Article
DOI: 10.1002/bkcs.11549 B. H. Le and Y. J. Seo BULLETIN OF THE KOREAN CHEMICAL SOCIETY

Transcription of Unnatural Fluorescent Nucleotides and their Application with Graphene Oxide for the Simple and Direct Detection of miRNA

Binh Huy Le[†] and Young Jun Seo[†,‡,*]

[†]*Department of Bioactive Material Sciences, Chonbuk National University, Jeonju 561-756, South Korea*
[‡]*Department of Chemistry, Chonbuk National University, Jeonju 561-756, South Korea.*
**E-mail: yseo@jbnu.ac.kr*
Received June 11, 2018, Accepted July 6, 2018

In this study we synthesized two differently sized fluorescent RNA nucleotides, **rUthioTP** and **rUpyrTP**, and examined their transcription ability using T7 RNA polymerase. The smaller **rUthioTP** could be incorporated and extended to produce a corresponding RNA sequence, but **rUpyrTP** could not. We then used this **rUthioTP**-containing fluorogenic transcription system, in conjunction with graphene oxide(GO), for the detection of **miRNA 146a** with high sensitivity and selectivity. This combination of a transcribed RNA product and GO is a simple *in situ* probing system for the detection of **miRNA 146a**—one that is less time consuming and more cost-effective.

Keywords: Probe, Fluorescent nucleotides, Transcription, miRNA, Graphene oxide

Introduction

MiRNAs are short (19–24 bases) single-stranded RNAs whose function is related to gene regulation focused on the untranslated region of mRNA.[1,2] Recently, many diverse diagnostic tools have been developed for the detection of miRNA as biomarkers, using different types of oligonucleotide probing systems, including Taqman probes,[3] molecular beacons (MBs),[4,5] and nanoparticle-attached oligonucleotide detection systems.[6–10] Most of these probing systems have employed oligonucleotides, modified with fluorophores and quenchers, that were prepared using solid phase synthetic methods. To obtain efficient versions of such probing systems, the oligonucleotides must be designed carefully to ensure secondary structures that align the fluorophore and quencher units appropriately.[11–17] Accordingly, the synthetic procedures for preparing oligonucleotide-based probing systems can be very complex. They can require several synthetic and purification steps, as well as complex diagnostic procedures in combination with other systems for the detection of miRNA; such processes can be time-consuming and expensive.[18,19]

To develop a simpler, rapid, and direct *in situ* diagnostic tool, we wished to apply *in vitro* transcription, a direct enzymatic oligonucleotide synthetic method mediated by T7 RNA polymerase, to incorporate and extend unnatural fluorescent nucleotides into RNA, and then use the transcribed fluorescent oligonucleotides, in conjunction with graphene oxide (GO), for the detection of miRNA.

Many different types of unnatural fluorescent nucleotides have been developed for the labeling of DNA or RNA for a variety of purposes. Nevertheless, it remains difficult to develop methods for the efficient incorporation and extension of fluorescent nucleotides into RNA, due to the active sites of polymerases being very tight.[20]

In this study we prepared unnatural fluorescent nucleotides based on deoxyuridine modified at the 5-position, which is tolerated by the active site of polymerases. We then employed T7 RNA polymerase to produce RNA oligonucleotides containing the fluorescent nucleotides, through *in vitro* transcription, because the transcribed RNA could be isolated from the DNA template and used directly for the detection of miRNA.

To develop the fluorescent nucleotides, we chose a pyrene fluorophore,[21–24] which is designed from our lab, displaying strong fluorescence and specific excimer behavior when aggregated, as well as a thiophene fluorophore, which is smallest fluorescent material, reported from Tor group.[24,25] We attached these fluorescent substrates at the 5′-position of deoxyuridine through Suzuki coupling and then prepared their corresponding fluorescent nucleotide triphosphates, **rUpyrTP** and **rUthioTP**, through the Yoshiuki method.[26,27] NMR spectroscopy and mass spectrometry confirmed the structures of these compounds (see Appendix S1, Supporting Information).

Experimental

General Procedure for Triphosphate Synthesis. Proton Sponge (1.5 equiv.) and the free nucleoside (1 equiv.) were dissolved in trimethylphosphate (0.3 M) and cooled to −20 °C. $POCl_3$ (1.5 equiv.) was added dropwise, and then the purple slurry was stirred at −20 °C for 2 h. Tributylamine (6.2 equiv.) was added, followed by a solution of

tributylammonium pyrophosphate (5.0 equiv.) in DMF (0.5 M). After 5 min, the reaction was quenched through the addition of 0.5 M aqueous $Et_3NH_2CO_3$ (20 vol equiv.) and then the resulting solution was lyophilized. Purification through reverse-phase (C18) HPLC (4–35% CH_3CN in 0.1 M $Et_3NH_2CO_3$, pH 7.5), followed by lyophilization, afforded the triphosphate as a solid.

Transcription Reaction. **ODN 1** and **ODN 2** were annealed (0.2 μM) by heating to 95 °C and then cooling slowly to room temperature. The rNTPs, **rUthioTP**, and **rUpyrTP** (5 mM) were added to samples, which contained 1× DTT and 1× T7 RNA polymerase buffer. All samples were incubated at 37 °C for 5 h.

Preparation of transcribed RNA interacted with GO. An appropriate amount of GO solution 0.2 μg (200 μL) was added into the tube containing **C-miRNA 146a** (0.1 μM) in 25 mM Tris–HCl buffer in DEPC water. The mixture was then vortexed for 2 min. To ensure that the **C-miRNA 146a** was completely absorbed on the surface of the GO, the solution was centrifuged (13 000 rpm) at 4 °C for 30 min. The liquid phase was collected, and its fluorescence emission spectra measured. In other tube, the same amount of **C-miRNA 146a** (0.1 μM) was prepared in the same buffer condition, then GO 0.2 μg (200 μL) was added into the tube and vortexed for 2 min. After that, the target miRNA (0.1 μM and 1.5 μM) was added to this solution, then incubated at room temperature for 10 min. Next, the mixture was centrifuged (13 000 rpm) at room temperature for 20 min, the solid phase was removed, and the liquid phase was collected to measure fluorescence emission spectra.

Sample Preparation for Fluorescence and UV Spectroscopy. The fluorescence emission spectra of the RNAs were recorded at 25 °C with excitation at 354 nm using a quartz cuvette (path length: 1 cm) on a JASCO PF-6500 spectrofluorometer (Tokyo 193-0835, Japan). The UV spectra were recorded using a Cary Series UV–Vis spectrophotometer Agilent (Santa Clara, CA 95051, USA) and a quartz cell (path length: 1 cm).

Results and Discussion

For *in vitro* transcription of **rUpyrTP** and **rUthioTP**, we employed **ODN 1** and **ODN 2** as the primer and template, respectively, in conjunction with T7 RNA polymerase to produce fluorescent RNA oligonucleotide (Table 1).[28] Monitoring the transcription reactions through gel electrophoresis revealed (Figure 1) that **rUthioTP** was incorporated and extended well into the RNA, but **rUpyrTP** was not. The successful preparation of the transcribed fluorescent RNA was confirmed using DNase I to remove the DNA primer and template. We suspect that the small size of the fluorescent unit in **rUthioTP** resulted in less of a steric clash with the active site of T7 RNA polymerase, allowing its efficient incorporation and extension during transcription. In contrast, **rUpyrTP** presented a bulky polyaromatic group that presumably did not fit in the active site.

Table 1. Oligonucleotide sequences investigated in this study.

Name	Sequence
ODN 1	5′- GCC ATG GGG CTG ATC
ODN 2	5′- TGA GAA CTG AAT TCC ATG GGT TGA TCA GCC CCA TGG C
miRNA 146a	5′- uga gaa cug aau ucc aug ggu u
miRNA 21	5′- uag cuu auc aga cug aug uug a
miRNA 24-3P	5′- ugg cuc agu uca gca gga aca g
miRNA25b	5′- ucc cug aga ccc uaa cuu gug a

After confirming that **rUthioTP** could be transcribed well using T7 RNA polymerase, we applied its transcribed fluorescent RNA product (**C-miRNA 146a)**, in conjunction with GO as a quencher, for the detection of miRNA. From experiments in which we varied the ratio of the transcribed fluorescent RNA to GO (Figure 2), we found that 200 ng of GO and 0.1 μM of the RNA product provided the optimal quenching state.

We chose **miRNA 146a**, which is related to human cancer[29]and a *Staphylococcus aureus* enterotoxin for cows,[30] as the target miRNA for detection. When we added **miRNA 146a** into the quenched RNA/GO probe system at a ratio (**C-miRNA 146a** to **miRNA 146a**) of 1:1 we did not observe any significant increase in fluorescence. In contrast, when we increased the content of **miRNA 146a** up to 1:15, the fluorescence increased dramatically (Figure 3),

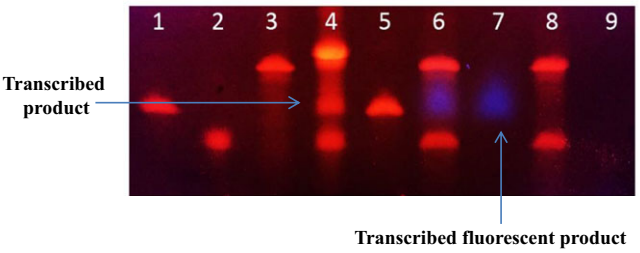


Figure 1. Denaturing polyacrylamide gel data, confirming the transcription reaction of **rUthiTP**. Lane 1: **miRNA 146a** (22mer); lane 2: **ODN 1; l**ane 3: **ODN 2**; lane 4: **ODN 1** + **ODN 2** + T7 RNA polymerase with rNTPs; lane 5: **ODN 1** + **ODN 2** + T7 RNA polymerase with rNTPs, treated with DNase I; lane 6: **ODN 1** + **ODN 2** + T7 RNA polymerase with **rUthiTP**, rCTP, rATP, and rGTP; lane 7: **ODN 1** + **ODN 2** + T7 RNA polymerase with **rUthiTP**, rCTP, rATP, and rGTP, treated with DNase I; lane 8: **ODN 1** + **ODN 2** + T7 RNA polymerase with **rUpyrTP**, rCTP, rATP, and rGTP; lane 9: **ODN 1** + **ODN 2** + T7 RNA polymerase with **rUpyrTP**, rCTP, rATP, and rGTP, treated with DNase I. **ODN 1:ODN 2** was annealed by heating at 95 °C and then cooled slowly to room temperature. T7 RNA polymerase (50 U) and its buffer containing DTT were added and incubated at 37 °C for 5 h. Reactions were quenched by adding 2 μL of 0.5 M EDTA. All transcription products were stained after gel electrophoresis using the ethidium bromide (EtBr) solution.

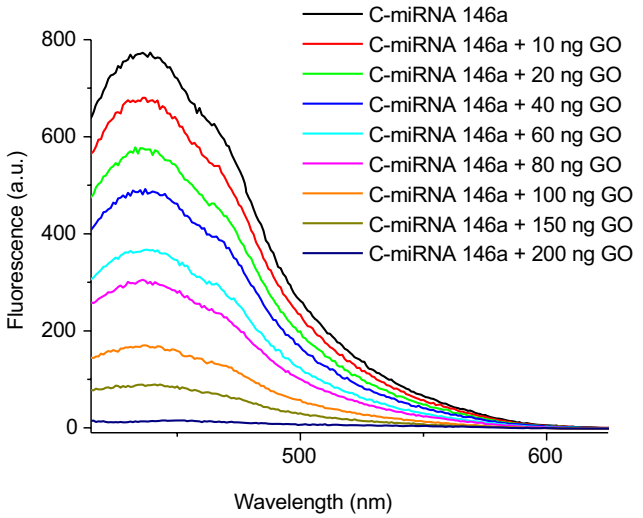


Figure 2. Fluorescence spectra recorded to optimize the quenching state formed between **C-miRNA 146a** and GO. All RNA solutions comprised, 0.1 μM (1 mL) of the RNA product in 20 mM Tris–HCl and various amounts of GO (10–200 ng). All samples were excited at 354 nm.

presumably because of displacement of the RNA oligonucleotide probe by the target **miRNA 146a** from the surface of the GO and subsequent formation of a duplex between the probe and the target RNA.[31] In this regard, we suspect that the RNA/GO probing system has less significant sensitivity relative to other probing system.

To examine the selectivity of this probe system, we tested the effects of various other **miRNA** sequences. A high increase in fluorescence occurred only in the presence of **miRNA 146a**; we could discriminate the target **miRNA 146a** from other random mismatched miRNAs which show low fluorescence intensity relatively (Figure 4). Thus, our transcribed RNA/GO-based probing system could be used to detect the target **miRNA 146a** selectively.

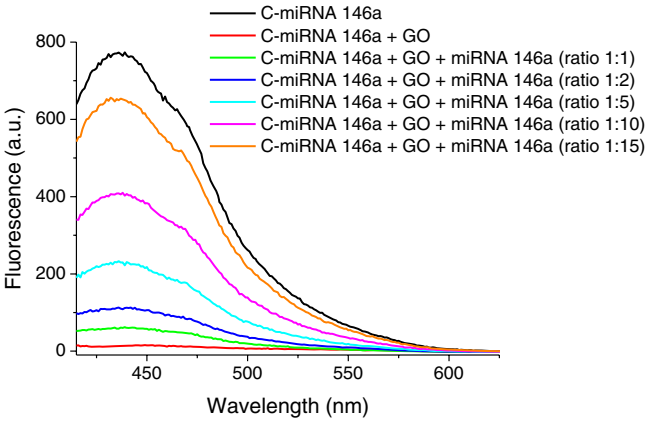


Figure 3. Fluorescence spectra recorded to examine the sensitivity of **C-miRNA 146a** as a probe. All samples contained 0.1 μM (1 mL) of **C-miRNA 146a** in 20 mM Tris–HCl as well as 200 ng of GO. The ratio of **C-miRNA 146a** to the target **miRNA 146a** was increased from 1:1 to 1:15. All samples were excited at 354 nm.

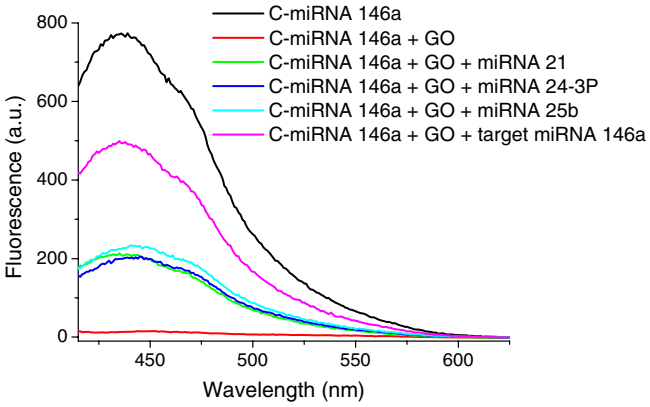
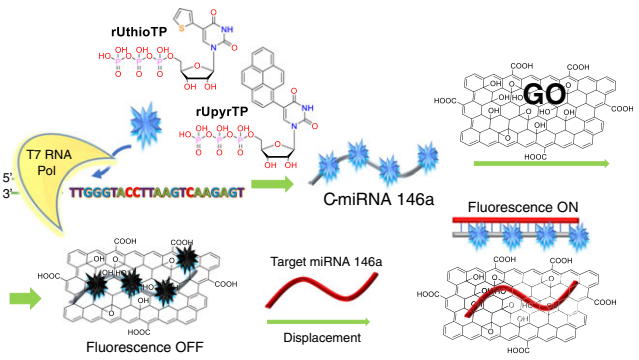


Figure 4. Fluorescence spectra recorded to examine the selectivity of the **C-miRNA 146a** probe. All samples contained 0.1 μM (1 mL) of **C-miRNA 146a** in 20 mM Tris–HCl as well as 200 ng of GO. **C-miRNA 146a** and the target (**miRNA 146a**) and mismatched (**miRNA21**, **miRNA24-3P**, **miRNA 25b**) miRNAs were added at a ratio of 1:15. All samples were excited at 354 nm.

Conclusion

We have synthesized the unnatural fluorescent nucleotides **rUthioTP** and **rUpyrTP** and examined their direct incorporation and extension into RNA through transcription using T7 RNA polymerase. Although **rUthioTP** could be incorporated and extended into the RNA, **rUpyrTP** could not, presumably because its greater bulk clashed sterically with the active site of the polymerase. We used the transcribed product containing **rUthioTP**, in conjunction with GO, for the detection of **miRNA 146a**. Because the fluorescence of the transcribed product containing **rUthioTP** was quenched dramatically in the presence of GO, we used this system for the direct *in situ* detection of **miRNA 146a**. We observed a change in fluorescence with a high discrimination factor when the ratio of the concentrations of the



Scheme 1. Schematic representation of the transcription of the fluorescent nucleotides **rUthioTP** and **rUpyrTP** into **C-miRNA 146a** and its direct application, with GO, in the detection of **miRNA 146a**.

transcribed RNA product and **miRNA 146a** was 1:15, presumably because **miRNA 146a** displaced the transcribed RNA product from the surface of the GO. This probing system displayed high selectivity, discriminating **miRNA 146a** from other random mismatched sequences. Thus, this transcribed RNA product combined with GO is a simple *in situ* probing system for the detection of **miRNA 146a**—in a less time consuming and more cost-effective manner.

Acknowledgments. This study was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF)(2017R1A2B4002398).

Supporting Information. Additional supporting information may be found online in the Supporting Information section at the end of the article.

References

1. V. Ambros, *Cell* **2001**, *107*, 823.
2. D. P. Bartel, *Cell* **2004**, *116*, 281.
3. P. M. Holland, R. D. Abramson, R. Watson, D. H. Gelfand, *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 7276.
4. S. Yagi, F. R. Kramer, *Nat. Biotechnol.* **1996**, *14*, 303.
5. C. Hong, A. Baek, S. S. Hah, W. Jung, D. E. Kim, *Anal. Chem.* **2016**, *88*, 2999.
6. P. Miao, B. Wang, F. Meng, J. Yin, Y. Tang, *Bioconjugate Chem.* **2015**, *26*, 602.
7. X. Zhang, H. Liu, R. Li, N. Zhang, Y. Xiong, S. Niu, *Chem. Commun.* **2015**, *51*, 6952.
8. J. Li, T. Deng, X. Chu, R. Yang, J. Jiang, G. Shen, R. Yu, *Anal. Chem.* **2010**, *82*, 2811.
9. W. Xu, X. Xie, D. Li, Z. Yang, T. Li, X. Liu, *Small* **2012**, *8*, 1846.
10. S. Tyagi, D. Bratu, F. R. Kramer, *Nat. Biotechnol.* **1998**, *16*, 49.
11. R. A. Cardullo, S. Agrawal, C. Flores, P. C. Zamecnik, D. E. Wolf, *Proc. Natl. Acad. Sci. USA* **1988**, *85*, 8790.
12. S. A. E. Marras, S. Tyagi, F. R. Kramer, *Clin. Chim. Acta* **2006**, *363*, 48.
13. G. T. Hwang, Y. J. Seo, B. H. Kim, *J. Am. Chem. Soc.* **2004**, *126*, 6528.
14. L. Wang, C. J. Yang, D. Colin, C. D. Medley, S. A. Benner, W. Tan, *J. Am. Chem. Soc.* **2005**, *127*, 15664.
15. P. Conlon, C. J. Yang, Y. Wu, Y. Chen, K. Martinez, Y. Kim, N. Stevens, A. A. Marti, A. S. Jockusch, N. J. Turro, W. Tan, *J. Am. Chem. Soc.* **2008**, *130*, 336.
16. H. S. Joshi, H. Y. Tor, *Chem. Commun.* **2001**, *6*, 549.
17. M. J. Gait, *Oligonucleotide Synthesis: A Practical Approach*, IRL Press, Washington, DC, **1984**, p. 1.
18. I. S. Kim, Y. J. Seo, *Bioorg. Med. Chem. Lett.* **2014**, *24*, 1589.
19. A. D. Maria, M. M. Javier, M. I. Adolfo, *Front. Chem.* **2016**, *4*, 18.
20. D. G. Lee, I. S. Kim, J. W. Park, Y. J. Seo, *Chem. Commun.* **2014**, *50*, 7273.
21. H. N. Joo, Y. J. Seo, *Chem. Commun.* **2015**, *51*, 2939.
22. I. S. Kim, Y. J. Seo, *Tetrahedron Lett.* **2014**, *55*, 1461.
23. H. N. Joo, Y. J. Seo, *Bioorg. Med. Chem. Lett.* **2014**, *25*, 5286.
24. M. S. Noé, R. W. Sinkeldam, Y. Tor, *J. Org. Chem.* **2013**, *78*, 8123.
25. S. G. Srivatsan, Y. Tor, *Chem. Asian. J.* **2009**, *4*, 419.
26. B. H. Le, J. C. Koo, H. N. Joo, Y. J. Seo, *Bioorg. Med. Chem.* **2017**, *25*, 3591.
27. H. Marcel, *Molecules* **2012**, *17*, 13569.
28. J. S. Thomas, A. Irina, *Nat. Rev. Microbiol.* **2011**, *9*, 319.
29. L. Li, X. P. Chen, Y. L. Li, *Scand. J. Immunol.* **2010**, *71*, 227.
30. F. Dilda, G. Gioia, L. Pisani, L. Restelli, C. Lecchi, F. Albonico, V. Bronzo, M. Mortarino, F. Ceciliani, *Vet. J.* **2012**, *192*, 514.
31. B. Liu, Z. Sun, X. Zhang, J. Liu, *Anal. Chem.* **2013**, *85*, 7987.

