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ORIGINAL PAPER

Investigation of the hybrid molecular probe for intracellular studies

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Abstract Monitoring gene expression in vivo is essential to the advancement of biological studies, medical diagnostics, and drug discovery. Adding to major efforts in developing molecular probes for mRNA monitoring, we have recently developed an alternative tool, the hybrid molecular probe (HMP). To optimize the probe, a series of experiments were performed to study the properties of HMP hybridization kinetics and stability. The results demonstrated the potential of the HMP as a prospective tool for use in both hybridization studies and in vitro and in vivo analyses. The HMP has shown no tendency to produce false positive signals, which is a major concern for living cell studies. Moreover, HMP has shown the ability to detect the mRNA expression of different genes inside single cells from both basal and stimulated genes. As an effective alternative to conventional molecular probes, the proven sensitivity, simplicity, and stability of HMPs show promise for their use in monitoring mRNA expression in living cells.

Keywords DNA detection · Fluorescence probes · Molecular beacons · Fluorescence resonance energy transfer (FRET) · mRNA monitoring

Introduction

DNA/mRNA detection techniques play an important role in disease diagnosis and gene expression studies. Traditional techniques, such as northern hybridization, reverse transcriptase polymerase chain reaction (RT-PCR), and southern blotting are time consuming and, in most cases, can only yield an average of millions of cells. To improve upon these methods, fluorescence probes, such as molecular beacons (MBs) and the hybrid molecular probe (HMP), have been developed and promise to have a significant impact in bioanalysis and biotechnology, including drug discovery, disease diagnosis, and gene expression studies [1–5].

MBs are single stranded DNA probe molecules that consist of a stem and loop structure [6–8]. The loop sequence is complementary to the single-stranded target DNA or RNA. The stem portion consists of five to seven base pairs complementary to itself such that, prior to binding target DNA sequences, the structure is in the closed state [8]. A fluorophore is covalently linked to one end, and a quencher is attached to the other end. The fluorophore and the quencher are kept in close proximity by the stem region of the MB. However, when a target hybridizes to the MB, the probe undergoes a conformational change resulting in the separation of the fluorophore and quencher, and this, in turn, results in the restoration of fluorescence.

These new molecular probes offer several advantages over traditional DNA/mRNA techniques. First, they have the ability to perform rapid and fully homogeneous analysis that requires no additional manipulation other than mixing the sample and test solution [9, 10]. Second, recognition of the target and optical reporting occur simultaneously, which is an advantage for homogeneous high-throughput assays and real-time analysis. Third, as a result of DNA hybridization to complementary sequences, which permits the detection of a single base mismatch, they are highly selective [11, 12]. On the other hand, the use of MBs for mRNA/DNA hybridization studies has several drawbacks. For example, it has been reported that the hairpin structure

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of MBs is not static and can fluctuate between different conformations [13]. The effectiveness of MBs is also hindered by degradation from cytoplasmic nucleases, which results in destabilization arising from protein interactions and false positive signals [9, 11]. Finally, MBs can simply open momentarily because of thermodynamic fluctuations, which is a flaw that results in a fluorescence background signal that can limit the sensitivity of the probe. To address these limitations, we have developed an alternative probe, the hybrid molecular probe (HMP), for gene expression studies [1]. In this paper, we report the investigation of the HMP for in vivo applications. In addition, a comparison between HMP and probes currently used for intracellular measurement was conducted.

In considering the requirements of molecular probes for intracellular mRNA monitoring, we developed an alternative probe, HMP, for single living cell studies. HMPs consist of two single strands of DNA with a polyethylene glycol (PEG) linker used to tether these two sequences together. A fluorescence donor is attached on the 3′ end of one DNA strand, and another fluorophore, acting as an acceptor, is attached to the 5′ end of the other strand. In the random unhybridized conformation of the probe, the two fluorophores are spatially separated from each other. However, when the target sequence containing the complementary sequences to both probes at adjacent positions is added, each strand binds to its corresponding target sequence, thus bringing the two fluorophores into close proximity, which allows fluorescence resonance energy transfer (FRET) to occur. It is this transfer of energy which results in the quenching of the donor fluorescence and corresponding fluorescence enhancement of the acceptor fluorophore. The working principle of the probe is shown in Fig. 1.

In this paper, HMP performance is further studied and then compared with the performance of MBs and linear probes under similar conditions. These experiments demonstrate the advantages of HMPs over MBs and linear probes for use in either hybridization studies or intracellular analysis inside single living cells.

Material and methods

Probe preparation

Molecular probes were designed based on the MnSOD and β-actin gene sequences. In addition, β-tubulin sequence from *Aplysia* genes, which are highly expressed within *Aplysia* cells, was used. The synthesized sequences are shown in Table 1. All the DNA reagents for the synthesis of HMP-tubulin, HMP β-actin, HMP MnSOD, MB-tubulin, MB-MnSOD, and targets were purchased from Glen Research. These probes and DNA targets were synthesized with an ABI3400 DNA/RNA synthesizer. FAM core pore glass (CPG) was used for all FAM-labeled probe synthesis, whereas Cy5 was labeled using Cy5 phosphoramidite. Phosphoramidite allows for direct labeling of synthetic oligonucleotides, which are generally used for the synthesis of short nucleic acids sequences. Specifically, spacer phosphoramidite 18 was used to incorporate PEG as a linker in the HMP probe. The MBs were synthesized using dabcyl CPG and FAM phosphoramidite. Based on the manufacturer's recommendations, sensitively labeled reagents, such as Cy5, require the use of bases with special protecting groups. Therefore, for all Cy5-labeled probes, monomers with phenoxyacetyl-protected dA, 4-isopropyl-phenoxyacetyl-protected dG, and acetyl-protected dC were used for the synthesis. Subsequently, deprotection of these monomers, along with the Cy5-labeled phosphoramidite, was performed using overnight incubation with 0.05 M K_2CO_3/methanol. The solutions that resulted from deprotection were then precipitated in ethanol, and the precipitates were dissolved in 0.5 mL of 0.1 M triethylammonium acetate (pH 7.0) for further purification with reversed-phase high-pressure liquid chromatography (RP-HPLC). RP-HPLC was performed on a ProStar HPLC Station (Varian, CA) equipped with a fluorescent and photodiode array detector. A C18 reversed-phase column (Alltech, C18, 5 μM, 250×4.6 mm) was used for separation purposes.

The reference probe, as well as the control MB (designs were based on published mRNA sequences), were synthe-

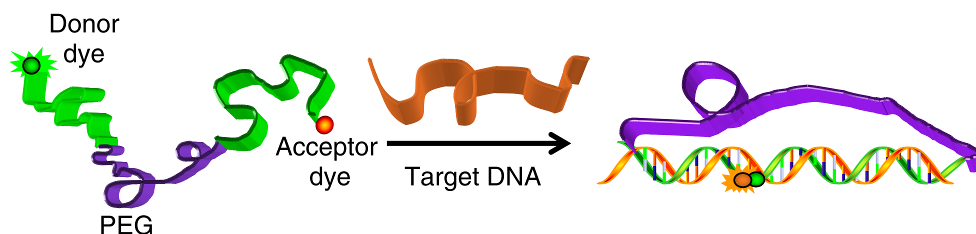


Fig. 1 Hybrid molecular probe (HMP). HMPs consist of two single strands of DNA (*green*) and a polyethylene glycol (PEG, *purple*) linker that is used to tether these two sequences together. A fluorescence donor is attached on the 3′ end of one strand (*green*), and another fluorophore, acting as an acceptor, is attached to the 5′ end of the other strand (*orange*). In the random unhybridized conformation of the probe, the two fluorophores are apart from each other. However, when a target (*orange* strand) containing the complementary sequences to both probes at adjacent positions is added, each strand binds to its corresponding target sequence, thus bringing the two fluorophores into close proximity, which allows energy transfer to occur

Table 1 Sequences used for the synthesis of the probes

	Sequences
Probes	
HMP Tub	5′-CY5-CTC ATT TTG CTG ATG ACG-$(X)_{16}$-TGT CTG GGT ACT CCT CC-FAM-3′ where X represent PEG
HMP MnSOD	5′-Cy5-TCT TAC ATT GAC -$(X)_{16}$-TTA GTT GAC CCC-FAM-3′
HMP β-actin	5′-Cy5AGA GCG CCT CAG GGC-$(X)_{16}$-GGA AGG AAG GCT GGA-Oregon green-3′
MB Tub	5′-FAM-CGC ACC TCC TCC CTC ATT TTG CTG GGT GCG-dabcyl-3′
MB MnSOD	5′ FAM-CCG AGC CAG TTA CAT TCT CCC AGT TGA TT G CTC GG-dabcyl -3′
MB control	5′-AF555-CCT AGC TCT AAA TCG CTA TGG TCG CGC TAG G-BHQ2-3′
Reference Probe	5′- TCT AAA TCG CTA TGG TCG C-AF488-3′
Targets	
β-Tubulin	5′-GCT CAT CAG CAA AAT GAG GGA GGA GTA CCC AGA CAG-3′
β-Actin	5′- GCC CTG AGG CGC TCT TCC AGC CTT CCT TCC-3′
MnSOD	5′-GTC AAT GTA AGA GGG TCA ACT AA- 3′

sized by Genomechanix (Gainesville, FL). Alexa Fluor 488 (AF488) and Alexa Fluor 555 (AF555) were purchased from Molecular Probes (Eugene, OR). The quencher used for the control MB was Blackhole Quencher 2 (BHQ2).

Spectrometer measurements

The in vitro experiments were performed using a SPEX Fluorolog spectrofluorometer from Horiba Jobin Yvon (Fisher Scientific Co., Pittsburgh, PA). The buffer for in vitro hybridization experiments consisted of 20 mM Tris, 50 mM of NaCl, and 5 mM of $MgCl_2$ with a pH of 7.5. The data were plotted and analyzed as the first derivative of the melting curve versus the temperature. The first derivative provides a mathematical formulation of the rate of change in fluorescence over the range of temperatures.

Thermal stability

The probe solutions were excited at 488 nm and monitored at 520 nm and 665 nm from 15 to 95 °C. Using a water bath (RTE-111 from Neslab), the melting temperatures were found for the respective hybridized probes. The instrument was settled in a way that increases by 1 °C and holds for 3 mins, reading the fluorescence at three different channels afterwards.

Cell preparation

MDA-MB-231 breast carcinoma cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's Modification of Eagle's Medium (DMEM, Fisher Scientific) with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 0.5 mg/mL Gentamycin (Sigma, St. Louis, MO) at 37 °C in 5% CO_2/air. Cells were plated in 35-mm glass-bottomed culture dishes and grown to 80% confluency (MatTek Corp., Ashland, MA) for 48 h prior to injection. To stimulate MnSOD mRNA expression, cells were incubated in 1 μg/mL lipopolysaccharide (LPS) from *E. coli* serotype 055:B5 (Sigma, St. Louis, MO) for 4 h prior to injection.

Fluorescence imaging

Fluorescence imaging was conducted with a confocal microscope setup consisting of an Olympus IX-81 inverted microscope with an Olympus Fluoview 500 confocal scanning system and three lasers, a tunable argon ion laser (458 nm, 488 nm, 514 nm), a green HeNe laser (543 nm), and a red HeNe laser (633 nm) with three separate photomultiplier tubes (PMTs) for detection. The cellular images were taken with a 40× 1.35 NA oil immersion objective. A Leiden microincubator with a TC-202A temperature controller (Harvard Apparatus, Holliston, MA) was used to keep the cells at 37 °C during injection and monitoring. An EXFO Burleigh PCS-6000-150 micro-manipulator was used for positioning the injector tip. An Eppendorf Femtojet microinjector with 0.5-μm Femtotips was used to inject the molecular probes and reference probe into the cells. The reference probe with AF488 was excited at 488 nm and collected at 520 nm. The control MB with AF555 was excited at 543 nm and collected at 570 nm. The Oregon green (donor)/Cy5 (acceptor) HMPs were excited at 488 nm and collected at 520 nm and 665 nm, respectively. Images were taken either 1 min for 15 min, or every 4 min for 60 min, including an initial image after a brief period during which the instrument was focused to yield the highest intensity for the probe. The images were assigned color representations and are not indicative of the actual emission wavelengths. The data collected from the confocal microscope were analyzed using the Fluoview analysis software. Ratiometric analysis of the HMP and single strands was performed by dividing the signal of Cy5 at 665 nm by the signal of Oregon green at 520 nm:

$$\text{Ratio} = I_{\text{Cy5 } 665\,\text{nm}} / I_{\text{FAM } 520\,\text{nm}}$$

where $I_{\text{Cy5 } 665\text{ nm}}$ and $I_{\text{FAM } 520\text{ nm}}$ refer to the intensities of Cy5 and FAM dyes that were collected at the 665 nm and 520 nm wavelengths, respectively.

On the other hand, the control MB data were analyzed using the following equation:

$$\text{Ratio} = (S_{\text{beacon}} - B_{\text{beacon}})/(S_{\text{reference}} - B_{\text{reference}})$$

where S_{beacon} is the signal of the beacon in the open state form (570 nm), $S_{\text{reference}}$ is the signal of the reference probe collected at 520 nm, and B_{beacon} and $B_{\text{reference}}$ are the backgrounds of the beacon and reference probe, respectively. The B_{beacon} and $B_{\text{reference}}$ signals were obtained from a region outside of the cell monitored in the 570-nm and 520-nm channels, respectively. Ratiometric analysis has the advantage of normalizing the fluorescent intensities by compensating for the instrumental and experimental variations inherent in intracellular analysis.

Results and discussion

Comparison of HMP with and without PEG as a linker

The main objective of this experiment was to investigate how the polymer linker affects HMP hybridization and equilibrium. To accomplish this, HMP for β-tubulin sequence from *Aplysia* genes was synthesized (Table 1). These genes are highly stable and highly expressed within *Aplysia* cells. Subsequently, the same sequence was used to synthesize the probes without PEG as a linker, i.e., two separate DNA probes. A calibration curve (Fig. 2) was performed for both DNA probes where different concentrations of targets were used. The concentration used for the probes was 300 nM. Figure 2 shows that HMP was able to detect concentrations as low as 0.70 nM target solution, whereas the single-stranded DNA probes only reached 7.2 nM. Also, these probes were found to differ in their respective dynamic ranges. Specifically, HMP, the probe with the linker, has a larger dynamic range than the probe without the linker. This is explained by the fact that the PEG linker allows the two DNA strands to remain together which allows binding of the HMP to the same target, even at high probe to target ratios. In contrast, the DNA strands without the PEG linker showed a decrease in fluorescence, even when the ratio of probe to target was 1:1. This response is explained by the tendency of the two single strand probes to bind to different targets sequences when the target is in excess, thereby decreasing the energy transfer of the single stranded probes.

On the contrary, the response of HMP, upon addition of various concentrations of targets, showed that the tethering of the probes facilitates the hybridization with the target. This behavior of HMP is explained as when one strand binds to the target the other strand becomes closer to the same target, allowing hybridization and therefore energy transfer.

Thermal stability

Evaluation of duplex melting temperature is one of the most widely used approaches to accurately and reliably predict the stability of DNA-based probes and targets along with

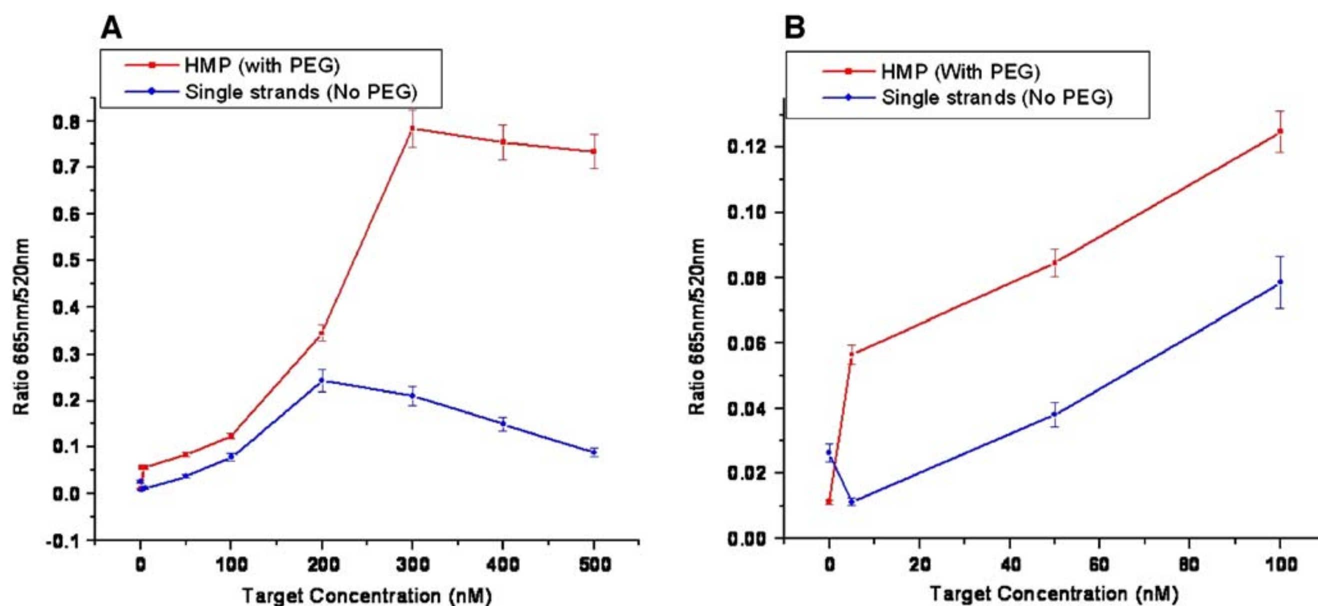


Fig. 2 Comparison of the influence of target concentration during hybridization between the single strand probe (no PEG linker) and HMP (with PEG linker). **a** HMP shows better hybridization efficiency at higher concentrations of target. **b** Hybridization of the probes at lower concentrations. Ratiometric analysis of the probes was performed by dividing the fluorescence signal of Cy5 at 665 nm by that of FAM at 520 nm. Three replicates were performed for the different target concentrations

the resultant duplex. Therefore, in order to evaluate the stability of the probes at room and physiological temperatures, the thermal stability of the probes was investigated. The DNA probes were hybridized using a 1:1 probe to target ratio at a final concentration of 300 nM. Both HMP and the two single DNA strand probes were excited at 488 nm and monitored at 520 nm and 665 nm. The fluorescence intensity for each probe was measured from 15 to 95 °C at intervals of 1 °C with holding times of 3 min. The melting temperature for both probes is shown in Fig. 3. The melting temperatures of the HMP and the probe with no linker were 67 and 30 °C, respectively. The higher melting temperature of the HMP indicates increased stability over the probe without the polymer linker, especially at physiologically relevant temperatures. Since PEG dramatically increases the local concentration of one DNA strand to another, its importance as a linker is clearly demonstrated. Furthermore, the local concentration effect actually facilitates probe hybridization and stabilizes the hybridized product. This is important in intracellular applications taking place at 37 °C because the duplex of the HMP and its target will be stable at that temperature, while stability of the duplex of the target and the probe with no linker will decrease, resulting in a corresponding decrease in sensitivity.

Comparison experiments between the HMP and MBs

As mentioned in the previous discussion, MBs have already been used for hybridization studies with some degree of success. Therefore, in the following set of experiments, HMP will be compared with MBs on the basis of such parameters as kinetics and stability, both in vitro and in vivo. In particular, and as a further test of their utility, the performance of each type of probe inside single living cells will be explored.

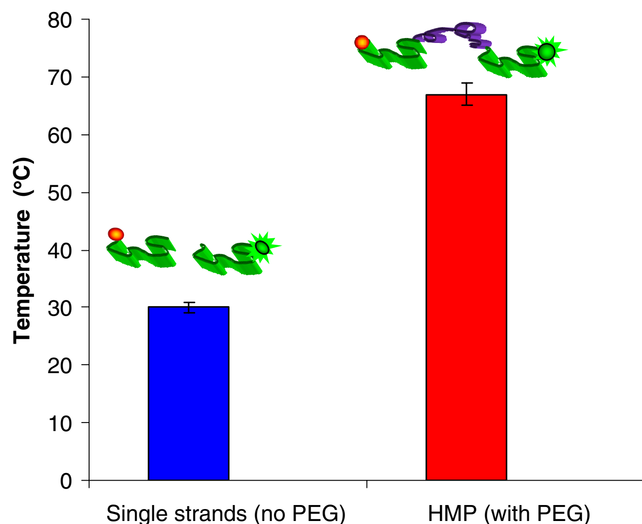


Fig. 3 Melting temperature for HMP (with PEG) and single-stranded probes (no PEG). Higher melting temperature of the HMP indicates higher stability of the duplex over the single-stranded probes without PEG. Calculation of the melting temperature values for each probe was determined as described in the Material and methods section

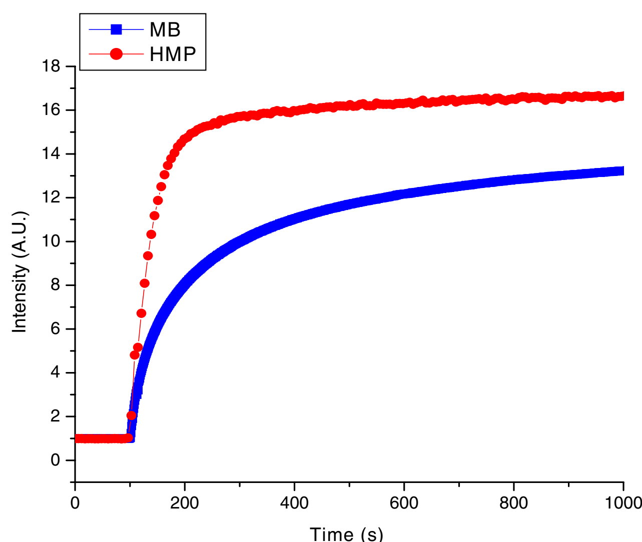


Fig. 4 Hybridization rate of MBs and HMP upon addition of target. A faster rate of hybridization was observed for the HMP when compared with that of the MBs

Kinetics

The aim of this experiment was to compare the kinetics of the HMP and the MB probes. Since the equilibrium measurement correlates to the overall mRNA expression, it is important that the signals reach equilibrium as fast as possible. If the probe requires excessive time to reach equilibrium, then its effectiveness as an intracellular probe is compromised. Hence, for intracellular experiments, faster hybridization kinetics is desirable. A concentration of 300 nM was used for the probes and the target in buffer solution. HMP tubulin and MB tubulin sequences were used in these experiments (see Table 1). The hybridization of the probe was done in a real-time experiment, and the data are recorded in Fig. 4.

As Fig. 4 demonstrates, the HMP shows a faster hybridization rate compared with that of the MB. Hybridization of the HMP reached maximum intensity in less than 400 s upon target addition. On the contrary, intensity of the MBs continued to increase, even at 600 s after target addition. Compared with MBs, the HMP exhibits a faster and more efficient hybridization mechanism, possibly because HMP, unlike MBs, has a flexible linker allowing its two strands to stay together in a single open state. In the MB, the stem portion of the probe must open prior to the loop hybridization with the target. Consequently, MBs require additional time to transition from the closed state of the beacon to the open state in order to hybridize. This is

not the case for the HMP, which is always in an open state, and thus requires less time to hybridize to its target and reach a state of equilibrium. Faster hybridization times are more advantageous for real-time monitoring, as the hybridization rate of the probe controls the precision of time-based measurements.

Biological stability of the probes

MBs and HMPs were developed as tools for nucleic acid target detection. These fluorescence probes have relatively good selectivity and are designed to recognize target molecules based on Watson–Crick base pairing. However, DNA-based probes can also be affected by a number of factors, such as protein binding and nuclease degradation in the complex environments found inside cells. Therefore, the study of molecular probe stability inside a living cell is highly significant because biological processes, including nonspecific DNA–protein interaction, can interfere with target recognition when the probes are used in an intracellular environment. Consequently, we investigated the biological stability of the probes against: (1) the presence of deoxyribonuclease (DNase), (2) the effects of protein binding, and (3) cell lysate interactions. Two different sequences were synthesized and tested for the MBs and HMP: tubulin and MnSOD (see Table 1).

Figure 5 shows a comparison of the response of MBs and HMPs to the target and a variety of proteins and compounds in buffer solution. The probes were evaluated with two proteins known to interact with DNA, lactate dehydrogenase (LDH) and DNase I. LDH is an enzyme in the glycolytic cycle that catalyzes the reversible interconversion of lactate and pyruvic acid. DNase I cleaves at the phosphodiester linkage adjacent to pyrimidine nucleotides, yielding 5′-phosphate-terminated polynucleotides with a free hydroxyl group on the 3′ position. The probes were also tested in cell lysate to evaluate their overall stability in a complex environment. In the case of HMP probes, there was no significant difference in signal enhancement in the presence of cell lysate, LDH or DNase interaction. However, addition of LDH to the MB solutions induced an increase in the fluorescence intensity resulting from the separation of the fluorophore and quencher. As long as the degradation or binding of the MB results in the separation of the fluorophore and quencher, a fluorescent signal is produced that is indistinguishable from target hybridization. Similarly, when the DNase was added to the MB solutions, high fluorescence signals were detected. These results demonstrate that MBs in the presence of DNase give false positive signals. This is explained by the fact that DNase cleaves the DNA in the probe and destroys the integrity of the stem structure, which then separates fluorophore and quencher, thus giving false positive signals. Consequently, the fluorophore is no longer quenched by the quencher, and the fluorescence of the dye is restored. It is interesting to note that the signal from MBs caused by DNase was even higher than that of cDNA for MB β-tubulin sequence. The MBs also produced a false positive signal when mixed with the cell lysate. In this case, the absence of target indicates that the signals originated from interactions in the cellular lysate itself, which then led to false positive signals. Although HMP may also interact with the some of the proteins tested, it does not generate false positive signals, which is a significant advantage for hybridization studies and/or intracellular measurements.

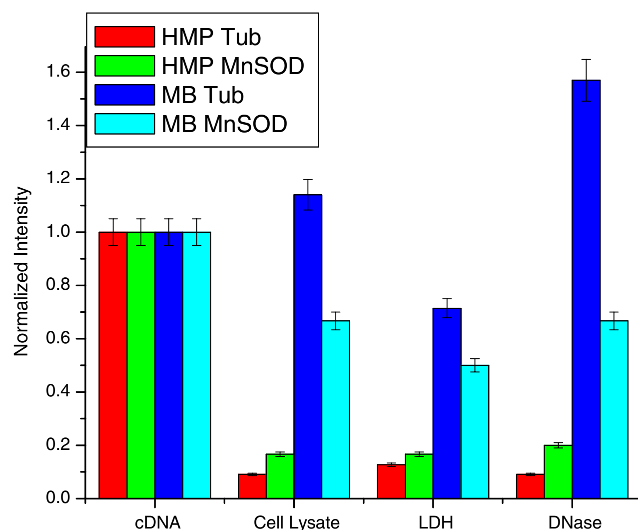


Fig. 5 Stability of HMP and MB in the presence of cell lysate, LDH, and DNase I. The fluorescence intensity was normalized with respect to the maximum signal obtained in the presence of target at a concentration of 300 nM. Two different DNA sequences were studied: tubulin and MnSOD

Intracellular measurements with HMP

The experiments reported above only investigated the behavior of the HMP in vitro, but it is more important to determine the performance of these probes in a cellular environment. Therefore, the next goal was to monitor the stability of the probes inside a living cell. In order to accomplish this, both the HMP and MB were delivered into human breast carcinoma cells and monitored every 4 min for 1 h (see Fig. 6). Neither the HMP nor the MB is complementary to a target inside the cell that is expressed at a detectable level. Ratiometric measurements were performed for the probes, preventing variability of the fluorescence signal inside cells, such as scattering in the sample, excitation source fluctuations, variation of cell volume, and variation in microinjection delivery. A reference probe was injected along with the control MBs. The reference probe emits a stable and constant signal that acts as an internal standard for analyzing the signal from the

MBs. Using this technique, signals originating from different cells can be directly compared. The probes had a concentration of 1 μM, and the signals from the cytoplasm were then measured along with those of the background. In each channel, the background signal was subtracted from both the MB signal and the reference signal, after which the ratio values were calculated. This experiment was repeated multiple times, and the results are shown in Fig. 6. In all the cells, the control MB exhibited a significant increase in the fluorescence signal with time. As no target exists inside the cell to hybridize with the control MB, the resulting fluorescence signal must have resulted from the degradation of the probe or interaction with cellular proteins during monitoring. As noted earlier, such conditions can result in false positive signals, which is a concern when using MBs for intracellular measurements. Unlike the control MB, HMP remained dark inside the cells, as no signal enhancement was appreciable without a target. Therefore, the high stability of HMP inside the cells is shown to be yet another of the attractive features that this probe offers for hybridization studies.

In the following experiment, our main goal was to investigate the potential of the HMP for hybridization studies inside a cell using human breast cancer cells. Figure 7a shows cellular measurements for the HMP for hybridized (top) and unhybridized (bottom) states inside a cell. β-Actin was used as a target sequence, as it is highly expressed within the cells (see Table 1). The images on the left side in Fig. 7a represent the channel for the detection of Oregon green, whereas the images on the right side are for the detection of Cy5 at 520 nm and 665 nm, respectively. In this experiment, Oregon green was used instead of FAM because Oregon green is more stable in the cellular environment; however, both dyes have the same spectral properties. The control HMP was monitored to prevent any nonspecific binding; however, the signal remains at very low levels and is stable throughout the monitoring period in the absence of its complement. On the contrary, the injected HMP β-actin showed an increase in the fluorescence signal in the presence of the highly expressed mRNA sequence. The negative response of the control HMP is significant because it indicates that the signal produced from the β-actin HMP inside the cell is a result of the hybridization with its mRNA complement and not a result of probe degradation inside the cell. If degradation had been taking place during the monitoring period, then the control HMP, as well as the β-actin HMP, would have been affected by equally producing the FRET signal. Figure 7b shows a bar graph for HMP hybridization using the average equilibrium values of five cells from the images. The enhancement of HMP with the target resulted in more than ninefold signal enhancement compared with the control HMP without the target, which demonstrates the effectiveness of HMP for use in hybridization studies in vivo. To verify that the control HMP was functioning properly, its complement inside the cells was stimulated. The control HMP is complementary for manganese superoxide dismutase (MnSOD). However, in cancer cells, the expression of MnSOD is significantly down-regulated and below the limit of detection for MBs and the HMP. Therefore, the gene expression of MnSOD can be stimulated through incubating the cells with lipopolysaccharide (LPS). In the LPS-induced cells, the expression of MnSOD was readily detectable and showed a signal in excess of six times higher than the cells at basal expression levels. These experiments demonstrate that the HMP can detect gene expression at the single level and that the HMP is a viable molecular probe for intracellular mRNA expression.

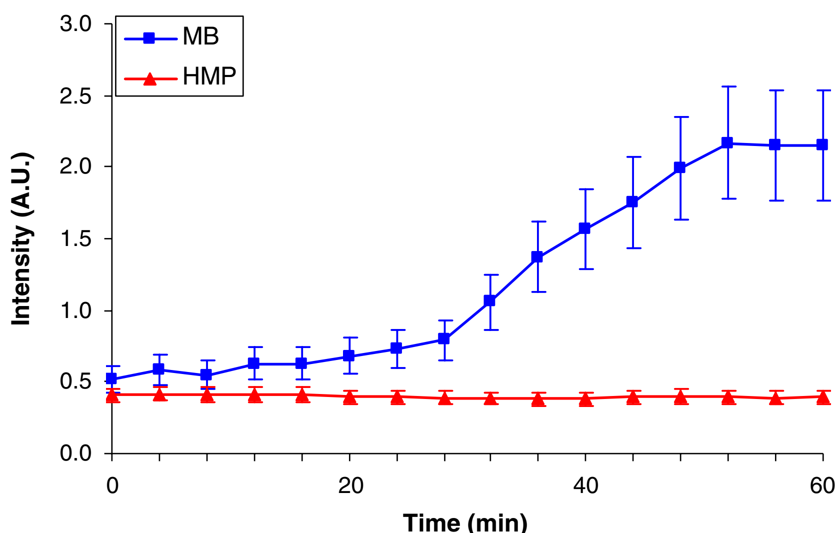
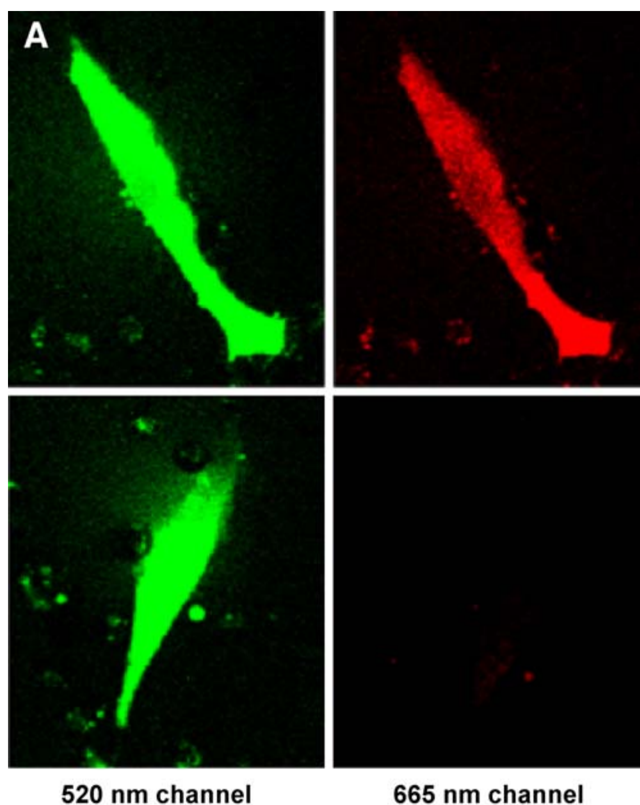


Fig. 6 Real-time monitoring of the MBs and HMP to test the stability inside single cells. HMPs showed high stability throughout the monitoring, whereas MBs exhibited an increase in the fluorescence signal (false positive signal) resulting from degradation of the beacon structure



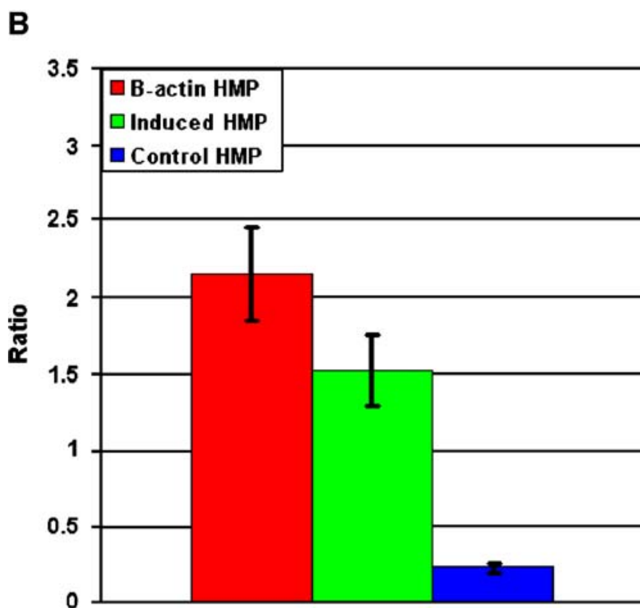


Fig. 7 HMP hybridized and unhybridized states inside the cell. **a** *Top* Confocal images of the HMPs before and after hybridization with its target; *bottom* Control HMP with no target inside the cell. **b** Bar graph representation of β-actin HMP in the presence of the target (*red*), induction of HMP MnSOD (*green*), and control HMP (*blue*) where no induction was performed

Conclusion

In this paper, we have demonstrated the viability of using the HMP for hybridization studies both in solution and inside the cell. Essentially, the probe takes advantage of a fluorescent signal transduction mechanism that enables a very sensitive analysis at low concentrations and sample volumes. This enables the HMP to overcome major problems associated with traditional methods of gene expression analysis. Specifically, the HMP signal can reach more than 20-fold, is very stable in a cell-like environment, and has the further advantage of detection without separation. Although MBs have been used with relatively acceptable performance, their tendency to false positive signals is a significant problem when used for intracellular measurements, as it is impossible to differentiate between target hybridization and false positive signals. In contrast, we have established in this report that the HMP has far less propensity for false positive signals and thus performs better than MBs inside single living cells. Overall, in this work, we have both characterized HMP and demonstrated its potential for both in vitro and in vivo analyses and as a tool for hybridization studies. Among its many advantages, the HMP has also exhibited the ability to detect the mRNA expression of different genes inside single cells from both basal and stimulated genes.

Future applications of the HMP involve its use in the study of disease states to investigate gene expression in single living cells. This includes issues of medical relevance, such as investigating the effects on gene expression in human breast carcinoma cells after treatment with chemotherapeutic drugs. Other issues of biological importance involve investigating mRNA expression patterns in single neuron cells as a means of exploring the processes involved in memory and learning. Furthermore, plans to incorporate nuclease resistance bases into HMPs would allow extended measurements inside living cells in the order of hours or even days. Ultimately, utilizing HMPs linked to cell-penetrating peptides may allow the delivery of HMPs to thousands of cells simultaneously. Development of such techniques might eventually permit clinical laboratories to utilize HMPs in fluorescent plate readers for diagnostic and drug sensitivity assays in cells obtained from human biopsy specimens representing a variety of disease states.

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