 °C the amplitude of fluorescence recovery was lesser without a major difference between LNA and DNA induced release. This observation was attributed to unstable LNA or DNA duplexes at 75 °C.

Finally, the specificity of DNA or LNA molecules was studied with GO using a mutated oligonucleotide with a single base mismatch. To do this we introduced a mutation in the middle

of the sequence, replacing a “G” with a “C”. The fluorescently labeled full complementary and mutated DNA molecules adsorbed on the graphene oxide surface with the same affinity, Fig 6a and b. After the adsorption was observed anti-miRNA DNA or LNA molecules were added into the complex to monitor the hybridization. Fig 6a indicates that full complementary anti-miR-10b DNA induced a fast release from the graphene oxide surface, which plateaued shortly after half an hour. The total fluorescence recovery was ~50% at the end of the 90 minute study, Fig. S6a. The mutated sequence induced a similarly fast release from the graphene oxide surface and plateaued. However the extent of the release was significantly less at 38%. The noncomplementary sequence was used as a control to show displacement-induced release from the surface.

The anti-miR10b LNA-induced release was also monitored in similar fashion to understand the specificity of LNA towards to the full complementarity and a mutation. Fig 6b shows that the anti-miR-10b LNA induced a significant release of adsorbed DNA molecules on graphene oxide surface. The mutated sequence similarly induced a release from the surface with lesser extent, Fig S6b. The total release at the end of the 90 minute study showed ~48% with full complementary and ~35% with single mutation. Data indicates that it was possible to distinguish miR-10b DNA analog from a mutated sequence with a single mismatch using either DNA or LNA.

Overall DNA and LNA showed similar hybridization-induced release on the GO surface, however unlike DNA, the rate of the fluorescence recovery with LNA is slightly slower but continuous, Fig 6. This same trend was observed with full complementary and the mutated sequence.

4. Conclusion

In conclusion, we demonstrate that GO serves as a superb nano-platform for monitoring DNA:LNA and DNA:DNA interactions, which have essential implications in miRNA imaging and therapeutic technologies. We were able to observe the similarities and differences in LNA and DNA hybridization kinetics using GO’s rapid quenching and adsorbing properties. The stability of the DNA-GO complex at a wide range of temperatures suggests that it is an attractive nanomaterial for monitoring hybridization and de-hybridization of oligonucleotides. It further implies that GO could serve as a suitable template for designing a rapid real-time oligonucleotide-based biosensor at climates with extreme temperatures. We were able to

determine approximate melting temperatures of duplexes using nano-sized GO, which suggests that even though GO is much larger than a small molecule quencher, it provides significant information about the melting temperatures of duplexes with or without LNA base modifications.

Moreover, GO possess the advantages of serving as a platform for numerous biomedical and environmental applications while providing kinetic and spectroscopic information about these biological events.

Furthermore, we demonstrated that once the melted single strands were adsorbed on the GO, they do not re-hybridize even though the temperature drops below the melting temperatures. Finally, it was possible to determine a single base mismatch using sequences with or without LNA modification. Due to the extraordinary quenching, fast adsorption and desorption properties, GO represents a remarkable platform for monitoring biological events and could serve as a promising material for biomedical applications.

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Figure Legends:

Fig. 1 Adsorption of FITC-labeled miR-10b DNA on graphene oxide and its desorption with complementary oligonucleotides with or without LNA modification. (a) A schematic representation of adsorption and desorption events. (b) Adsorption of FITC-labeled DNA was observed by a decrease in fluorescence, while release was observed as an increase with addition of anti-miR-10b DNA (blue curve), LNA (red curve), and non-complementary DNA (green curve). Experiments were performed in triplicate.

Fig. 2 Thermal desorption of FITC-labeled miR-10b DNA between 25 and 90 °C after adsorption on the graphene oxide surface at RT, ($n=3$). No significant desorption is observed.

Fig. 3 Real time denaturation of DNA:DNA and DNA:LNA duplexes using graphene oxide (a) Schematic description of the methodology, (b) The melting curves of miR-10b and anti-miR-10b DNA (red curve) or LNA (blue curve) duplexes were observed by fluorescence measurements at 518 nm, ($n=3$). Higher melting temperatures were observed in LNA incorporated oligonucleotides.

Fig. 4 Desorption and re-hybridization of melted and adsorbed oligonucleotide strands on GO between 90 and 45 °C. FITC labeled miR-10b DNA and anti-miR-10b DNA (red curve) or LNA (blue curve) shows no desorption and re-hybridization upon cooling to temperatures below melting temperatures, ($n=3$).

Fig. 5 Complementary oligonucleotide with or without LNA modification induced desorption of FITC labeled miR-10b DNA at 70 °C, ($n=3$). Oligonucleotides with LNA base modification induce greater release of complementary miR-10b DNA on graphene oxide due to higher duplex stability.

Fig. 6 Adsorption and desorption of FITC-labeled miR-10b DNA and a mutated oligonucleotide with a single base mismatch on graphene oxide using complementary oligonucleotides with or without LNA modification. Graphene oxide can distinguish one-base mismatch in DNA:DNA and LNA:DNA interaction. Adsorption and hybridization-induced desorption of full complementary and single mismatch oligos on graphene oxide is monitored with (a) anti-miR-10b DNA and (b) anti-miR-10b LNA. Experiments were performed in triplicate.

Figures:

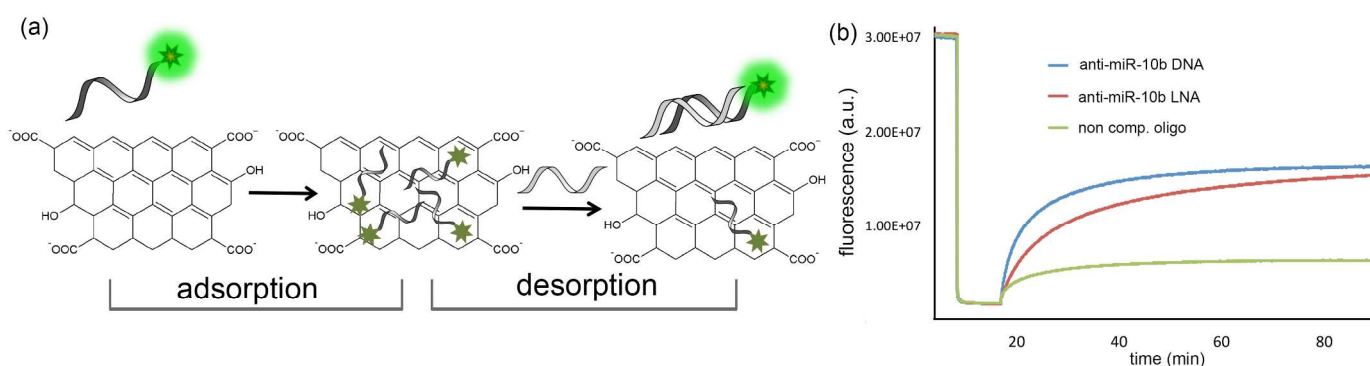


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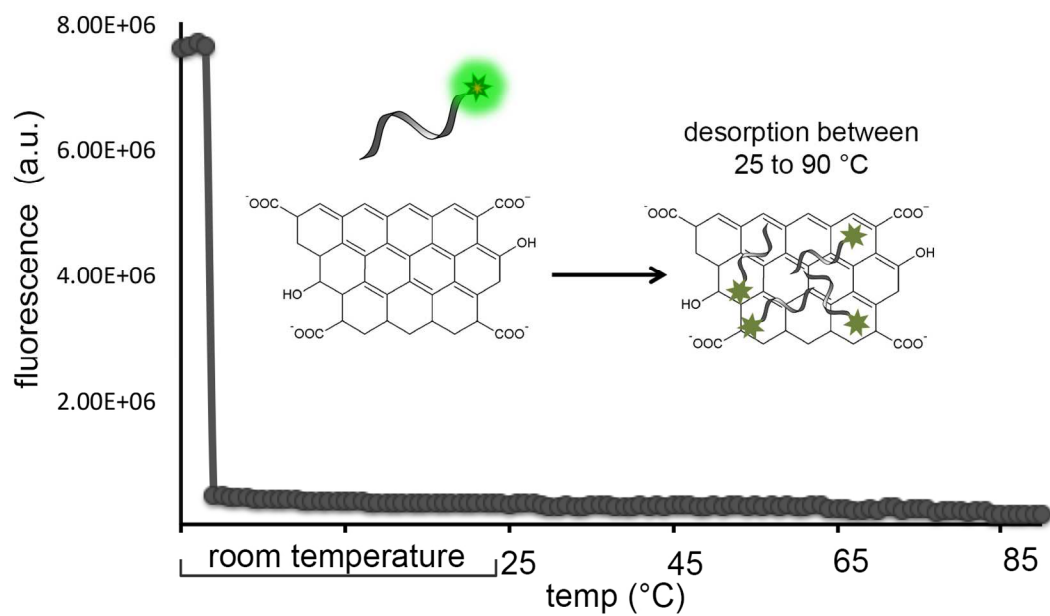


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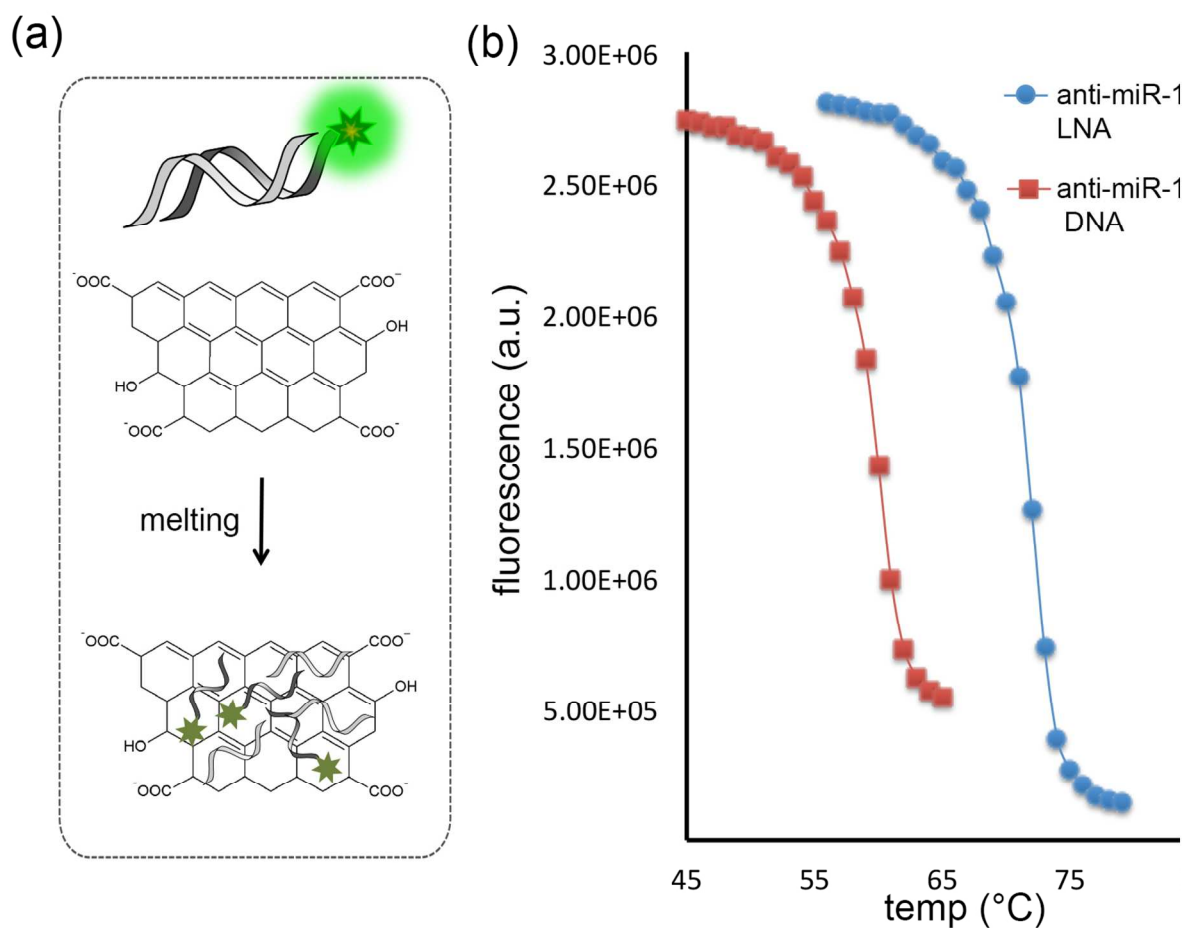


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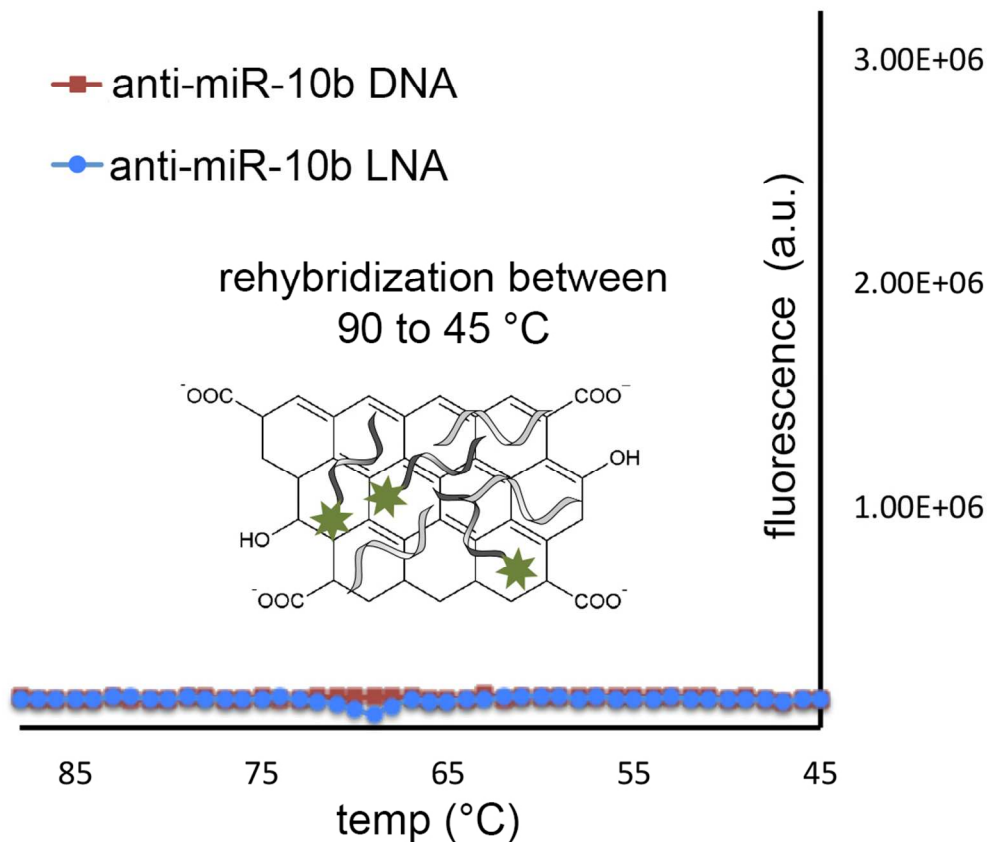


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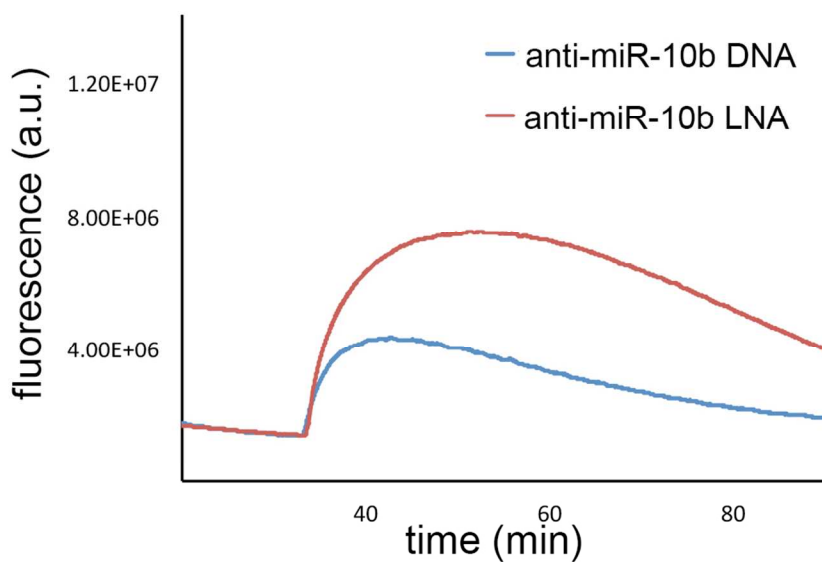


Fig. 5 Complementary oligonucleotide with or without LNA modification induced desorption of FITC labeled miR-10b DNA at 70 °C, ($n=3$). Oligonucleotides with LNA base modification induce greater release of complementary miR-10b DNA on graphene oxide due to higher duplex stability.

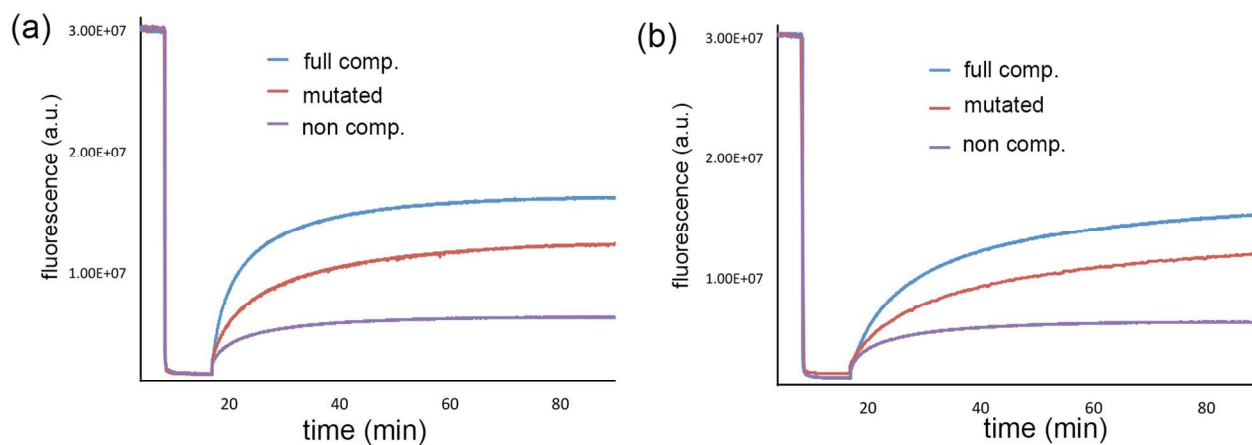
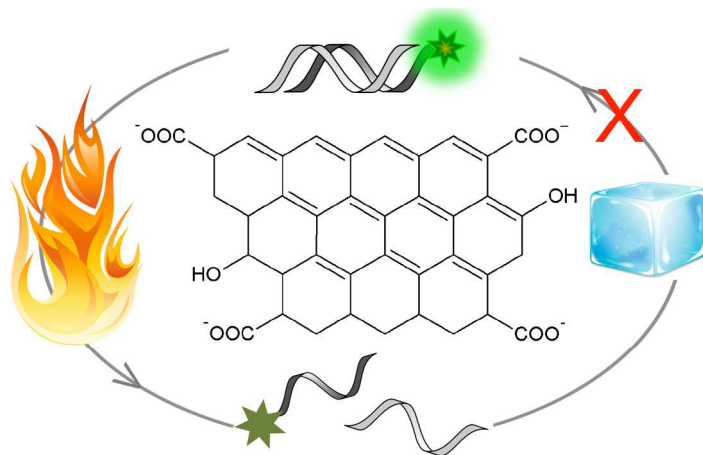


Fig. 6 Adsorption and desorption of FITC-labeled miR-10b DNA and a mutated oligonucleotide with a single base mismatch on graphene oxide using complementary oligonucleotides with or without LNA modification. Graphene oxide can distinguish one-base mismatch in DNA:DNA and LNA:DNA interaction. Adsorption and hybridization-induced desorption of full complementary and single mismatch oligos on graphene oxide is monitored with (a) anti-miR-10b DNA and (b) anti-miR-10b LNA. Experiments were performed in triplicate.

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Graphene oxide serves as a stable nano-platform for adsorption of nucleic acids with or without LNA base modifications while providing kinetic and spectroscopic information about the interaction.