Bioorganic & Medicinal Chemistry Letters xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Direct incorporation and extension of a fluorescent nucleotide through rolling circle DNA amplification for the detection of microRNA 24-3P

Binh Huy Le [a], Young Jun Seo [a,b,*]

[a] Department of Bioactive Material Sciences, Research Center of Bioactive Materials Chonbuk National University, Jeonju 561-756, South Korea
[b] Department of Chemistry, Chonbuk National University, Jeonju 561-756, South Korea

ARTICLE INFO

Article history:
Received 15 February 2018
Revised 16 April 2018
Accepted 24 April 2018
Available online xxxx

Keywords:
Fluorescent nucleotide
RCA
Direct labeling
miRNA
Virus

ABSTRACT

We designed and synthesized several fluorescent nucleotides from thiophene, anthracene and pyrene, which have different sizes, and screened their incorporation and extension capability during the rolling circle amplification of DNA. The thiophene-based fluorescent nucleotide (**dUthioTP**) could highly incorporate and extended into the rolling circle DNA product, while other fluorescent nucleotides (**dUanthTP**, and **dUpyrTP**) could not. This **dUthioTP** fluorescent nucleotide could be used for the detection of **miRNA 24-3P**, which is related PRRSV. This direct labeling system during rolling circle DNA amplification exhibited an increased fluorescence signal showing gel formation for the detection of **miRNA 24-3P**. This direct labeling system is a very simple and cost-efficient method for the detection **miRNA 24-3P** and also exhibited highly sensitive and selective detection properties.

© 2018 Published by Elsevier Ltd.

MiRNAs are small endogenous single-stranded noncoding RNAs that regulate gene expression with a focus on the translation.[1] There have been many different types of miRNAs reported related to diverse cell processes, such as cell proliferation, differentiation, stress resistance, and apoptosis.[2,3] This diverse relationship of miRNAs in cell processes could allow miRNA to be used as a biomarker, particularly targeting several human diseases as well as animal diseases.[4–7] miRNA also has a critical key role in viral infections by regulating the expression of virus RNA or DNA. Porcine reproductive and respiratory syndrome virus (PRRSV) induces a severe disease in pigs and is one of the most significant viruses that causes large economic losses.[8] **miR-24-3P** promoted PRRSV replication and could be used as a biomarker for the detection of PRRSV.[9]

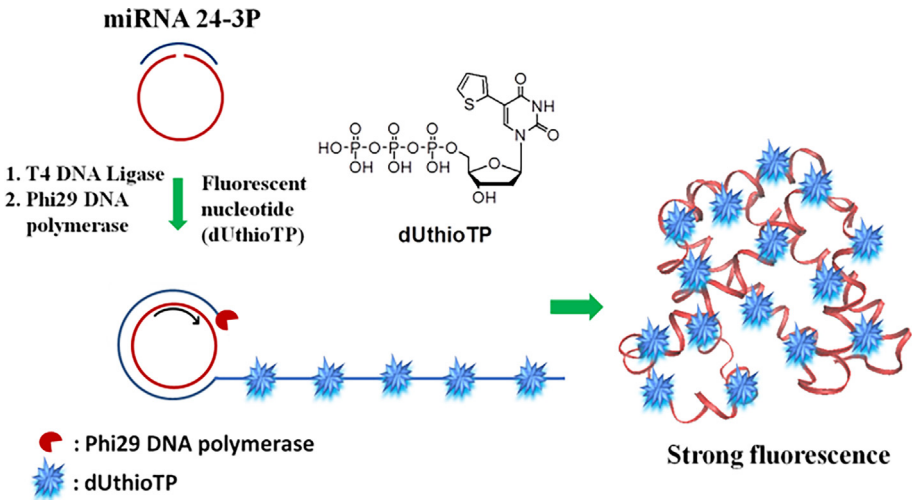
For the detection of miRNAs, many different types of probing systems have been developed, such as real time PCR[10,11], northern blot[12], and antibody.[13] However, for the practical detection of miRNA, the extremely low number of copies of miRNA in the blood is limiting and causes low sensitivity. Thus, researchers have tried to amplify the signal by employing a degradation enzyme such as exonuclease III[14] or duplex specific enzyme.[15] The rolling circle amplification (RCA) method[16] is another alternative efficient method for signal amplification. Based on the rolling circle amplification system, many different types of visualization methods have been developed using intercalative dye[17], SYBR Green[18], fluorescent primers[19], stem-loop molecular beacons[20,21], a G-quadruplex-gold combination system[22], or a graphene oxide based probing system.[23]

However, most of these visualization methods are indirect signal amplification methods and use complicated processes, which are time-consuming and have a high cost. Our goal, with regards to that point of view, was to develop a simple direct labeling system during the RCA process without further complicated labeling processes (Scheme 1). For this purpose, we developed a fluorescent nucleotide triphosphate, which could be incorporated and extended into the rolling circle amplified DNA showing a fluorescence signal increase. For this purpose, the most important process is to find a fluorescent nucleotide that could be recognized by Phi29 DNA polymerase and incorporated and extended into the RCA DNA. This is an exceedingly challenging and promising task because the active site of DNA polymerase is very tight and restrictive against mutated nucleotides.

We screened three different sized fluorophores including pyrene (four-membered aromatic ring), anthracene (three-membered aromatic ring), and thiophene (one aromatic ring) based on deoxy uridine.[24] Pyrene is a large-sized efficient fluorophore, which shows unique excimer-fluorescence properties when they organize together.[25] Anthracene is mid-sized fluorophore, which has a light-induced dimerization property.[26] Thiophene is the smallest fluorescent nucleotide, which shows a weak fluorescence property.[27]

* Corresponding author at: Department of Chemistry, Research Institute of Physics and Chemistry, Chonbuk National University, Jeonju 561-756, South Korea.
E-mail address: yseo@jbnu.ac.kr (Y.J. Seo).

https://doi.org/10.1016/j.bmcl.2018.04.058
0960-894X/© 2018 Published by Elsevier Ltd.



Scheme 1. Concept of rolling circle amplification with fluorescent nucleotide.

First, we synthesized fluorescent nucleotide triphosphate based on the Yoshiuki method[28] via several steps and confirmed the synthesis using NMR and mass spectrometry (see Supporting Information). Then, we examined the enzymatic incorporation properties of these fluorescent nucleotides using the RCA system. For this, we designed **RCT 1** as a rolling circle template, which is the complementary sequence-targeting **miRNA 24-3P** (Table 1). **RCT 1** hybridized with **miRNA 24-3P** and could form a circular DNA with ligation using T4 DNA Ligase. To confirm the circular DNA formation, we used EXO 1 enzyme, which could cleave single-stranded DNA selectively but could not cleave the circular DNA. Figure 1a shows clear evidence of **RCT 1** circular DNA formation.

Table 1
Oligonucleotide sequences that were used for this study.

Name	Sequences
RCT 1	5′-[a]Pho-TGA ACT GAG CCA ACT GCT GCT GCT GCT GCT GCT GCT GCT GAC TGT TCC TGC
miRNA 24-3P	5′- ugg cuc agu uca gca gga aca g
ORN 1	5′- ugg cuc agu ucu gca gga aca g
miRNA 21	5′- uag cuu auc aga cug aug uug a

[a] Pho:Phosphate

To validate the rolling circle amplification using our unnatural fluorescent nucleotides, we added phi 29 DNA polymerase with dATP, dGTP, and dCTP including each fluorescent nucleotide (**dUpyrTP**, **dUanthTP**, and **dUthioTP**) into the **RCT 1** circular DNA, which is complementary to the **miRNA24-3P** sequence. We examined agarose gel electrophoresis to confirm the RCA product by using enzymatic synthesis (Fig. 1b). Interestingly, it showed a size-dependent incorporation and extension pattern. From **dUpyrTP** and **dUanthTP**, we did not observe any amplified DNA product, while we could observe amplified DNA product using **dUthioTP**. Only by using **dUthioTP** did we observe over 10 kb RCA DNA product. From this result, we noted that only the

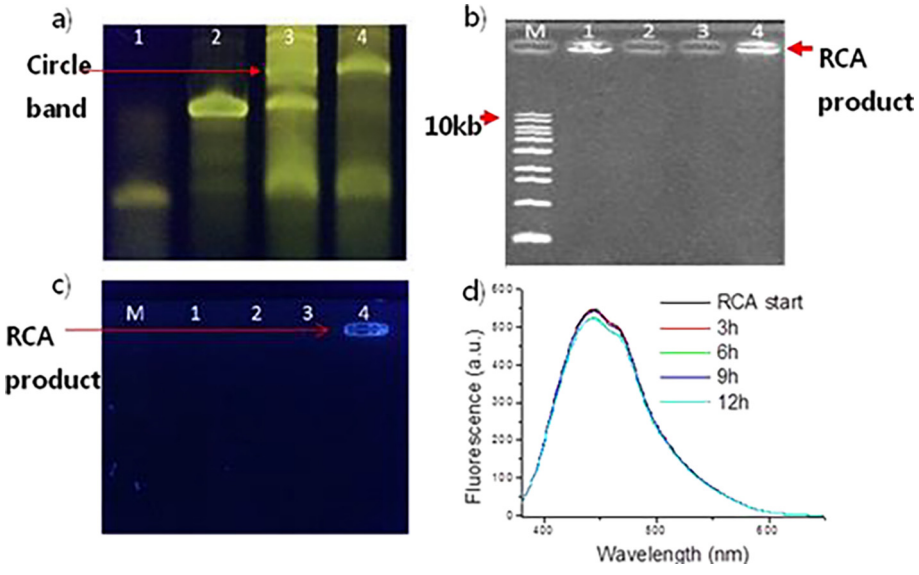


Fig. 1. RCA reaction using **dUpyrTP**, **dUanthTP**, and **dUthioTP**. **a)** Denaturing polyacrylamide gel data to confirm the ligated reaction. Lane 1: **miRNA 24-3P**; Lane 2: template **RCT 1**; Lane 3: **miRNA 24-3P + RCT 1** ligated with T4 ligase; Lane 4: **miRNA 24-3P + RCT 1** ligated with T4 ligase and treated with Exo I nuclease. **b)** and **c)** Agarose gel data, confirming the success of the RCA reaction (see Supporting Information for detail RCA reaction condition). M: Marker 1 kb; lane 1: **miRNA 24-3P** + **RCT 1** + T4 ligase and RCA reaction with dNTP; lane 2: RCA reaction with **dUpyrTP**, dATP, dCTP, dGTP; lane 3: RCA reaction with **dUantTP**, dATP, dCTP, dGTP; lane 4: RCA reaction with **dUthioTP**, dATP, dCTP, dGTP. **d)** Time dependent fluorescence spectra to determine the effect of **dUthioTP**. All RCA reactions were prepared with dNTP and including 5 mM dUthio. All samples were excited at 354 nm.

Please cite this article in press as: Le B.H., Seo Y.J. Bioorg. Med. Chem. Lett. (2018), https://doi.org/10.1016/j.bmcl.2018.04.058

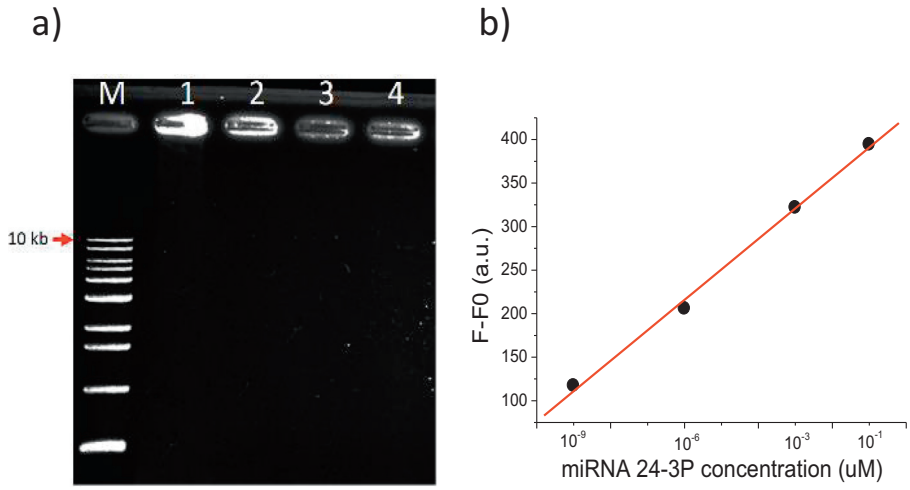


Fig. 2. Sensitivity data of **dUthioTP-RCA** system for the detection of **miRNA24-3P**. a) Agarose gel data confirming the RCA reaction (M: Marker 1 kb; Lane 1: 10^{-1}; Lane 2: 10^{-3}; Lane 3:10^{-6}; Lane 4: 10^{-9} μM of target **miRNA 24-3P**; b) concentration-dependent (10^{-9}, 10^{-6}, 10^{-3} and 10^{-1} μM of target **miRNA 24-3P**) fluorescence data using **dUthioTP-RCA**. All samples were excited at 354 nm. Fluorescence signal change ($F-F_0$), where F (with target **miRNA 24-3P**) and F_0 (without target **miRNA 24-3P**) are the fluorescence intensities from the RCA product.

small-sized fluorescent nucleotide could be recognized by the phi 29 DNA polymerase and may be used for the direct labeling of the RCA reaction.

For the direct labeling, we examined the fluorescence properties of this **dUthioTP-RCA** system (Fig. 1c). Interestingly, it showed a fluorescence signal corresponding to DNA amplification product. Interestingly, we also observed the DNA gel formation during the RCA reaction. Thus, we checked if this fluorescence signal is from the incorporation and extension of our **dUthioTP** during the rolling circle amplification or just from the simple interaction of **dUthioTP** monomer with the DNA gel. To demonstrate this, we simply added dUthio nucleoside monomer with dNTP during the rolling circle amplification. However, we could not observe any increased fluorescence (Fig. 1d). From this experiment, we confirmed that the fluorescence is from the incorporation and extension of **dUthioTP** into the RCA DNA.

Next, to evaluate the sensitivity of this **dUthioTP-RCA** probing system, we measured the fluorescence spectra depending on

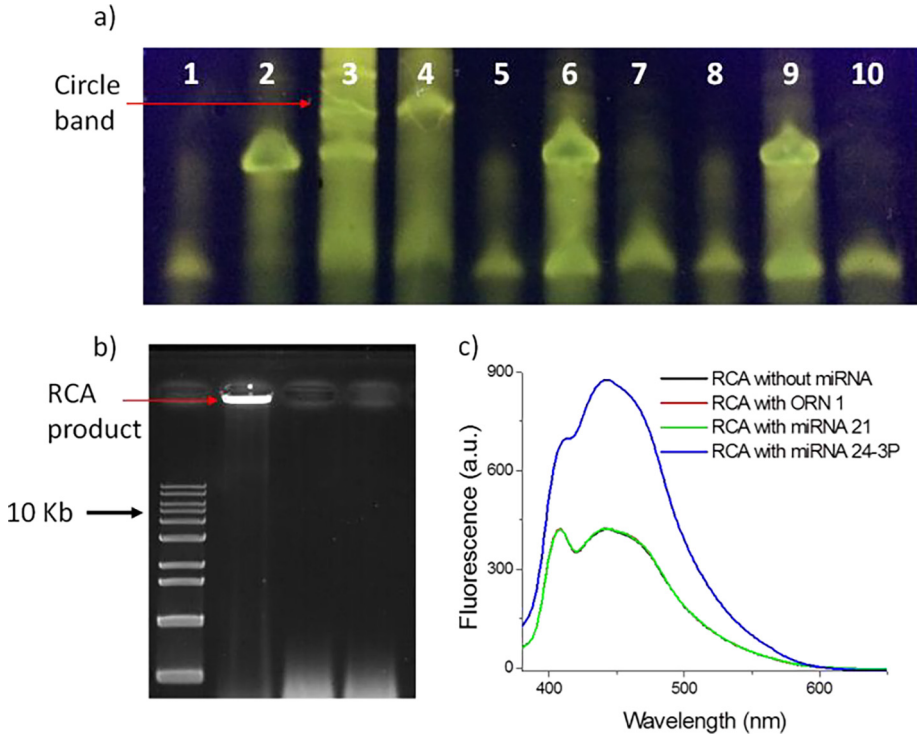


Fig. 3. Selectivity data of **dUthioTP-RCA** for the detection of **miRNA 24-3P**. **a**) Denaturing polyacrylamide gel data confirming the circular DNA structure of **RCT 1** (lane 1: **miRNA 24-3P**, lane 2: **RCT 1**; lane 3: **miRNA 24-3P** + **RCT 1** + T4 ligase; lane 4: **miRNA 24-3P** + **RCT 1** + T4 ligase + Exo I nuclease; lane 5: **ORN 1**; lane 6: **ORN 1** + **RCT 1** + T4 ligase; lane 7: **ORN 1** + **RCT 1** + T4 ligase + Exo I nuclease; lane 8: **miRNA 21**; lane 9: **miRNA 21** + **RCT 1** + T4 ligase; lane 10: **miRNA 21** + **RCT 1** + T4 ligase + Exo I nuclease. **b)** Agarose gel data, confirming the success of the RCA reaction (M: Marker 1 Kb; lane 1: **miRNA 24-3P** + **RCT** 1 + T4 ligase + phi 29 DNA polymerase; lane 2: **ORN 1** + **RCT** 1 + T4 ligase + phi 29 DNA polymerase; lane 3: **miRNA 21** + **RCT 1** + T4 ligase + phi 29 DNA polymerase). **c**) Fluorescence spectra of **dUthioTP-RCA** system alone and in the presence of the target **miRNA 24-3P**, mismatched miRNA (**ORN 1)** and random target (**miRNA 21**). All samples were excited at 354 nm.

Please cite this article in press as: Le B.H., Seo Y.J. Bioorg. Med. Chem. Lett. (2018), https://doi.org/10.1016/j.bmcl.2018.04.058

different amounts of **miRNA24-3P** from uM to aM. To measure this, we checked whether the RCA product depends on the concentration of **miRNA 24-3P** (Fig. 2a). Figure 2a shows that the amount of RCA product depends on the amount of **miRNA24-3P**. We also measured that the fluorescence spectra, with the expectation that the **dUthioTP** incorporation induces different fluorescence signals depending on the concentration of **miRNA24-3P** (Fig. 2b). Figure 2b exhibited a clear increased fluorescence signal even using a low concentration of **miRNA24-3P**. From this result we confirm that our direct incorporation of **dUthioTP** with the RCA probing system could discriminate the signal without and with the target **miRNA 24-3P** even at low concentrations.

Finally, to assess specificity, we designed and synthesized **ORN 1** and **miRNA21** as a one base mismatch and other miRNA sequences. Figure 3a shows the selective circular DNA formation. We observed circular DNA formation with **miRNA24-3P**, while we could not observe circular DNA formation with **ORN 1** and **miRNA 21**. We also observed RCA product with **miRNA24-3P**, but we could not observe any RCA product with **ORN 1** and **miRNA21** sequences, which shows clear evidence of the selective detection of target **miRNA24-3P**. Finally, we measured the fluorescence spectra to confirm the selectivity, and we could not observe any fluorescence signal increase with **ORN 1** or **miRNA21**, while we could observe a dramatic fluorescence increase with target **miRNA24-3P**. From all of these results, we believe that our direct labeling system using **dUthioTP-RCA** could be used for the detection **miRNA24-3P** with simple processing and low cost.

In conclusion, to achieve a simple process for the detection of **miRNA 24-3P**, we tried to develop a fluorescent nucleotide that is recognized by phi29 DNA polymerase. For this purpose, we designed and attached several different sizes of fluorescent substrate at the 5′ position of deoxy uridine bases such as thiophene (**dUthioTP**), anthracene (**dUanthTP**), and pyrene (**dUpyrTP**). We screened the incorporation and extension capability of these fluorescent nucleotides during rolling circle amplification using phi 29 DNA polymerase. Among these, only **dUthioTP** exhibited a high incorporation and extension capability into the rolling circle DNA product. This **dUthioTP-RCA** system could be used to detect the **miRNA 24-3P**, which is related with PRRSV. We also examined the sensitivity and selectivity using this **dUthioTP-RCA** probing system and confirmed that this system could detect **miRNA 24-3P** with high sensitivity. We also observed that this **dUthioTP** probing system could discriminate the **miRNA 24-3P** from one base (**ORN 1**) and random **miRNA 21**. Thus, we believe that **dUthioTP-RCA** probing system is very efficient for the detection of **miRNA 24-3P** with high sensitivity and selectivity, and does not require any other extra labeling step, which reduce a complicated time-consuming process and high cost.

Acknowledgments

We thank the NRF project of Korea (2017R1A2B4002398) for financial support.

A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmcl.2018.04.058.

References

1. Ambros V. *Cell*. 2001;107:823.
2. Bartel DP. *Cell*. 2004;116:281.
3. Harfe BD. *Curr Opin Genet Dev*. 2005;15:410.
4. Esquela-Kerscher A, Slack FJ. *Nat Rev Cancer*. 2006;6:259.
5. Tricoli JV, Jacobson JW. *Cancer Res*. 2007;67:4553.
6. Zhang Y, Yang Q, Wang S. *Cancer Chemother Pharmacol*. 2014;74:1105.
7. Rabinowits G et al. *Clin Lung Cancer*. 2009;10:42.
8. Lunney JK, Fang Y, Ladinig A, et al. *Annu Rev Anim Biosci*. 2016;4:129.
9. Shuqi X, Xue W, Huaibao N, et al. *J Virol*. 2015;89:4494.
10. Chen C, Ridzon DA, Broomer AJ, et al. *Nucleic Acids Res*. 2005;33:e179.
11. Raymond CK, Roberts BS, Garrett-Engele P, Lim LP, Johnson JM. *RNA*. 2005;11:1737.
12. Valoczi A, Hornyik C, Varga N, Burgyan J, Kauppinen S, Havelda Z. *Nucleic Acids Res*. 2004;32:175.
13. Quavi AJ, Kindt JT, Gleeson MA, Bailey RC. *Anal Chem* 2011;83:5949.
14. Qinfeng X, Anping C, Ling FZ, Chun YZ. *Anal Chem*. 2012;84:10845.
15. Bin CY, Yu QL, Bang CY. *J Am Chem Soc*. 2012;134:5064.
16. Mohsen MG, Kool ET. *Acc Chem Res*. 2016;49:2540.
17. Zhou Y, Huang Q, Gao J, Lu J, Shen X, Fan C. *Nucleic Acids Res*. 2010;38:e156.
18. Taku M, Jun S, Makoto K. *Nucleic Acids Res*. 2009;37:19.
19. Jonstrup SP, Koch J, Kjems J. *RNA*. 2006;12:1747.
20. Mestdagh P, Feys T, Bernard N, et al. *Nucleic Acids Res*. 2008;143:32.
21. Wang L, Luo YZ, Zhang L, Jiao XM, Wang MB, Fan YL. *Nucleic Acids Res*. 2008;36:149.
22. Li J, Deng T, Chu X, et al. *Anal Chem*. 2010;82:2811.
23. Hong C, Baek A, Hah SS, Jung W, Kim DE. *Anal Chem*. 2016;88:2999.
24. Le BH, Koo JC, Joo HN, Seo YJ. *Bioorganic Med Chem*. 2017;25:3591.
25. Hurley DJ, Tor Y. *J Am Chem Soc*. 2002;124:3749;
Mahara A, Iwase R, Sakamoto T, Yamana K, Yamaoka T, Murakam A. *Angew Chem Int Ed*. 2002;41:3648;
Kim IS, Seo YJ. *Bioorg Med Chem Lett*. 2014;24:1589;
Park JW, Seo YJ, Kim BH. *Chem Commun*. 2014;50:52;
Kim IS, Seo YJ. *Tetrahedron Lett*. 2014;55:1461.
26. Guntas G, Hallett RA, Zimmerman SP, et al. *Proc Natl Acad Sci USA*. 2015;112:112.
27. Robin MP, O'Reilly RK. *Chem Sci*. 2014;5:2717;
Noé MS, Sinkeldam RW, Tor Y. *J Org Chem*. 2013;78:8123.
28. Hollenstein M. *Molecules*. 2012;17:13569.