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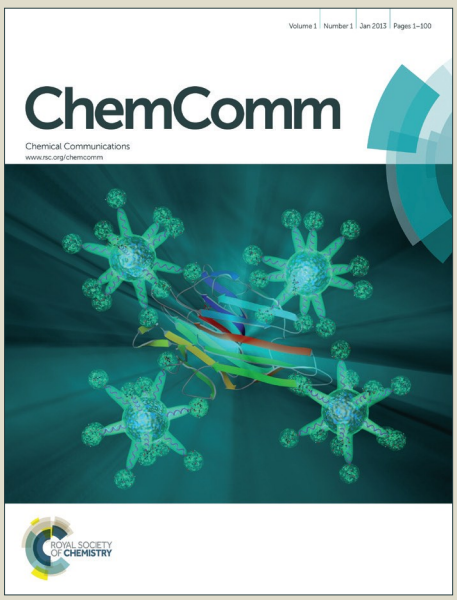

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ARTICLE TYPE

Multiplex Fluorophore Molecular Beacon; Detection of Target sequence Using Large Stokes Shift and Multiple Emission Signal Properties

Han Na Joo[a] and Young Jun Seo[a,b]*

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We have developed multiplex fluorophore Molecular Beacon (mfMB) with fluorophores located at its end to produce unique FRETs (Fluorescence Resonance Energy Transfer). It exhibited diverse fluorescence properties depending on the mixing pattern, such as large stokes shift emission and multiple colors, namely, blue, green and red using one excitation wave length. Our mfMB also worked in probing target perfect matched sequence with exonuclease III.

Fluorescence is one of the most popular and commonly used tools for molecular recognition in bioimaging thanks to its high resolution and sensitivity.[1] Molecular Beacon (MB) is a well-known detection tool for recognition of DNA or RNA with diverse applications such as real-time PCR, *in vitro* diagnostics and *in vivo* DNA and RNA imaging.[2] Traditional MB is composed of a hairpin loop targeting the DNA and a stem region to attach a linker and conjugate a fluorophore and a quencher,[3] or two fluorophores for FRET.[4] If the target DNA meets the MB probe, the hairpin structure is changed to the open state and the FRET is broken with an increasingly strong emission signal.

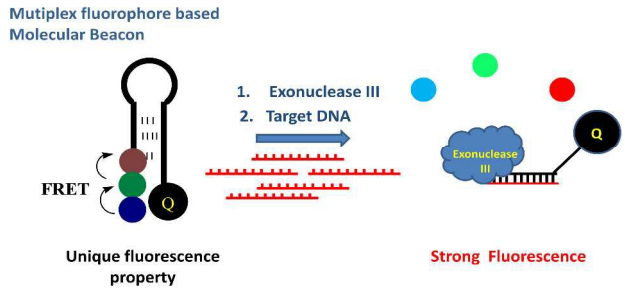
However, there are many limitations like low quantum yield of fluorophore, limited number of fluorophores to apply for multiple targets and a small stokes shift fluorescence property for *in vivo* imaging.[5]

Our goal was to design and develop a novel MB system that can exhibit a unique emission signal system using a limited number of fluorophores.

An alternative approach would be incorporation of multiple fluorophores with different mixing patterns to create photophysically or electronically distinct interactions because development of new fluorescence material is time-consuming and costly.[6] This mixed fluorescence system could be sensitive to environment changes such as conformational change of DNA and may incorporate unique fluorescence properties into the mfMB depending on the structural state.[7]

To make this unique multiplex fluorophore based MB (**mfMB**) system we used three dyes, namely, **dA^{py}** for blue signal that we had developed in our lab, **dT^{Fam}** for green signal and **dT^{TAMRA}(dT^{Tam})** for red signal. We choose **EBQ** as a universal quencher, which is provided by Bioneer company (Figure S1).

The excitation and emission λ_{max} wavelengths for **dA^{py}**, **dT^{Fam}** and **dT^{TAMRA}** are 386 nm, 465 nm, 550 nm and 450 nm, 520 nm, 580 nm, respectively. **dA^{py}** could be used as a donor for **dT^{Fam}** and **dT^{TAMRA}**. **dT^{Fam}** could be used as a bifunctional fluorophore being an acceptor from **dA^{py}** and a donor for **dT^{TAMRA}**. **dT^{TAMRA}** could only be used as an acceptor (Figure S2).



Scheme1. Schematic presentation of multiplexed fluorophore based Molecular Beacon (mfMB)

These fluorophores were mixed in different patterns for each MB to produce diverse fluorescence properties. We used exonuclease III(EXO 3)(an enzyme for specific duplex DNA degradation) to detect the target sequence.[8]

Our hypothesis was that our **mfMB** is not changed with single-stranded DNA because it is not degraded by exonuclease III, but if it binds the target sequence (perfect matched sequence) it works and degrades mfMB to exhibit an increased fluorescence signal.

Table 1. Designed oligonucleotide and **mfMB** systems

Name	Sequence
mfMB 1	dA^{py}dT^{Fam}-GCU GAG AAG TTA GAA CCT ATG CTC AGC-EBQ
mfMB 2	5'-dA^{py}dA^{py}dT^{Tam}-GCU GAG AAG TTA GAA CCT ATG CTC AGC-EBQ
mfMB 3	5'-dA^{py}dT^{Fam}dT^{Tam}-GCU GAG AAG TTA GAA CCT ATG CTC AGC-EBQ
mfMB 4	5'-dT^{Fam}dA^{py}dT^{Tam}-GCU GAG AAG TTA GAA CCT ATGCTCAGC-EBQ
ODN 1	5'-CAT AGG TTC TAA CTT
mfMBP	5'-dT^{Fam}dA^{py}dT^{Tam}-GCT GAG AAG TTA GAA CCT ATG CTC AGC TTT TTT T-**EBQ**
ODN R1	3'-CGA CTC TTC AAT CTT GGA TAC GAG TCG AAA AAA A-5'
ODN R2	3'-GGA CTC TTC AAT CTT GGA TAC GAG TCG AAA AAA A-5'
ODN R3	3'-GCA CTC TTC AAT CTT GGA TAC GAG TCG AAA AAA A-5'

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We synthesized various DNA MBs from **mfMB1** to **mfMB4** with different mixing patterns of $\mathbf{dA^{py}}$, $\mathbf{dT^{Fam}}$, and $\mathbf{dT^{TAMRA}}$ at the 5' end and **EBQ** at the 3' end as a universal quencher. This synthesis was made possible using general phosphoramidite chemistry and a solid-phase DNA synthesizer (Table 1).[9]

This procedure was simple because we incorporated several dyes into the MB system without any external conjugation step with a linker.[3]

First, we checked the UV/Vis absorption properties for each **mfMB** (Figure 1). Interestingly, for all cases, UV/Vis absorption data showed a strong acceptor fluorophore band. The observed λ_{max} for **mfMB1** was 445 nm, which is the characteristic band for $\mathbf{dT^{Fam}}$. The observed λ_{max} for **mfMB2** and **mfMB3** was 550 nm, which is the characteristic band for $\mathbf{dT^{TAMRA}}$. However, for **mfMB4**, we observed both 360 nm and 550 nm, which are for $\mathbf{dA^{py}}$ and $\mathbf{dT^{TAMRA}}$, respectively.

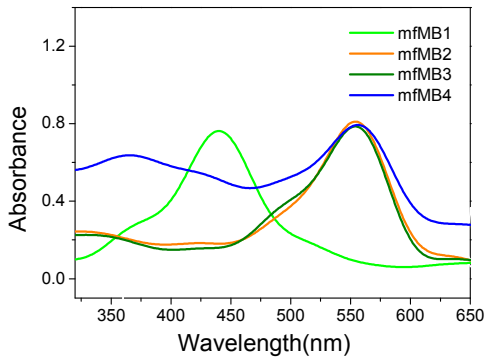

Fig 1. UV/Vis absorption property of **mfMB1**, **mfMB2**, **mfMB3**, and **mfMB4**. All DNA samples were prepared at the concentration of 1μM in 100mM Tris-HCl buffer in 25 °C at pH 7.2 (1mM $MgCl_2$)

Next, we tested the fluorescence properties of each **mfMB** (Figure 2). Interestingly, each **mfMB** exhibited different fluorescence patterns. **mfMB1** exhibited strong emission at 450 nm and 520 nm, which are characteristic bands for $\mathbf{dA^{py}}$ and $\mathbf{dT^{Fam}}$. This means no strong FRET from $\mathbf{dA^{py}}$ to $\mathbf{dT^{Fam}}$. **mfMB2** had three different bands, namely, 450 nm for $\mathbf{dA^{py}}$, 530 nm as a new band and 575 nm for $\mathbf{dT^{TAMRA}}$. **mfMB3** exhibited a 450 nm band for $\mathbf{dA^{py}}$ and almost no band at 520 nm ($\mathbf{dT^{Fam}}$) and a strong band at 575 nm for $\mathbf{dT^{TAMRA}}$.

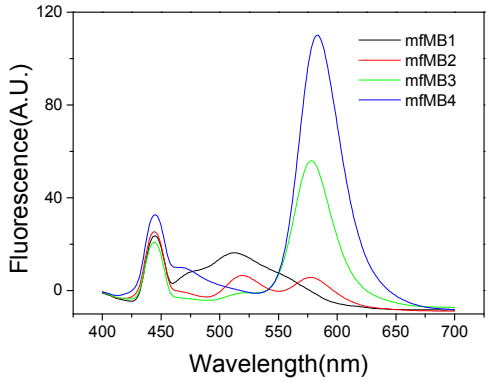

Fig 2. Fluorescence spectra of **mfMB1**, **mfMB2**, **mfMB3**, and **mfMB4**. All DNA samples were prepared at the concentration of 1μM in 100mM Tris-HCl buffer in 25 °C at pH 7.2 (1mM $MgCl_2$). All samples were excited at 386nm

mfMB4 had a weak band at 450 nm for $\mathbf{dA^{py}}$ and strong band at 575 nm for $\mathbf{dT^{TAMRA}}$ but no $\mathbf{dT^{Fam}}$ signal at 520 nm. From these data, we postulated a weak FRET from $\mathbf{dA^{py}}$ to $\mathbf{dT^{Fam}}$ and a strong FRET from $\mathbf{dT^{Fam}}$ to $\mathbf{dT^{TAMRA}}$.

Based on these findings, we tested for the possibility of an MB with target sequence. We added **ODN1**, which is a 15mer perfect matched sequence to each **mfMB.** However, most MB systems did not show a dramatic fluorescence change (Figure 3). We could not obtain a dramatic discriminated emission signal in duplex (open state) with target sequence compared to the hairpin state (closed state). We assumed that the distance between the fluorophore and the quencher is still close even in open state with FRET, which leads to the absence of discrimination.

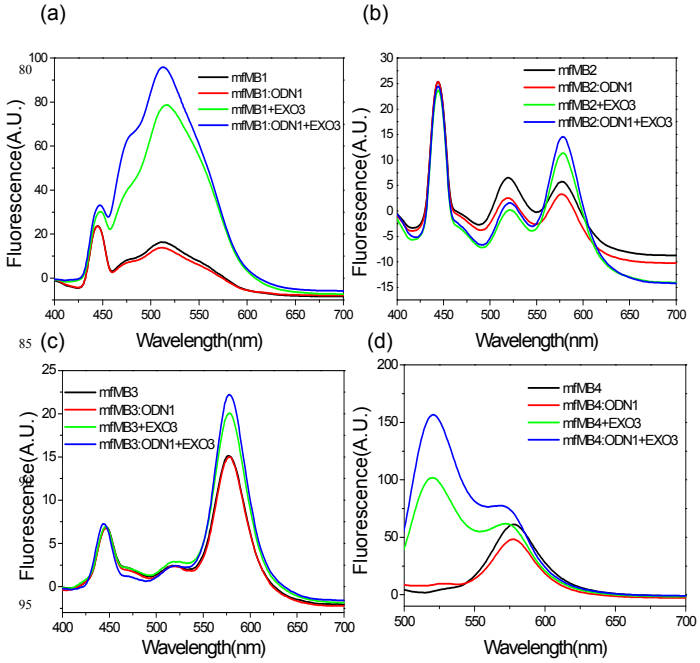

Fig 3. Fluorescence spectra of (a) **mfMB1**, **mfMB1:ODN1**, **mfMB1** with exonuclease III and **mfMB1:ODN1** with exonuclease III. (b) **mfMB2**, **mfMB2:ODN1**, **mfMB2** with exonuclease III and **mfMB2:ODN1** with exonuclease III. (C) **mfMB3**, **mfMB3:ODN1**, **mfMB3** with exonuclease III and **mfMB3:ODN1** with exonuclease III. (D) **mfMB4**, **mfMB4:ODN1**, **mfMB4** with exonuclease III and **mfMB4:ODN1** with exonuclease III. All DNA samples were prepared at the concentration of 1μM in 100mM Tris-HCl buffer in 25 °C at PH 7.2 (1mM $MgCl_2$). All samples were excited at 386nm except **mfMB4** which was excited at 485nm.

We also tested the Circular Dichroism and melting temperature to confirm a secondary structure of each **mfMB** with target compound **(mfMBs:ODN1)**. Circular Dichroism data showed duplex characteristics 250 nm negative and 275 nm positive bands with target oligonucleotide **ODN1** (Figure S5).[10]

The melting temperature was also tested to distinguish between the hairpin and duplex structures (Table 2). All samples had a melting temperature of around 37 °C for hairpin state (Figure S3) and 52 °C for duplex(Figure S4), which shows reasonable stability for hairpin and duplex structures.

Next, we considered treatment with exonuclease III, which is a specific enzyme for duplex degradation. This enzyme releases mononucleotides from the recognition site of 3'-hydroxyl termini of duplex in a stepwise manner.[8,11]

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We added this enzyme to the duplex state with target DNA and we observed a dramatic emission signal change (Figure 3). **mfMB1** exhibited an extremely increased fluorescence signal with a high discrimination factor (Figure 3a). **mfMB1** probe exhibited a large stokes shift signal. We used 386 nm for excitation and obtained a strong emission signal at 525 nm (139 nm stokes shift). **mfMB2** exhibited three emission signals, namely, 446 nm for $\mathbf{dA^{py}}$, 580 nm for $\mathbf{dT^{TAMRA}}$ and an extra band at 520 nm that could be from a pyrene excimer,[12] which is previously reported from our group (Figure 3b). This property is potentially applicable for using one excitation wavelength and reading three signals using different emission filters.

We observed a more dramatic signal pattern for **mfMB3** and **mfMB4**. They showed a dramatic FRET property especially from $\mathbf{dT^{Fam}}$ to $\mathbf{dT^{TAMRA}}$. For both **mfMBs** we did not observe $\mathbf{dT^{Fam}}$ signal but we observed a strong $\mathbf{dT^{TAMRA}}$ signal, which is an evidence of FRET from $\mathbf{dT^{Fam}}$ to $\mathbf{dT^{TAMRA}}$. Another unique signal pattern for these **mfMB** systems was a large stokes shift at 580 nm from the excitation at 386 nm (194 nm stokes shift). **mfMB4** showed a dramatic change after adding a target sequence with exonuclease III (Figure 3d). With an excitation at 485 nm we could observe a highly discriminated emission signal at 525 nm compared to the hairpin state (discrimination factor is 5.8 times).

Table 2. Absorption, Fluorescence, T_m and discrimination factor of the designed oligonucleotides.

Compound	Discrimination factor[a]	$\lambda_{ab\ max}$	$\lambda_{em\ max}$	T_m °C
mfMB 1	1.0	445	446,515	41.9
mfMB 1:ODN 1	0.7	445	446,515	52.6
mfMB 2	1.0	550	448,520,580	40.1
mfMB 2:ODN 1	0.9	550	448,520,580	51.5
mfMB 3	1.0	550	450,575	36.6
mfMB 3:ODN 1	0.9	550	450,575	52.4
mfMB 4	1.0	365,550	450,580	36.9
mfMB 4:ODN 1	0.8	365,550	450,580	51.5
mfMB 1+EXO3	1.0		446,515	N
MfMB 1:ODN 1+EXO3	1.3 at 515nm		446,515	N
mfMB 2+EXO3	1.0		448,520,580	N
MfMB 2:ODN 1+EXO3	1.5 at 580nm		448,520,580	N
mfMB 3+EXO3	1.0		450,575	N
mfMB 3:ODN 1+EXO3	1.2 at 580nm		450,575	N
mfMB 4+EXO3	1.0		450,520,580	N
MfMB 4:ODN 1+EXO3	1.7 at 525nm			N
mfMBP	1.0		450,520, 580	58.4
mfMBP:ODN R1	13.6 at 485nm		580	66.5
mfMBP:ODN R1+ EXO3	3.1 at 555nm		580	N

See supporting information for detail T_m conditions. N: No data. [a]: x-fold f luorescence enhancement in fluorescence intensity in the presence of the target.

Finally, we tested the discrimination factor between the **mfMB:ODN1** duplex compare to a background signal from cleavage of **mfMB** hairpin probe by exnuclease III(Table 2).[13,14] Our exonuclease III treated **mfMB:ODN1** systems still showed large discrimination factor from 1.2 to 1.7, while the discrimination factor is not observed for general MB system (Table 2).

To increase a discrimination factor we designed and synthesized new Molecular Beacon(**mfMBP**) which has prolonged oligo T sequence at the end of hairpin stem structure based on **mfMB4** fluorophore combination (Table 1) and we checked discrimination factor with perfect matched sequence **ODN R1**. Interestingly, we could observed large enhanced signal to noise ratio with target sequence (discrimination factor 13.6 at 485 nm excitation)(Figure 4a and Figure S7).We also checked SNP(single nucleotide polymorphism)typing capability of this **mfMBP** with perfect matched sequence **ODN R1**, one base mismatched **ODN R2** and two base mismatched sequence **ODN R3** (Figure 4a and Figure S7). According to result, it shows large discrimination factor with perfect matched sequence relative to mismatched sequences. And also we measured amplified fluorescence DNA detection capability using exonuclease III.[13] It was demonstrated that exonuclease III based MB system (**mfMBP**) could be used for amplified DNA detection even in femto molar concentration of target sequence (discrimination factor 3.1 at 555nm excitation) (Figure 4b, Figure S9).

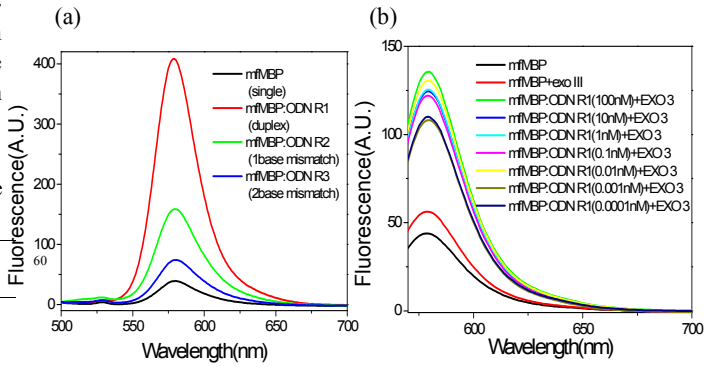

Fig 4. Fluorescence spectra of (a) **mfMBP**, **mfMBP:ODN R1**(Perfect match), **mfMBP:ODN R2**(1 base mismatch), **mfMBP:ODN R3**(2 base mismatch) at 485nm excitation, and (b) **mfMBP** with different concentration of target sequence(**ODN R1**) in exonuclease III at 555nm excitation. All DNA samples were prepared at 100nM for (a) and different concentration for (b) in 100mM Tris-HCl buffer at 25°C at PH 7.2(1mM MgCl2).

Conclusions

We made **mfMBs** which exhibit a diversity of new fluorescence properties with a limited number of fluorophores that is not shown in individual monomeric fluorophores. This simple mixing approach of fluorophore produce novel fluorescence properties by shuffling the fluorophores at the 5' end of MB DNA. They showed a wide range of FRET properties including large stokes shifts and multiple fluorescence properties at single excitation wavelength. This **mfMB** system could also detect the perfect matched target sequence using exonuclease III. We could observe different FRET patterns depending on the mixing order at the hairpin stem region. When these structures bind the target and forms a duplex, they could be degraded by exonuclease III that can specifically cleave the duplex DNA. It broke the FRET pattern and showed a diversity of signal changes. This approach is unique and the first reported on **mfMB** with exonuclease III. It creates diverse fluorescence properties and could expand the limitation of fluorephore numbers in MB signalling systems.

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Notes and references

[a] *Department of Chemistry, Chonbuk National University, Jeonju 561-756, South Korea. Fax: +82-63-270-3408; Tel.: +82-63-270-3417; E-mail: yseo@jbnu.ac.kr*
[b]*Department of Bioactive Material Sciences, Research Center of Bioactive Materials, Chonbuk National University, Jeonju 561-756, South Korea.*

†Electronic Supplementary Information (ESI) available: The sample preparation details, UV absorption, fluorescence spectra for both the monomers and oligonucleotides, and melting temperature for each **mfMBs** system.

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