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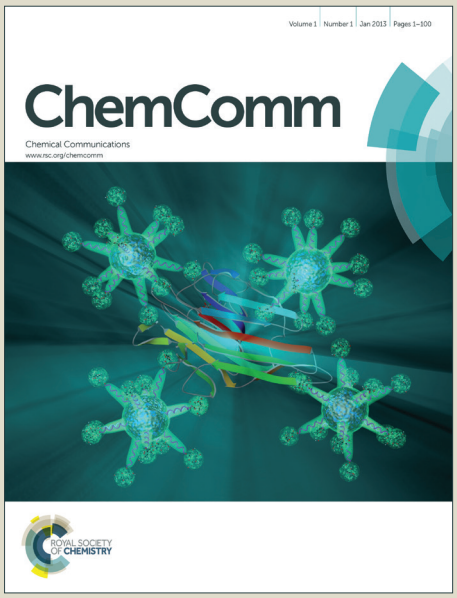

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A Biosensor for the Detection of Single Base Mismatches in microRNA

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Jieon Lee[a,b], Ginam Park[b], and Dal-Hee Min*[,a,b,c]

Graphene oxide quenches fluorescence corresponding to only mismatched target due to selective denaturing of the thermo-unstable duplex composed of probe peptide nucleic acid and single base mismatched target RNA and thus, the fluorescence signal only from perfectly matched target RNA is measured.

Nucleic acid detection is a crucial tool in clinical diagnosis and monitoring of viral infection,[1] cancer[2] and diseases associated with genetic alterations.[3] In general, most of the conventional detection methods such as blotting methods,[4] fluorescence in situ hybridization (FISH),[5] polymerase chain reaction (PCR)[6] and DNA microarray[7] are based on the sequence specific recognition by using single stranded nucleic acid as a probe, through Watson-Crick base pairing. Basically, the hybridization of probe with target occurs due to sequence specificity, but one or two nucleotide substitution in target is insufficient for distinguishing the strand from perfectly matched target by using the identical complementary probe. A single mismatched base pair (bp) within DNA duplex shows little decrease of melting temperature (T_m), less than 3 ℃.[8]

Precise recognition of few base differences in sequences of nucleic acid is important because it provides valuable information on genetic mutation,[9] DNA damage[10] and single-nucleotide polymorphism (SNP).[11] Recently, to overcome the drawback of natural nucleic acid as a sensing probe, structurally modified unnatural nucleic acid probes were developed and harnessed in many sensor systems.[12] For examples, peptide nucleic acid (PNA) has neutral amide bond backbone instead of phosphate, which enables rapid formation of thermodynamically more stable duplexes of PNA with complementary target.[13] Locked nucleic acid (LNA) possessing a methylene bridge between the 2'-oxygen of ribose and the 4'-carbon also exhibits strong affinity to target.[14] Typically, a single mismatched bp in hybrid duplex of artificial probe and natural target causes much lower binding affinity compared to perfectly matched duplex, resulting in a wide decline of T_m upto 15 ℃.[8,15] The larger decrease of T_m compared to natural probes allows precise nucleic acid detection with high sequence specificity. In the sensing systems based on modified unnatural probes, discrimination of one or more mismatches was generally accomplished by adjusting the hybridization condition such as temperature, ionic strength, formamide concentration, or other dissociation conditions of the probe and target duplex.[16] However, most of these systems require cumbersome optimization of the discriminating condition and separation of unbound probe molecules. In recent decades, advanced strategies have been developed by employing molecular beacon (MB), enzymes involved in nucleic acid metabolism and nanomaterials interacting with DNA.[17-19]

Here, we demonstrate a new strategy for discriminating one base difference among nucleic acid strands using graphene oxide (GO) and PNA. GO,[20] a water-soluble derivative of graphene, has been actively harnessed in diverse biological applications such as drug delivery,[21] catalysis,[22] enzyme activity assay[23] and biosensor.[24] General strategy in these applications relies on the strong affinity of single stranded nucleic acid and/or hydrophobic molecules with GO through pi-pi stacking and/or hydrogen bonding interactions and the fluorescence-quenching capability of GO. As a probe, we chose PNA, one of the artificial nucleic acid to obtain high sequence specificity. In addition to the widely known properties described above, PNA could interact with negatively charged GO with higher affinity than negatively charged nucleic acid probes and thus, gives more stable PNA/GO complex resulting in less non-specific desorption of probes from GO.[25]

In this study, the highly efficient discrimination of single nucleotide substitution was achieved by minimizing the false signal generated from unstable duplex of PNA and mismatched target. Upon pre-incubation of fluorescent dye conjugated PNA (dye-PNA) with totally or partially complementary RNA in a solution, the PNA/RNA duplex was formed in a few minutes and the hybridization yield was inversely proportional to a number of mismatched pair and the site. By elevating the temperature of the hybridized PNA/RNA duplex in the presence of GO, the thermo-unstable duplex possessing mismatched target was denatured and

[a] Center for RNA Research, Institute for Basic Science, Seoul National University, Seoul, 151-747, Korea.
[b] Department of Chemistry, Seoul National University, Seoul, 151-747, Korea.
[c] Lemonex Inc., Seoul, 151-742, Korea.
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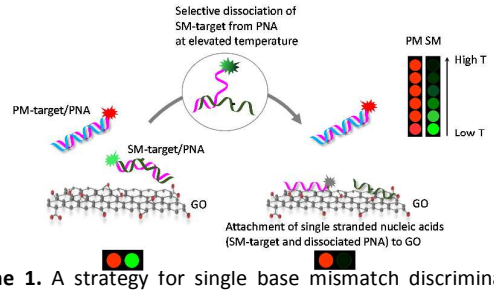
the temporarily dissociated ssPNA from mismatched target was adsorbed on GO. Finally, fluorescence signal corresponding to mismatched target was quenched by GO but dye-PNA maintaining the hybridization with perfectly matched target showed strong fluorescence emission (Scheme 1).



Scheme 1. A strategy for single base mismatch discrimination in nucleic acid using PNA and GO. (PM-target: perfectly matched target, SM-target: single mismatched target).

We first prepared an aqueous suspension of GO according to a modified Hummers method[26] of which procedure was described in supporting information. The formation of single layer GO sheet was characterized by atomic force microscopy (AFM) and the height profile (Figure S1a). We further characterized GO by using Raman, UV-Vis absorption and Fourier transform infrared (FT-IR) spectroscopies (Figure S1). Collectively, the data confirmed that GO was successfully prepared.

To demonstrate our strategy, we chose three microRNAs (miRNAs) in let-7 miRNA family, let-7a, let-7c and let-7f RNA, as a perfectly matched (PM) target (let-7a) and two types of single mismatched (SM) targets (let-7c and let7-f), respectively. The let-7 miRNA family members are conserved across diverse animals in sequence and function, and its abnormal expression leads to a less differentiated cellular state and the development of serious diseases such as cancer.[27] let-7c RNA and let-7f have one nucleotide substitution from let-7a RNA in different site and thus, they are suitable model targets for evaluating the discrimination ability to distinguish single nucleotide differences as well as interesting biomarkers for sensor system (Figure 1a). We next prepared carboxyfluorescein (FAM) labeled ssPNA probe of which sequence was fully complementary to let-7a RNA and determined the optimal GO concentration for quenching the fluorescence of FAM-PNA probe. We found that the fluorescence of FAM-PNA probe (10 pmol) was quenched up to ~98% in the presence of GO (0.1 μg), giving maximum quenching efficiency under the experimental condition (Figure S2).

Prior to the full-scale studies for single base mismatch discrimination, we performed nucleic acid detection using FAM-PNA/GO complex and synthetic RNAs to confirm the sequence specificity at room temperature according to the previously reported protocol by our group.[25b] The FAM-PNA probe was first mixed with GO solution to construct PNA/GO complex and then, each miRNA target was added at room temperature. The fluorescence intensities of the mixtures increased upon addition of both perfectly and partially complementary targets (let-7a, let-7c and let-7f RNA), whereas addition of scrambled RNA showed little increase in fluorescence. Upon addition of let-7c and let-7f RNA, the fluorescence of FAM-PNA probe showed 53 and 73% of fluorescence recovery compared to let-7a RNA, respectively (Figure S3). This result indicated that the previously established nucleic acid sensing system was not enough to use in the applications requiring extremely high sequence specificity such as miRNA profiling and SNP detection, unfortunately.

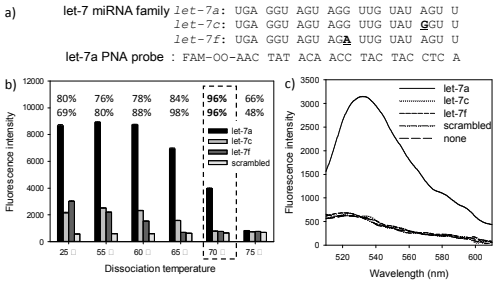


Figure 1. (a) Sequences of target miRNAs and FAM conjugated let-7a PNA probe. (b) Fluorescence intensity of FAM-PNA in the presence of each miRNA after thermal dissociation step in the presence of 0.1 μg GO. The discrimination efficiencies were shown above the bar graphs. (c) Fluorescence spectra of FAM-PNA with target miRNA after thermal dissociation at 70°C in the presence of 0.1 μg of GO.

We next carried out temperature dependent single base mismatch discrimination test to optimize the condition for dissociation of PNA from mismatched target. First, FAM-PNA was pre-incubated with three synthetic miRNAs in buffered solutions for hybridization, and then, GO was added to the solution containing duplexes. The mixture was heated at high temperature (55 ℃ –75 ℃) for 5 min to induce dissociation of less stable PNA/RNA duplex and adsorption of the generated ssPNA onto GO. At room temperature without heating, let-7c and let-7f RNA showed fluorescence increase corresponding to 20 and 31% of that of let-7a, respectively, which is much higher than background signal obtained from addition of totally scrambled RNA sequence (Figure 1b). Here, we define 'discrimination efficiency for single base mismatch discrimination' as the difference in relative fluorescence intensity generated by perfectly matched target and single mismatched target (discrimination efficiency (%)=100 x (F - $F_{scrambled}$)/($F_{perfectly\ matched}$-$F_{scrambled}$)). Therefore, the discrimination efficiency at 25 ℃ in the present system is 80 and 69% for let-7c and let-7f, respectively. However, upon heating, the discrimination efficiency increased depending on the temperature and reached upto 96% at 70 ℃. Under the experimental condition at 70 ℃, high sequence specificity was confirmed by showing almost undistinguishably overlapped fluorescence emission spectra of mixtures containing let-7c and let-7f miRNA with scrambled RNA (Figure 1c). The optimized temperature for selectively denaturing the unstable mismatched duplex is dependent on its T_m value and therefore, could vary with the length of PNA probe.

To evaluate the performance of the present sensor towards DNA targets, we next investigated the temperature dependent base-mismatch discrimination using ssDNA targets possessing the same sequences with let-7a, let-7c, let-7f RNA but thymine in place of uracil. We screened a wide range of dissociation temperature using

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DNA targets and found that the optimized discrimination efficiency of higher than 95% was obtained at 65 ℃, which is 5 ℃ lower than RNA targets (Figure S4). This shift of dissociation temperature was demonstrated by the previously reported result in which T_m value of PNA/DNA duplex was 4 ℃ lower than PNA/RNA duplex.[15] The data suggested that the present GO based sensing platform is highly sensitive to T_m of duplexes and suitable for discrimination of differences in T_m caused by single base substitution in DNA as well as RNA.

We next performed quantitative fluorometric detection of target RNA with the procedure containing thermal treatment to evaluate detection limit of the present sensor. Various concentrations of target let-7a RNA solutions ranging from 0 to 100 nM were mixed with FAM-PNA probe and then, GO solution was added. The mixed solutions of target RNA, FAM-PNA and GO underwent the thermal treatment step for selective dissociation at 70 ℃ for 5 min. The fluorescence emission spectra of the mixture showed the RNA concentration dependent fluorescence increase (Figure S5a). This sensor showed a linear fluorescence increase between 0 and 6.25 nM with the detection limit of 230 pM according to the equation, LOD = 3.3 (SD / S) (LOD: limit of detection, SD: standard deviation, S: slope of the calibration curve) (Figure S5b).

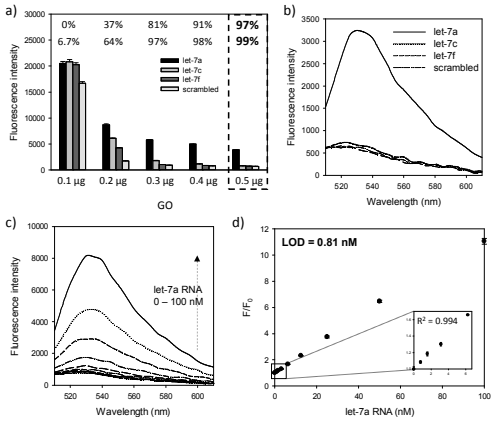

Figure 2. Single base mismatch discrimination towards spiked miRNAs in cell lysates. (a) Fluorescence intensity of FAM-let-7a PNA probe in the presence of each spiked let-7 miRNA in HeLa cell lysate after thermal dissociation at 70 ℃ with various amounts of GO. (b) Fluorescence spectra of FAM-PNA with target miRNA after thermal dissociation in the presence of 0.5 μg of GO. (c) Fluorescence spectra and (d) Fluorescence intensity enhancement (F/F_0) of PNA probe in the presence of various concentrations of spiked let-7a miRNA in HeLa cell lysate after thermal dissociation step upon addition of 0.5 μg of GO. (F: fluorescence intensity of PNA probe with target, F_0: fluorescence intensity of PNA probe without target).

To investigate the applicability of highly sequence specific nucleic acid detection in biological samples, we carried out the same experiment using RNA spiked cell lysate (10,000 HeLa cells per sample). We found that mixtures of FAM-PNA and RNA including 0.5 μg of GO showed high efficiency of discrimination over 95% (Figure 2a, b). The amount of GO was raised from 0.1 to 0.5 μg in this application because under the GO concentration established in sensing in PBS solution, let-7a RNA could not be sufficiently discriminated against single base mismatched target, let-7c or let-7f RNA even in a whole range of dissociation temperature (Figure S6). One plausible explanation for this phenomena might be found in the interaction between biomolecules (e.g., nucleic acids, proteins) of cell lysate and the surface of GO, which prevents the adsorption of mismatched target and PNA probe denatured from unstable duplex at high temperature. The fluorescence emission spectra showed slightly higher fluorescence intensity compared to that from solutions prepared in 1XPBS with same conditions (Figure 2c). This finding agrees with previously reported result in which addition of proteins to GO based nucleic acid sensor system can enhance the target specific signal by blocking nonspecific adsorption of duplex.[28] Under the condition, the sensor also exhibited a linear fluorescence increase in the concentration range of target RNA (0 ~ 6.25 nM) with a slightly higher LOD of 810 pM (Figure 2d).

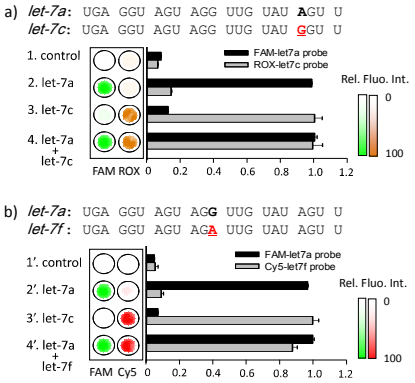

Figure 3. Fluorescence images and bar graphs showing relative fluorescence intensity of a) FAM-let-7a probe/ROX-let-7c probe after dissociation step at 71 ℃, b) FAM-let-7a probe/Cy5-let-7f probe after dissociation step at 69 ℃ in the presence of each target miRNA.

Finally, we demonstrated the capability of multiplexed detection in a solution using multiple RNA targets possessing only one nucleotide difference each other. We chose two sets of RNA targets, let-7a/let-7c RNA and let-7a/let-7f RNA. Each complimentary sequenced PNA probe for let-7a, let-7c and let-7f RNA was conjugated with three different fluorescent dyes—FAM, ROX (6-carboxyl-X-rhodamine) and Cy5 (cyanine 5) which showed emission maxima of 518 (green), 608 (orange) and 670 nm (red) with excitation at 492, 587 and 650 nm, respectively. For two kinds of RNA detection in a solution, each target RNA or a combination of two RNAs were added to the mixture of each corresponding dye labelled PNA probe (100 nM, each), followed by addition of optimized amount of GO (Figure S7). Then, we screened the temperature from 65 to 75 ℃ with the interval of 2 ℃ for 5 min to optimize the temperature for effective dissociation of two different PNA probe from mismatched target in a solution. As a result, let-7a/let-7c RNA and let-7a/let-7f RNA were satisfactorily discriminated at 69 and 71 ℃, respectively (Figure S8). This tendency of optimized temperatures agrees well with the order of T_m of each PNA probe with perfectly matched target (82 ℃ for let-7c pair > 79 ℃ for let-7a pair > 76 ℃ for let-7f pair). At each

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optimized temperature, green, orange and red fluorescence emission spectra and images were obtained only in the sample containing each corresponding RNA target (Figure 3).

In conclusion, we developed a new strategy for highly sequence specific nucleic acid detection using PNA and GO. This work provides the first example of highly effective and reliable discrimination of single base pair mismatch among many GO based sensors. The present system utilized remarkable properties of PNA probe and GO. First, PNA probe provides high binding affinity and sequence specificity to target due to its neutral backbone. A mismatched pair in a duplex of PNA and target nucleic acid induces a wide decline of Tm, which enables selective denaturation upon elevating temperature. Second, GO serves as a scavenger for adsorption of ssPNA dissociated from mismatched target as well as a superquencher for the dye conjugated to the PNA. In addition, owing to the strong interaction between PNA and GO, quantitative target detection with high sequence specificity was enabled even in the biological samples such as cell lysates without a wide variation of LOD. The present sensor is technically straightforward compared to the SNP detection based on PNA molecular beacon (see ESI for further explanation) and compatible with accurate multiplexed sensing formats using sequentially similar targets. Many samples can be simultaneously prepared, and the quantitative multiplex detection with high sequence specificity can be conducted without complicated optimization of experimental condition and separation of mismatched target. We believe that this new strategy for reliable discrimination of single base mismatch using PNA probe and GO will become an important tool in basic research of nucleic acid biology and the field of personalized diagnostics of diseases.

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