Novel Nanoprobes to Detect mRNA *in situ,* Directed Against Mouse Pyruvate Dehydrogenase

Pallavi Cheguru[1], Michael Østergaard[2], Madhusudhan R. Papasani[1], Sai V.V.R[2], Jesper Wengel[3], Patrick J. Hrdlicka[2], and Rodney A. Hill[1]

[1]Dept of Animal and Veterinary Science, [2]Dept of Chemistry, University of Idaho, Moscow, Idaho
[3]Nucleic Acid Center, Dept of Chemistry, University of Southern Denmark, Odense M, Denmark
Corresponding author email: rodhill@uidaho.edu
Presenting author email: pcheguru@vandals.uidaho.edu

ABSTRACT

Novel nanoscale synthetic locked nucleic acid (LNA) probes incorporating molecular beacon (MB) technology were developed. These novel MBs contained 2'-*N*-(pyren-1-yl)carbonyl-2'-amino-LNA monomers incorporated in the loop region. Specificity and sensitivity of MB designed to target the mRNA of mouse pyruvate dehydrogenase complex, component X (Pdhx) mRNA were tested. Using antisense and sense RNA strands of mouse Pdhx synthesized by *in vitro* transcription, binding specificity of MBs was examined. Incubation of antisense mRNA with the MB showed a dose-response relationship and linear increase. Cellular uptake of MBs by mouse 3T3-L1 cells and in cell fluorescence of MB-target mRNA complexes was demonstrated in a dose dependent manner. A linear increase in fluorescence emitted from the cells was observed with increasing dose of MB. In addition, we examined the stability of the MB fluorescence signal after MB were taken up by the cells. We observed that fluorescence emission was stable even at the maximum time-point tested (48 h). Finally, to test the effect of the backbone structure of MB on the resulting fluorescent signal intensity, MB having phosphorothioate (PS-DNA) or 2' O-Me RNA as backbone elements were compared. We demonstrate that MB with 2' O-Me RNA backbone provide enhanced fluorescence, compared to MB containing PS-DNA backbone.

Keywords: molecular beacons, pyrene monomers, 2'O-Me RNA, phosphorothioate DNA, mRNA

INTRODUCTION

Detecting a specific mRNA sequence or any nucleotide sequence within cells has always been challenging and has not yet been accomplished satisfactorily *in situ.* Ever since molecular beacons (MBs) were discovered by Tyagi *et al.* in 1996 (1), they have been extensively used in certain biological applications. Due to its sensitivity and specificity in detecting target sequences this technique was used for the quantitative detection of target mRNAs, in various species or cell lines (2-4). However, there are very few studies in which MBs have been used for the *in situ* detection of mRNA in cultured, living cells. Sokol *et al.* in 1998 microinjected human leukemia cells with MBs and observed the fluorescent signal within the cells (5). In other studies, cells were transfected using liposomes (6) or through Lipofectamine transfection (7). These studies provided an initial indication that MBs might be useful for the detection of specific mRNA in living cells. However, these studies lacked evidence to demonstrate that MBs were truly binding to the target complementary sequence of the mRNA within the cells.

In the present study, we have utilized two types of MBs that differ in their backbone structure, one having a phosphorothioate DNA backbone (PS-DNA) and the other having a 2'O-Methyl RNA backbone. These MBs have four 2'-*N*-(pyren-1-yl)carbonyl-2'-amino-LNAmonomers incorporated, opposite to a Thymidine base in the loop region of the molecular beacon sequence. These pyrene groups brightly fluoresce upon hybridization of the probe to the target sequence (8).

Pyruvate dehydrogenase complex (PDC) plays an important role in cellular respiration process and is expressed abundantly in cells. In the present study, the specific sequence for molecular beacons was designed to target the mRNA of mouse PDC, component X (Pdhx) mRNA.

MATERIALS AND METHODS

1. Binding of *in vitro* transcribed RNA with MB:

A partial sequence of Pdhx (441 bp) that encompasses the location of target complementary region of MB was PCR amplified using primers specific to mouse Pdhx cDNA. The PCR product was ligated in to pGEMT-Easy vector (Promega, Madison WI), as per the manufacturer's instructions. Plasmids with correct orientation of insert were selected.

pGEM-T Easy vector containing the partial cDNA sequence of Pdhx was linearized by restriction digestion using either SpeI or SacII restriction endonucleases. Sense and antisense strands of RNA were synthesized by *in vitro* transcription (Roche Applied Science) using either Sp6 or T7 RNA polymerase, as per the manufacturer's instructions. The RNA transcripts were further purified using NucAway Spin columns (Ambion) and quantified.

Individually, T7, SP6 synthesized RNA transcripts and MB were diluted to an equal concentration. Either T7 or Sp6 synthesized RNA were used in increasing amounts of 0 ng, 125 ng, 250 ng, 500 ng, 1000 ng and 2000 ng in a black frame clear bottom 96 well plate along with 500 ng of MB. The final volume was made up to 100 µl with PBS. RNA and MB were incubated at 37˚C for 2 h. Fluorescence emission was measured using a fluorometric assay [Wallac Victor 2], at a fluorescence excitation wavelength of 355nm and emission detection at 420nm. Data represented was normalized to the control treatment (0 ng).

2. MB uptake and fluorescence detection in living cells:

2.1. Transfection:

3T3-L1 cells were grown in 24 well plates on glass cover slips in Dulbecco's Modified Eagle's Medium (DMEM) with 10 % fetal bovine serum. Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad CA), as per the manufacturer's instructions. At 24 h post-transfection, cells were washed twice with PBS and fixed in 3 % formaldehyde. MB fluorescent signal measurements were taken directly from the cells using a fluorometric assay [Wallac Victor 2], at a fluorescence excitation wavelength of 355 nm and emission detection at 420 nm. Following this assay, the glass cover slips were removed from the wells and inverted on to a glass slide with mounting medium, to observe using a fluorescence microscope.

2.2. Fluorescence Microscopy:

Images were obtained using a Nikon Eclipse E1000 Fluorescent Microscope equipped with Hamamatsu C4742-95 monochrome digital camera. Fluorescent images were acquired by excitation at 358 nm and emission at 460 nm. Quantification of images was performed with Metamorph software.

3. Dose response study:

Five different doses of MBs 0 ng, 125 ng, 250 ng, 500 ng, 1000 ng were prepared and mixed with Nt-DNA (non target DNA) to make up to a final total dose of 1000ng of oligonucleotides. In addition, a dose of 1000 ng of Nt-DNA per well was used as negative control. For all treatments, a total of 1000ng of the oligonucleotides were mixed with 2 µl of Lipofectamine per well. Cells were transfected and MB fluorescence was quantified as described above. Fluorescence emission values were normalized to control values.

4. Time course study:

3T3-L1 cells were transfected, as described in section 2.1, with 500 ng of either MB or Nt-DNA as control. Medium was changed at 6 h post transfection. Subsequently, cells were fixed and fluorescent images were acquired as described in section 2.2 at the following time points: 8h, 16 h, 24 h, 32 h, 40 h and 48 h post transfection.

5. Comparison of 2' O-Methyl RNA MBs with PS-DNA MBs:

Cells were transfected with either 2' O-methyl RNA (2' O-Me RNA) MB or phosphorothioate DNA (PS-DNA) MB or PS-DNA MB with no pyrenes (control), as described in section 2.1. Fluorescent images were acquired and quantification of images was performed as described in section 2.2.

6. Statistical Analysis:

SAS 9.0 software was used to perform statistical analysis. All data were expressed as mean ± SE (n=3). Data were compared using one-way analysis of variance (ANOVA). Tukey's Studentized Range (HSD) test was used to find the significant differences among the different means. Differences when $P<0.05$, were considered statistically significant.

RESULTS

1. Incubation of *in vitro* transcribed RNA with MB:

Fluorescence data of absolute values of antisense mRNA incubation with DNAMB are reported. Increasing doses of sense and antisense mRNA were incubated with a constant amount of DNAMB. Incubation of MB with sense strand mRNA resulted in minimal fluorescence. However, incubation of antisense mRNA with DNAMB showed a dose-response relationship (Fig 1).

Binding of the highest dose of antisense mRNA (2000 ng) to DNAMB resulted in a fluorescent signal that was 14 times greater than the signal resulting from incubation of the smallest amount of antisense mRNA tested (125 ng). The fluorescent signal from incubation of DNAMB with either antisense or sense mRNA (range 125-2000 ng) resulted in significantly greater fluorescence for each antisense mRNA concentration in comparison to an equal concentration of sense mRNA tested.

The difference in fluorescent signal between three highest antisense mRNA tested (500, 1000 and 2000 ng) were significant. With increasing dose of antisense mRNA (doubling doses between 125 and 2000 ng), the resulting fluorescence signal increased between 1.6 and 2.6 fold. Thus the signal showed an approximately linear increase with antisense mRNA concentration.

2. Dose response study of fluorescence of molecular beacon in 3T3-L1 cells.

To demonstrate the fluorescent signal generated in DNAMB-transfected 3T3-L1 cells, the signal intensity was investigated in a dose response design. 3T3-L1 cells were transfected with increasing doses of MBs. It was hypothesized that an increase in fluorescence would be observed with increasing doses of transfected MBs.

Quantification of fluorescence intensity of images showed an increase in the fluorescence intensity with increase in the amount of MB transfected (Fig 2). The amount of fluorescence emitted from each treatment was

normalized to the control, i.e., without MB. These observations were consistent with the fluorescence microscopy images (images not shown) which showed that visually there was increasing amount of fluorescence with the increase in the amount of the MB transfected.

3. Time course study:

Next, we investigated the stability of the fluorescence signal in cellular conditions. Cells transfected with MBs showed fluorescence at each time point. The fluorescence emission was stable even until 48 h post-transfection (Fig 3). There was no fluorescence observed in the control treatment (images not shown). This demonstrates that the pyrene monomers were able to fluoresce at least until 48 h in cellular environment.

4. Effect of backbone structure on the stability of MBs in the cells:

To investigate the influence of backbone structure of MBs in sustaining the cellular conditions and enzymatic degradation, 3T3-L1 cells were transfected with MBs having either 2'-O Me RNA or PS-DNA backbone structure, incorporated with pyrenes or MBs with no pyrenes. Fluorescence microscopy data demonstrated that 2'-O Me RNA MB and PS-DNA MB exhibited substantial fluorescence emission of the pyrenes in the cells transfected with either 2'-O Me RNA MB or PS-DNA MB, while there was no fluorescence emission observed in the cells treated with MBs with no pyrenes (Fig 4). Fluorescence intensity values of 2'-O Me RNA MB were almost three fold higher than fluorescence intensity of PS-DNA MB (data not shown) demonstrating the higher stability of 2'O-Me RNA MB over PS-DNA MB in the cellular environment.

DISCUSSION

Molecular beacons with 2'-*N*-(pyren-1-yl)carbonyl-2'-amino-LNA monomers incorporated in the loop region can emit fluorescence when bound to a complementary target sequence. These probes have excellent quantum yields (between 0.30 to 0.99) and mismatch discrimination (9). Binding of MB to either RNA from the cells or complementary DNA sequences provides excellent signal intensity. In the present study, the level of fluorescence of 2'-*N*-(pyren-1-yl)carbonyl-2'-amino-LNA monomers was examined in living cells and in solution.

There are several bio-applications of MBs (10), the most common being gene expression studies, using quantitative RT-PCR (3). In our study, specificity of MB to bind to RNA obtained from cells was tested. The absolute difference of MB fluorescence between the antisense and sense RNA strands was significant and well above background levels. *In vitro* transcribed RNA is much closer to the physiological form of RNA (11) compared to chemically synthesized analogs. Thus, the specificity of MB binding to *in vitro* transcribed RNA was tested through MB incubation with sense or antisense RNA.

Next, to visualize the fluorescence levels of pyrene monomers in living cells, a dose response study was performed. In several other studies, conventional MBs were delivered into cells through either electroporation (12) or microinjection (13). Nitin et al used TAT peptide conjugated to MB for delivery of MB into cells (14). We used Lipofectamine transfection as this is a standard approach for introducing siRNA or antisense oligonucleotides into cells. In fact, the present study is the first to contribute an understanding of the level of fluorescence emission by pyrene monomers in a dose-dependent manner under intracellular conditions.

Further, we evaluated the stability of pyrene monomers in the cellular environment in a time-course study. MBs with pyrene monomers were stable in the cells even at the maximum time-period tested i.e., 48 h. This suggests that pyrene-containing MBs can withstand the cellular environment and fluoresce at least until 48 h. Hence, probes with pyrene monomers can function as novel potential tools for cell studies involving a prolonged period of molecular imaging.

Finally, to test the effect of the backbone structure on fluorescent signal intensity in the cellular environment, MB with the same sequence but with different backbone structures were tested. We demonstrated that oligonucleotides with 2' O-Me RNA backbone provide enhanced fluorescence, greater than MB containing PS-DNA backbone. 2' O-Me RNA MBs are more stable and resistant to enzymatic cellular conditions when compared to MBs with PS backbone structure.

REFERENCES

1. Tyagi, S., et al. (1996) *Nat Biotechnol* **14**, 303-308
2. Dong-Hun Lee, et al. (2005) *J Clin Microbiol* **43**, 5111-5116
3. Takacs T., et al (2008) *J Virol Methods* **149**, 153-162
4. Levy-Booth, D. J., et al. (2008) *J Agric Food Chem* **56**, 6339-6347
5. Sokol, D. L., et al. (1998) *Proc Natl Acad Sci U S A* **95**, 11538-11543
6. Matsuo, T. (1998). *Biochim Biophys Acta* **1379**, 178-184
7. Yang, L., et al. (2005) *Cancer Biol Ther* **4**, 561-570
8. Hrdlicka, P. J.,et al. (2004) *Chem Commun (Camb)*, 1478-1479
9. Hrdlicka, P. J., et al (2005) *J Am Chem Soc* **127**, 13293-13299
10. Li, Y., et al. (2008) *Biochem Biophys Res Commun* **373**, 457-461
11. Krieg, P. A., et al. (1984) *Nucleic Acids Res* **12**, 7057-7070
12. Chen, A. K., et al. (2008) *Nucleic Acids Res* **36**, e69
13. Perlette, J., et al. (2001) *Anal Chem* **73**, 5544-5550
14. Nitin, N., et al. (2004). *Nucleic Acids Res* **32**, e58

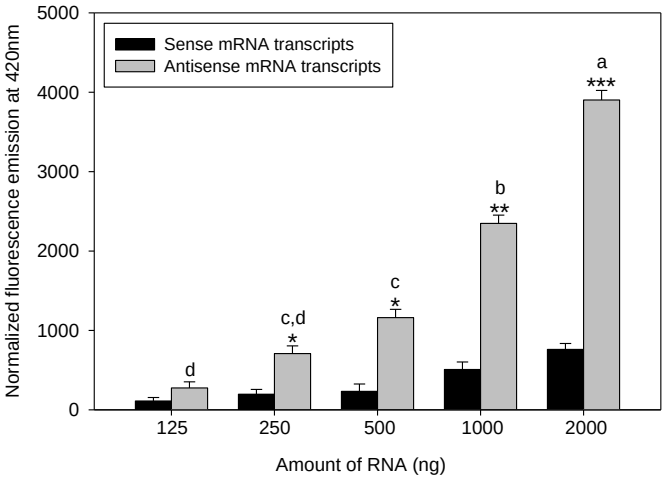

Fig 1: Binding of in vitro transcribed RNA with MB: Pdhx partial cDNA sequence was inserted into pGEM-T Easy vector and linearized with SpeI and SacII for synthesizing antisense and sense RNA transcripts using T7 and Sp6 RNA polymerases respectively. Individually, RNA transcripts were incubated in increasing amounts with 500 ng of MBs. Fluorescence emission was normalized to that of the control (0ng of RNA). Data were means ± SE (n=3). Different letters represent statistical significance of absolute differences of fluorescence emitted by sense and antisense transcripts at different doses (P<0.05). "*" represents statistical differences in pair-wise comparisons of fluorescence emission between sense and antisense transcripts at each particular dose (* represents P<0.01, ** represents P<0.001, *** represents P<0.0001).

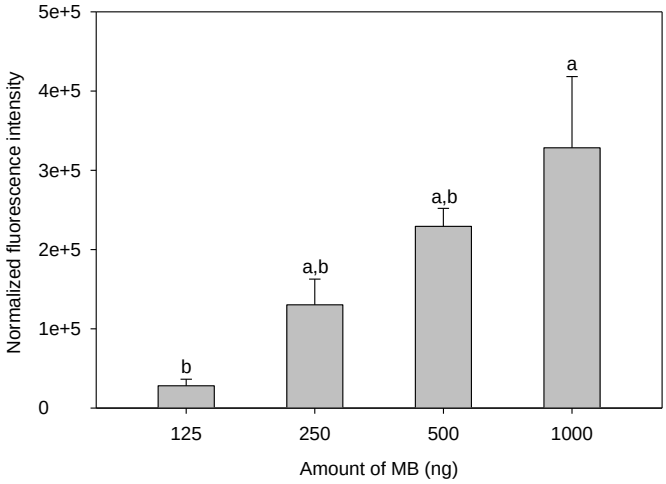

Fig. 2: Dose response study - Quantification of Photomicrograph Images: 3T3-L1 cells were transfected with increasing amounts of MBs and fluorescence emission from the cells was observed by fluorescence microscope. Fluorescence signal intensities from images were quantified (Metamorph). Values were normalized to the control (0ng of MB). Data were means ± SE (n=3). Different letters represent statistical significance of effect of different doses on the fluorescence intensities (P<0.05)

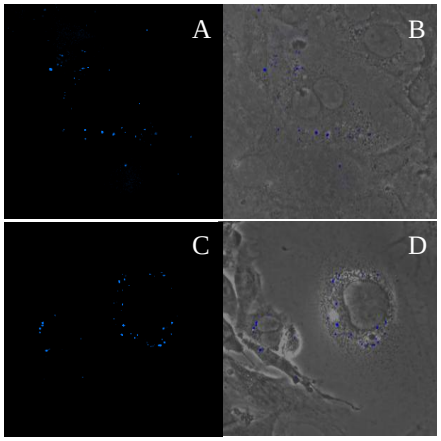

Fig 3: Time course study: 3T3-L1 cells were transfected with 500 ng of MBs and incubated for 6 h after which the media was changed. Cells were further incubated for the total indicated periods, fixed and fluorescence images were acquired at 20X magnification. Representative images of 100 µm in length and height were shown. Data presented as the fluorescent image followed by an overlay of fluorescent and transmitted light images. Panels A and C represent fluorescent images for treatments: 8 h, and 48 h respectively, with their corresponding

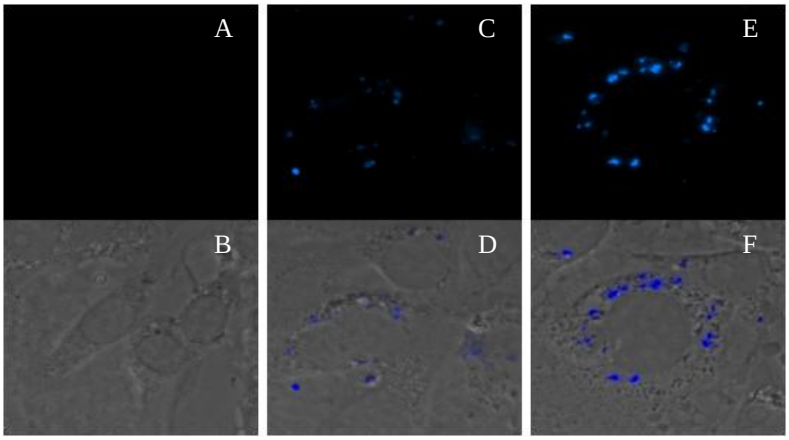

Fig 4: Effect of backbone structure on fluorescence signal from MBs in 3T3-L1 cells: 3T3-L1 cells were transfected with either PS-DNA MB or 2'O-Me RNA MB possessing pyrene monomers or PS-DNA MB without pyrenes. Fluorescence emission was observed at 24 h post-transfection by fluorescence microscopy at 60X. Each treatment was presented as the fluorescent image followed by an overlay of fluorescent image and transmitted light image. Representative images of 50 µm in length and height were shown. Panel A, C and E represent fluorescent images of control (PS-DNA MB with no pyrenes), PS-DNA MB and 2'O-Me RNA MB bound to pyrenes, respectively, followed by the corresponding overlay images (Panels B, D and F).