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Lab in a Tube: Sensitive Detection of MicroRNAs in Urine Samples from Bladder Cancer Patients Using a Single-Label DNA Probe with AIEgens

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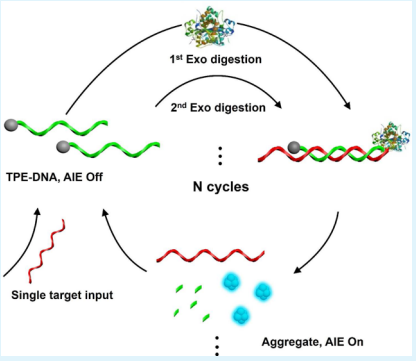
Supporting Information

ABSTRACT: We demonstrate an ultrasensitive microRNA detection method based on an extremely simple probe with only fluorogens but without quencher groups. It avoids complex and difficult steps to accurately design the relative distance between the fluorogens and quencher groups in the probes. Furthermore, the assay could accomplish various detection limits by tuning the reaction temperature due to the different activity of exonuclease III corresponding to the diverse temperature. Specifically, 1 pM miR-21 can be detected in 40 min at 37 °C, and 10 aM (about 300 molecules in 50 μL) miR-21 could be discriminated in 7 days at 4 °C. The great specificity of the assay guarantees that the real 21 urine samples from the bladder cancer patients are successfully detected by our method.



KEYWORDS: *AIEgens, DNA, miR-21, exonuclease III, urine samples*

INTRODUCTION

Abnormal expressions of microRNAs have been implicated in various vital disease states.[1−3] Detection microRNAs, therefore, are crucial in early detection of diseases, for example, varieties of cancers in recent years.[4−7] Fluorescent bioprobes are powerful tools for qualitative and quantitative analytical sensing for microRNAs, which could be divided into two categories according to the ratio between the targets (microRNAs[8−11]) and the probes (DNA[12−19] and RNA probes[20−22]). Initially, each target strand hybridizes with only a single copy of the probe,[23−27] and then the mission of the target is over. Thus, the ratio between the targets and the probes is 1:1. In addition to that, polymerase chain reaction (PCR) is a milestone[28] in the microRNA detection, and isothermal amplification assays have also been established recently.[29−36] The ratio between the targets and the probes then sharply tuned to 1:N (N > 1), which distinctively enhances the detection limit to single molecule or single cell level detection. Unfortunately, almost all of the bioprobes in the above assays contain both fluorogens and quencher groups. The target binding with the probe tunes the distance between the fluorogens and quencher groups, the fluorescence signals either increase (signal-on models) or decrease (signal-off models). Dual-modification (fluorogens and quencher groups) in the probe induces the difficulties for the probe-design and the complex probe-synthesis steps prone to false signals. Thus, simplification of the bioprobe structure is a potential solution to the above limitations.

Herein, we proposed an ultrasensitive one-pot microRNA detection assay that uses bioprobes with only fluorogens and without quencher groups' enzymatic amplification to overcome the aforementioned limitations in microRNA detection. Moreover, our proposed assay has high specificity, which is suitable for the direct detection of microRNAs in the urine samples from bladder cancer patients. The fluorogens modified with the DNA probe in our assay are the unique aggregation-induced emission (AIE) fluorogens, which emit very weakly in solutions but display strong fluorescence in their aggregate state.[37−39] AIE fluorogens are widely applied in biosensing and bioimaging fields due to their advanced features, such as, super absorptivity, high luminosity, and high photobleaching resistance.[40−44] Specifically, tetraphenylethylene (TPE), an iconic AIE fluorogen, is chemical modification with DNA

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probe using click-reaction (TPE-DNA composite probe in Figure 1a and Scheme S1 of the Supporting Information). S1

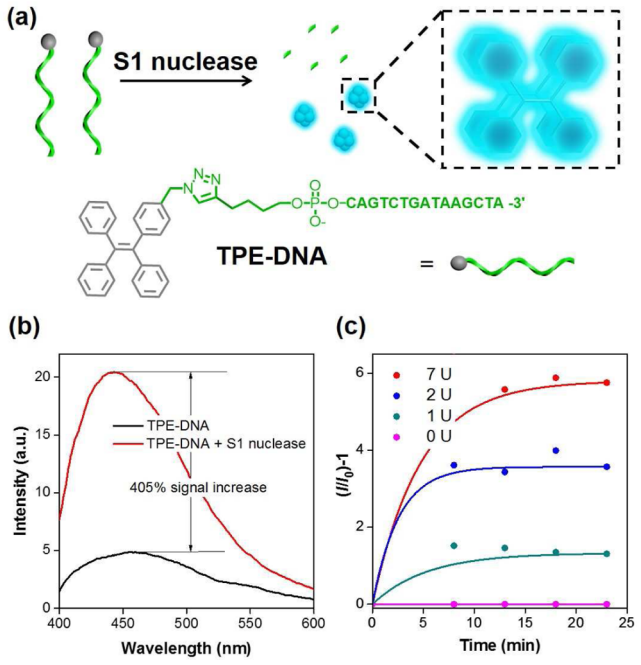

Figure 1. (a) TPE-DNA composite probe is composed of two parts, hydrophobic AIEgens and hydrophilic DNA, which is an amphiphile molecule. The DNA probe is only single labeled with florescence but without a quencher group. AIEgens are characterized by their propeller-shaped rotorlike structures, which undergo low-frequency torsional motions as isolated molecules and emit very weakly in solutions (the composite probe in buffer before being digested by S1 nuclease). Their aggregates show strong fluorescence mainly due to the restriction of their intramolecular rotations in the aggregate state (the composite probe in buffer after being digested by S1 nuclease). (b) The fluorescence intensity of the composite probe in the presence of S1 nuclease (2 U) is 405% higher than that in the absence of it. (c) The higher the concentration of enzyme, the faster the rate of signal increase rate.

Nuclease, a single-strand-specific endonuclease that hydrolyzes single-stranded RNA or DNA into 5′ mononucleotides, is chosen to test the bioactivity and biocompatibility of the composite probe. The DNA part of the composite probe is digested by S1 nuclease, and TPE and TPE with short DNA residues are released into the solution, which aggregate together due to the reduction of their solubility.

Then the aggregations result in the RIM process, which thus blocks the radiationless relaxation pathways of TPE and activates its fluorescence (Figure 1b and Figure S1 of the Supporting Information).[44] Furthermore, the process time is also dependent on the concentration of S1 nuclease (Figure 1c), which demonstrates that TPE modification does not affect the bioactivity and biocompatibility of DNA in the composite probe.

EXPERIMENTAL SECTION

Materials. Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl under nitrogen immediately prior to use. Sodium ascorbate, dimethyl sulfoxide (DMSO), copper(I) bromide, and acetonitrile were purchased from Aladdin and used as received without further purification. Water is purified by a Millipore filtration system. Other chemicals were purchased from Sigma-Aldrich and used as received without further purification. The exonuclease III, NEB buffer 1, RNase inhibitor, Thrombin, Bst DNA polymerase, S1 nuclease, and DEPC-treated water were purchased from TaKaRa Bio Inc. (Dalian, China; DEPC = diethylpyrocarbonate). The exonuclease III specifically catalyzes the stepwise removal of mononucleotides from 3′-hydroxyl termini of duplex DNA. RNA RNase inhibitor and RNase A form 1:1 complex, and then inhibit RNase activity. S1 nuclease is a single-strand-specific endonuclease that hydrolyzes single-stranded RNA or DNA into mononucleotides. MicroRNAs and other oligonucleotides were synthesized by Sangon Biotech (Shanghai, China). Patient samples were donated by the First Affiliated Hospital of Nanchang University.

Synthesis of TPE-DNA Conjugates via Click Reaction. The synthetic route to TPE-DNA is shown in Scheme S1 of the Supporting Information. TPE-N_3 was successfully synthesized according to the previously published procedures.[44] Mass Spectrum of TPE-N_3 is shown in Figure S2 of the Supporting Information. Click conjugation of custom oligonucleotides with TPE-N_3 was carried out by preparing an aqueous solution of alkyne-labeled custom oligonucleotide sequence (5′-alkyne-CAG TCT GAT AAG CTA-3′, thereafter named DNA-A 65 nmol) and preparing an aqueous solution of TPE-N_3 (325 nmol). Freshly prepared aqueous solution of sodium ascorbate (1495 nmol) was added to the mixture above, followed by cuprous bromide (747.5 nmol). The mixture was stirred overnight at room temperature before reverse HPLC purification. The product is obtained as a colorless solid in 32%, which were kept at −20 °C. Deionized water was added to dissolve the TPE-DNA to yield a stock solution with known concentrations.

DNA Cleavage Reaction with S1 Nuclease. A total volume of 50 μL of reaction mixture containing TPE-DNA (1.0 μM), 1X SI buffer, and different amounts of S1 nuclease was incubated at 37 °C for 8, 13, 18, and 23 min, respectively. Then, the reaction mixture was added to the buffer solution for fluorescence spectral measurements.

Amplified Detection of MicroRNAs. The reaction buffer is 1X NEB buffer 1. The exonuclease III amplification was performed in 50 μL of 1X NEB buffer 1 which contains 10 μM TPE-DNA probe, 300 U of exonuclease III, and varying concentrations of DNA target at 37 °C for 3 h.

For experiments at 4 °C, the exonuclease III amplification was performed in 50 μL of 1X NEB buffer, which contains 10 μM TPE-DNA probe, 300 U of exonuclease III, and varying concentrations of DNA target at 4 °C for 7 days.

RESULTS AND DISCUSSION

For the microRNAs detection process, exonuclease III is chosen in our assay, which catalyzes the stepwise removal of mononucleotides from 3′-hydroxyl termini of double strand DNA but does not with single strand DNA. More significantly, exonuclease III does not need a specific recognition site, therefore, our assay is distinctively different from other amplification methods using nicking endonucleases enzymes. For the proof-of-concept experiment, we selected miR-21, one of the first mammalian microRNAs identified, which is associated with a wide variety of cancers, including that of breast,[45] lung,[46] liver,[47] brain,[48] prostate,[49] pancreas,[50] and bladder.[51] The TPE-DNA composite probe is only modified with a TPE molecule at its 5′ terminus without the quencher group, which contains exonuclease III resistant 3′ protruding termini. At this stage, the solubility of the composite probe makes the TPE disaggregated with weak emission. The initial detection step begins from the hybridization between the miR-21 and the corresponding TPE-DNA composite probe, which induces the conformation change from random coil single-DNA (TPE-DNA probe) to duplex DNAs (TPE-DNA probe with miR-21) with a blunt 3′ terminus. Thus, exonuclease III catalyzes the stepwise removal of mononucleotides from 3′ terminus, releasing TPE molecules before finally liberating the

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miR-21. The liberated target then hybridizes with the second TPE-DNA probe, starting a new cycle. Therefore, a single copy of the miR-21 generates many TPE molecules, which get aggregated inducing the RIM process, and thus blocks the radiationless relaxation pathways of TPE and activates its fluorescence (Figure 2a).[63] Mass spectra of the composite probe before (Figure 2b) and after (Figure 2c) miR-21 detection illustrates that almost all of the composite probes are hybridized with miR-21 and then digested by exo III (Figure S3–S5 of the Supporting Information). After treatment with miR-21, particles with an average diameter of 529 nm are formed along with the increase of solution fluorescence (Figure S6 of the Supporting Information). This is further confirmed by confocal images as shown in Figure S7 of the Supporting Information.

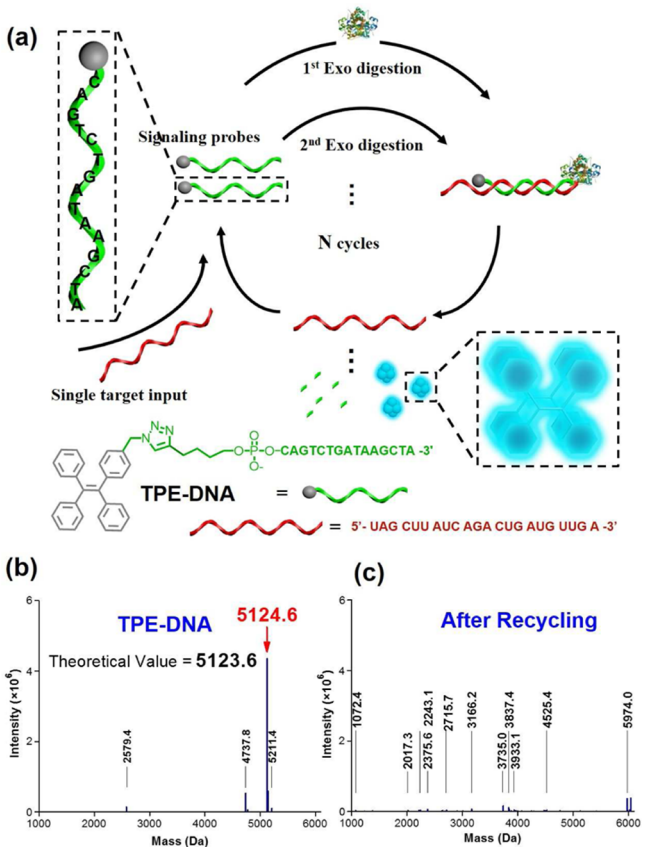

Figure 2. (a) The hybridization between the composite probe and miR-21 initiates the detection process. The exo III digests the composite probe and releases the intact miR-21, which will hybridize with another composite probe and begins the second cycle. The TPE is also liberated from the composite probe. Finally, a single copy of miR-21 generates many TPE molecules that get aggregated, inducing the RIM process, turning on the fluorescence signal. Mass spectra of the composite probe before [(b) the main peak at 5124.6 corresponding to the theoretical value of 5123.6)] and after [(c) there is no distinctive peak any more] the miR-21 detection, which illustrates that almost all of the composite probe are hybridized with miR-21 and then digested by exo III.

We first conducted the assay in 37 °C, which is the optimized working temperature for exonuclease III. Ten nanomolar miR-21 in the presence of the TPE-DNA probe and exonuclease III contributes a 485% increase in the final fluorescence signal. It is clear that this assay is dependent on the exonuclease III, as no signal increase is observed when it is omitted or replaced by other enzymes, such as thrombin and Bst DNA polymerase (Figure 3a). Under the above conditions, the TPE-DNA probe-based enzyme-assisted assay performs rapidly, responding to its target miR-21 within 40 min (Figure 3b).

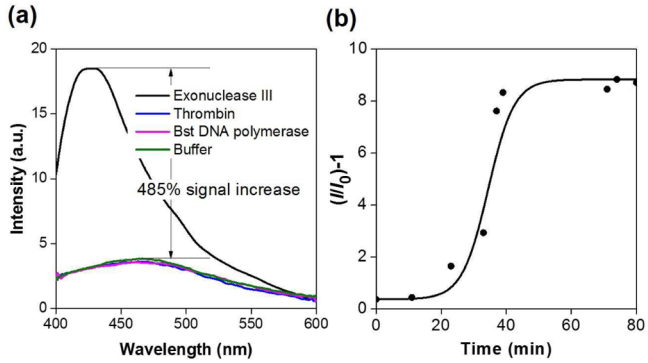

Figure 3. (a) It confirms that exo III induces the cycle detection, not the buffer, thrombin and Bst DNA polymerase. (b) The time-dependent curve shows that the miR-21 detection process is almost completed in 40 min at 37 °C.

The assay is sensitive (Figure 4a) and specific (Figure 4b). The calibration curve indicates a 1 pM detection limit within 40 min at 37 °C. The fluorescent intensity also increases with the miR-21. For the specificity evaluation test, the assay is challenged by 3 bases mismatched and even 1 base mismatched targets (Table S1 of the Supporting Information). The results show the signal for perfect match target is 245% better than that of 1 base mismatched targets, which indicates the great specificity of our assay.

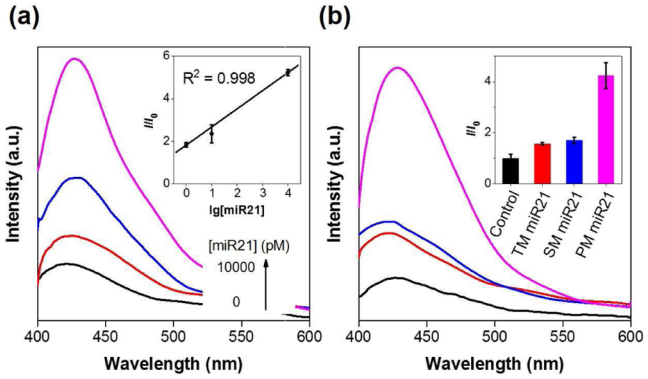

Figure 4. (a) Emission spectra in response to different concentration of miR-21 (0, 1, 10, and 10000 pM) at 37 °C. Inset: The calibration curve of miR-21 shows the detection limit is 1 pM. (b) The specificity curve reveals that the assay based on the TPE-DNA composite probe could discriminate the perfect matched targets and the single/triple mismatched targets. Inset: The column diagram of specificity. Error bars indicate standard deviation of triplicate tests.

Our previous results demonstrated that the residual exonuclease III activity against the single-stranded TPE-DNA probe increases our detection background and reduces our signal gain at 37 °C,[52] which is almost entirely abolished at 4 °C.[53] Although activity of the enzyme decreases at 4 °C, the reaction time is prolonged, so that the background fluorescence decreases distinctly. Therefore, the assay performs great with high signal gain, and the calibration curve shows the detect

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range from 10 aM to 1 fM, with a 10 aM detection limit corresponding to about 300 molecules in 50 μL (Figure 5a).

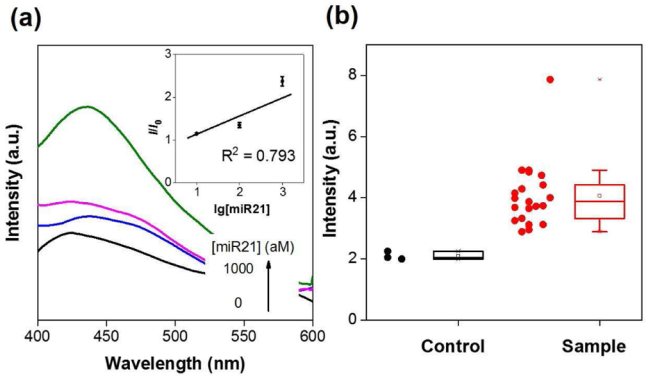

Figure 5. (a) Emission spectra in response to different concentration miR-21 (0, 10, 100, and 1000 aM) at 4 °C. Inset: The calibration curve of miR-21 shows the detection limit is 10 aM, corresponding to about 300 molecules in 50 μL. (b) 21 Urine samples from bladder cancer patients are tested by the TPE-DNA composite probe. 100% of the results show the positive results, which proves the specificity of our assay. Error bars indicate standard deviation of triplicate tests.

Although the sensitivity is highly enhanced in the lower temperature, it takes a much longer reaction time: 7 days for the detection limit of 10 aM at 4 °C in comparison with 40 min for 1 pM detection limit at 37 °C. The concentrations of microRNAs vary in different organs we could choose, corresponding to the reaction temperature to process the suitable assay.

Since our assay illustrates great sensitivity and specificity mainly due to the very simple structure of our TPE-DNA probe with only fluorogens but without quencher groups. The assay is challenged by the clinical samples: 21 urine samples from the bladder cancer patients which contain miR-21 (Figure 5b and Figure S8 and Table S2 of the Supporting Information). The results from our assay is fully consistent with that in the clinic, which shows the great potential for early cancer diagnosis with accuracy and selectivity.

CONCLUSION

In conclusion, we have demonstrated an ultrasensitive microRNA detection method based on an extremely simple probe with only fluorogens without quencher groups, which avoids the complex and difficult steps to accurately design the relative distances between the fluorogens and quencher groups in the probes. In addition, the assay could accomplish various detect limits by tuning the reaction temperature due to the different activity of exonuclease III, corresponding to the diverse temperature. Specifically, 1 pM miR-21 could be detected in 40 min at 37 °C, and 10 aM (about 300 molecules in 50 μL) miR-21 could be discriminated in 7 days at 4 °C. The super specificity of the assay guarantees that the real 21 urine samples from the bladder cancer patients are successfully detected by our method. Aside from the sensitivity and specificity, our assay requires only one step to realize cycle amplification for ultrasensitive detection of microRNAs, without any multiple self-assembly steps as required in fluorogens-and-quencher groups dependent amplification assays[54] or complicated operations as required in the PCR.[55] The novel assay based on TPE-DNA probes, therefore, should be a potential method for microRNA studies in the near future, for instance, the early diagnosis of vital pathema with the help of microRNAs.

ASSOCIATED CONTENT

Supporting Information

Detailed description of the experimental procedures, DNA sequences, comparison of fluorescence intensities for TPE-DNA in the presence of different amounts of S1 nuclease, mass spectrum of TPE-N_3, chemical structure and mass spectrum of TPE-DNA, mass spectrum of TPE-DNA after the presence of S1 nuclease and after the miR-21 detection, light scattering measurement of TPE-DNA after the miR-21 detection, confocal images of TPE-DNA in the absence and presence of miR-21, results of TPE-DNA-based miR-21 detection in real urine samples, and classification of patients' urine samples. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.5b04821.

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Author Contributions

‖X.M. and Y.Z. contributed equally.

Notes

The authors declare no competing financial interest.

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