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A Targeted, Self-delivered and Photocontrolled Molecular Beacon for mRNA Detection in Living Cells

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Abstract

Messenger RNA (mRNA) is, by its nature, transient, beginning with transcription and ending with degradation, but with a period of processing and transport in between. As such, the spatiotemporal dynamics of specific mRNA molecules are difficult to image and detect inside living cells, and this has been a significant challenge for the chemical and biomedical communities. To solve this problem, we have developed a targeted, self-delivered, and photocontrolled aptamer-based molecular beacon (MB) for intracellular mRNA analysis. An internalizing aptamer connected via a double-stranded DNA structure is used as a carrier probe (CP) for cell-specific delivery of the MB designed to signal target mRNA. A light activation strategy was employed by inserting two photolabile groups in the CP sequence, enabling control over the MB's intracellular function. After being guided to their target cells via specific binding of aptamer AS1411 to nucleolin on the cell membrane, light illumination releases the MB for mRNA monitoring. Consequently, the MB is able to perform live-cell mRNA imaging with precise spatiotemporal control, while the CP acts as both a tracer for intracellular distribution of the MB before photoinitiation, and an internal reference for mRNA ratiometric detection.

The expression level and subcellular distribution of specific mRNA can be modulated by cells when responding to their internal genetic programs or external stimuli.[1,2] Therefore, intracellular mRNA monitoring and detection can yield valuable information for biological study, medical diagnosis, adaptive therapy and drug discovery. In this regard, live-cell imaging is a powerful approach because it promises high spatiotemporal resolution for the analysis of mRNA dynamics.[1,3] Of the several current live-cell imaging methods for mRNA,[3-8] molecular beacons (MBs) may be the most attractive since they are easy to make, simple to use, and do not involve the complicated genetic manipulations of GFP-tagged methods[9]. Furthermore, they have a low background without the need to wash away unbound probes, as well as high multiplexing potential by labeling with different dyes. Based on their inherent signal-transduction mechanism, MBs are able to monitor RNAs in

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Supporting Information
Detailed experimental procedures, DNA sequences, and supplementary data. This material is available free of charge via the Internet at http://pubs.acs.org.

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real time with high sensitivity and selectivity.[10] To visualize and track mRNA in specific living cells with high spatiotemporal resolution, the probe must initially meet two important requirements: specific delivery into the target cell's cytoplasm, where the mRNAs are located, as well as good spatial and temporal resolution of the probe.

MBs are negatively charged hydrophilic macromolecules; as such, they cannot freely permeate the lipophilic cell membrane.[11] To solve the problem of MB delivery, several methods have been attempted, such as microinjection, transfection using liposomes and cationic polymers, and peptide-assisted delivery.[12] Despite wide investigation, all of these methods have their limitations. For example, microinjection is tedious and invasive with low throughput, while oligonucleotide probes tend to sequester in the nucleus rapidly and thoroughly by such a single pulse. Although peptide-assisted delivery can deliver probes efficiently, the necessary conjugation between the peptide and the DNA makes probe synthesis complicated and costly. More importantly, most methods reported to date show no cell specificity.

Thus, alternative delivery strategies with the advantages of high throughput, cell-type specificity, low cost, and facile preparation are being sought. In recent decades, aptamers have attracted much attention in biological research, owing to their intrinsic advantages of high affinity and selectivity, simple synthesis, convenient modification with functional groups, lack of immunogenicity, and good stability. Aptamers are single-stranded oligonucleotides selected through an *in vitro* method known as SELEX on the basis of their specific binding to target molecules.[13] Particularly, those aptamers that can be internalized directly into cells have been widely used for targeted drug delivery.[14] As nucleic acids, aptamers can be easily integrated with MBs through hybridization or one-pot synthesis in the DNA/RNA synthesizer. In addition, it has been reported that specific protein/aptamer binding can protect the DNA strand from enzymatic degradation, which would be valuable for studying living systems with DNA probes.[15] Surprisingly, the incorporation of internalizing aptamers into MBs for living cell assays has not yet been reported.

Once the MB is internalized into the cytoplasm by specific aptamer binding with its target cell, it is necessary to achieve spatiotemporal control of the probe's function. The synthesis and degradation of mRNAs, as well as their subcellular sorting and location, are finely timed and tightly organized in living cells.[1,16] For example, the *bicoid* mRNA is concentrated at the anterior tip of oocytes within the developmental stage and early embryo until the formation of blastoderm cells.[17] The expression level of manganese superoxide dismutase (MnSOD) mRNA is closely related to the malignant phenotype and tumor proliferation.[18] Accordingly, methods that allow us to study specific mRNAs with high temporal and spatial resolution are expected to provide more information about the mechanisms underlying these biological events. In this regard, light irradiation is a clean form of energy, with the further advantages of easy, remote and accurate control. The photodecaging strategy is a typical example, essentially because molecular activity is masked by a photoresponsive caging group and then recovered by specific light irradiation at a designated time and location.[19] With the advantages of light stimulus and high efficiency of the photodecaging reaction, this technology allows us to determine when and where to perform the desired molecular activity in living systems.

Herein, we make use of the aforementioned three key points to design and synthesize a cell-targeted, self-delivered, photocontrolled MB for spatiotemporal mRNA monitoring in living cells, using MnSOD mRNA as the model target. To achieve this goal, a double-stranded DNA probe was used. One strand is the MB, which is designed to signal the target mRNA, while the other is an internalizing aptamer AS1411[20] with an extended cDNA sequence, which functions as a carrier probe (CP) for the targeted cellular delivery of the MB (Scheme

1). This aptamer is known to specifically bind to nucleolin, which is commonly overexpressed on the membranes of tumor cells.[20] Since nucleolin shuttles between cytoplasm and nucleus, the MB, using aptamer AS1411 as its guidance system, can be translocated across the cell membrane into the cytoplasm. In contrast to single-stranded DNAs, it has been reported that the cell-uptake efficiency of antisense DNAs can be improved by delivery as double-stranded complexes.[21] By combining the internalizing aptamer with a duplex structure, the MB should enter the cytoplasm of specific cells efficiently. Next, to exert spatiotemporal control over the detection function of MB in living cells, two photocleavable linkers (PC-linkers), each containing an o-nitrobenzyl moiety, were inserted into the CP sequence (Table S1). In the initial stage, as shown in Scheme 1, a stable duplex structure with a protruding aptamer is formed by hybridization between MB and CP, causing the MB to remain in an inert state, even in the presence of target molecule. However, upon irradiation with a pulse of UV light, the PC-linkers are quickly cleaved, releasing the MB, thereby activating its sensitivity to the target mRNA. In this way, the MB is guided to the target cell, enters it, and performs its detection mission at a given time and location.

Furthermore, to monitor the photocleavage process and to measure the intracellular mRNA level, the MB was modified with a Cy3 fluorophore and a BHQ2 quencher, while the CP was labeled with a Cy5 fluorophore. Using such dye-labeling strategy, the photocleavage reaction can be evaluated by the FRET signal change between Cy3 and Cy5. As shown in Figure 1A, an increase in light irradiation time rapidly decreases the FRET signal of Cy5 (at 663 nm), whereas the Cy3 signal (at 563 nm) shows little change. Meanwhile, the FRET ratio, which is calculated by dividing the fluorescence intensity obtained at 563 nm by that obtained at 663 nm (λ_{Exc}=525 nm), drops steeply and reaches a plateau after 5 min, indicating that the MB is quickly released from CP to restore a self-quenched hairpin structure. Native polyacrylamide gel electrophoresis (PAGE) was performed to further confirm the photodecaging reaction, and it revealed cleavage of over 98% of CPs after a 5-min light irradiation (Figure S1). These data demonstrate that the photodecaging reaction occurs rapidly and efficiently.

To verify whether the detection of the CP/MB probe could be controlled by light, fluorescence measurements of this probe were first conducted in buffer solution. As shown in Figure 1B, without light irradiation, only a small fluorescence enhancement of MB-Cy3 is induced by the target DNA (curve c), compared with the blank sample (curve b). Presumably, this slight increase in Cy3 signal results from free MBs that were not initially captured by CPs, because the FRET signal of Cy5, which represents the ratio of the CP/MB hybrids, remains unchanged. In contrast, after exposure to UV light for 10 min, the Cy3 fluorescence of the target sample increases significantly, while the FRET signal of Cy5 decreases (curve e). However, under the condition of light irradiation in the absence of target, no increase of Cy3 signal is observed, and the FRET signal of Cy5 also decreases (curve d). These data prove that the inert MB can be activated to signal the presence of the target by light irradiation.

After confirming that the probe can work as expected in solution, we investigated its performance in MCF-7 cells whose membrane overexpresses nucleolin.[22] First, however, several prerequisites must be satisfied before performing living cell assays. Specifically, the probes should first enter the cells efficiently and stay largely in the cytoplasm. To confirm this, CP labeled with TAMRA (CP-TMR) was used to track its intracellular location and assess its internalization ability. The confocal microscopy images showed that AS1411s were distributed throughout the cytoplasm of MCF-7 after a 2-hr incubation, while the TAMRA signals from the nucleus were rather weak, as indicated in previous reports[22]. On the other hand, the control probe with a random sequence failed to enter the cells (Figure

S2), revealing the sequence-specific internalization of AS1411. Meanwhile, the much weaker fluorescence signal from HBE135, a normal bronchial epithelial cell line serving as the nucleolin-negative control cell, in comparison to that from the target cancer cell line (MCF-7), demonstrated the cell type-specific internalization of AS1411 (Figure S3). Analysis of internalization dynamics showed that AS1411s could efficiently enter MCF-7 cells. The whole cytoplasm was very bright after a 2-hr incubation with the probes, and higher internalization efficiency could be obtained by increasing the probe's concentration (Figure S4). The cellular delivery efficiency of the double-stranded complexes (CP/unmodified MB) was demonstrated to be higher than that of the single-stranded CP, and the exact mechanism for this is under investigation (Figure S5). Second, since unmodified oligonucleotides are usually unstable in living cells and tend to be digested by intracellular nuclease with a lifetime of ~30 min, it was necessary to test the stability of the probe in living cells. To accomplish this, we used a hybrid of CP-TMR and MB-Q having the same sequence as MB, but modified with DABCYL quencher at its 5' end. Unexpectedly, however, after incubation for 2 hr, the background signal from the cells with the CP-TMR/MB-Q probe was negligible, compared to that with the CP-TMR/unmodified MB probe (Figure S6), even when the cells were kept at 37 °C with 5% CO_2 for another 4 hr. This may be explained by the G-quadruplex structure of AS1411, which is able to resist enzymatic degradation,[22] as well as the specific protein/aptamer binding, which can protect the DNA sequence from exonuclease digestion.[15] Finally, the use of UV light could be a concern due to the potential damage to cells. However, as demonstrated, the condition of UV illumination used in this experiment (302 nm, 1.06 W, 10 min) had no negative impact on cells during the entire monitoring process. The cells were morphologically identical to those without UV treatment (Figure S7), and over 95 % of cells retained their viability (Figure S8), even when irradiated for 20 min and then held at room temperature for another 2 hr.

Having demonstrated the feasibility of CP/MB in live-cell as, says, we next studied the photoactivation and the mRNA detection ability of the probe in MCF-7 cells. Figure 2A presents the images of cells after incubation with the CP/MB probe at 37 °C for 2 hr. A FRET signal of Cy5 can be observed, while the Cy3 signal is negligible. After light irradiation, however, the Cy5 FRET signal disappeared, and the Cy3 signal slightly increased (Figure 2B), indicating that the photodecaging reaction of the probe performs well in cells and that the basal level of endogenous MnSOD mRNA is low. As controls, cordycepin[23] and lipopolysaccharide (LPS)[24] were used to downregulate and upregulate the expression level of MnSOD mRNA in MCF-7 cells, respectively. As demonstrated via *in situ* hybridization with MB, the MnSOD mRNA expression in cytoplasm changed as expected (Figure S9). Confocal fluorescence imaging was performed, and the results are shown in Figure 2C,D,G,H. Before light irradiation, MB remained in an inert state, and no detectable response signal can be observed in any sample. In contrast, illumination with UV light activated the ability of MB to detect its target mRNA. Specifically, the sample with LPS treatment produced the highest Cy3 fluorescence signal, while the lowest Cy3 signal was observed in the sample with cordycepin treatment, compared to that treated with PBS. These results are accordant with those obtained by the traditional method (*in situ* hybridization, Figure S9), demonstrating that the expression level and distribution pattern of MnSOD mRNA in MCF-7 cells revealed by this CP/MB sensing system is not an artifact. Moreover, the fluorescence images are sharper and more vivid than those observed in fixed cell assays, since a large number of the probes are constrained and opened in the nucleus during *in situ* hybridization, resulting in a high nuclear background. The low background in the mRNA-negative control sample also showed that the probe underwent ignorable degradation during this detection process (Figure 2C,D). To demonstrate further that the signal arises solely from the hybridization of the MB with the target, a control system with the same design, but having a "random" beacon with no match in the entire human genome (Table S1), was employed. No detectable Cy3 signals were observed from the LPS-

stimulated samples incubated with the control probe, irrespective of light illumination (Figure 2G, H). Meanwhile, without CP, MB itself cannot freely traverse the cell membrane to execute its detection mission, as indicated by the negligible signal from the MnSOD mRNA-positive cell sample, confirming the importance of CP for MB's cellular delivery (Figure S10).

Since confocal microscopy can present the images of only a few cells, the results of this technique will likely be skewed by the presence of experimental artifacts and cell-to-cell variation. Flow cytometry, which is able to collect information from thousands of cells per second, was therefore used for statistical analysis of a large number of cells incubated with the target and control probes. The flow cytometry results (Figure S11) are consistent with those of confocal microscopy. Upon light irradiation, the fluorescence intensity from the sample incubated with the CP/MB probe was over 2.5 times that of the control, while each sample produced a relatively low signal without light illumination. All these results demonstrate that the CP/MB probe is powerful for mRNA analysis in living cells.

Detection of mRNA in living cells is challenging, but important to the study of cellular events. To obtain more reliable results from MBs, a strategy of ratiometric measurements is usually performed by introducing a reference probe.[25] However, additional reference probes can increase the complexity of the sensing system. Therefore, in our scheme, the CP labeled with a Cy5 fluorophore was designed to be multifunctional. That is, the Cy5 signals not only report the amount internalized and the intracellular distribution of MBs, but also serve as an internal reference for ratiometric analysis. MnSOD mRNA expression was modulated by pretreatment with LPS for different lengths of time. After removing the LPS solution, the cells were uniformly incubated with the probe for 2 hr, irradiated by UV light for 10 min, and then imaged by confocal microscope. As shown in Figure 3A, the Cy5 signal intensities of CP were similar with different LPS treatments, indicating that this external stimulation had little impact on the internalizing ability of the probe. In contrast, the MB signal increased when LPS induction was prolonged. The imaging data were collected from these four samples and processed with NIH ImageJ software. The relative signal value was calculated by dividing F_{Cy3} by F_{Cy5}, where F_{Cy3} and F_{Cy5} represent the averaged signal intensities of Cy3 and Cy5, respectively, and the signal obtained from the sample without LPS stimulation is defined as 100%. Based on this CP/MB sensing system, the MnSOD mRNA expression level of MCF-7 cells treated with LPS for 1 h, 2 h, and 4 h increased to 163%, 197% and 294%, respectively (Figure 3B), matching the results of others.[24] These results demonstrate that this CP/MB system is capable of detecting target mRNA in living cells. With further photocontrollable processes and molecular engineering,[19,20,26] one should be able to realize quantitative cellular imaging of multiple analytes in living cells.

In summary, we have developed a self-delivered MB for photoinitiated real-time imaging and detection of mRNA in living cells via direct hybridization of an extended internalizing aptamer and a molecular beacon. Using this fluorescent aptamer as an internalizing carrier, the MB can be efficiently delivered into the cytoplasm of targeted cells, and the amount internalized as well as intracellular distribution can be tracked before photoactivation. Moreover, the carrier probe can serve as an internal reference for live-cell mRNA ratiometric detection, thus eliminating the need for additional control reference probes. Based on the photocleavage reaction, this probe allows us to control the detecting function of MB with high temporal resolution, while the spatial resolution in single cell level is potentially achievable by using proper light equipment, which is valuable for studying a myriad of biological processes. Furthermore, this internalizing aptamer/detection probe system can be expanded for analysis of other biomarkers in living cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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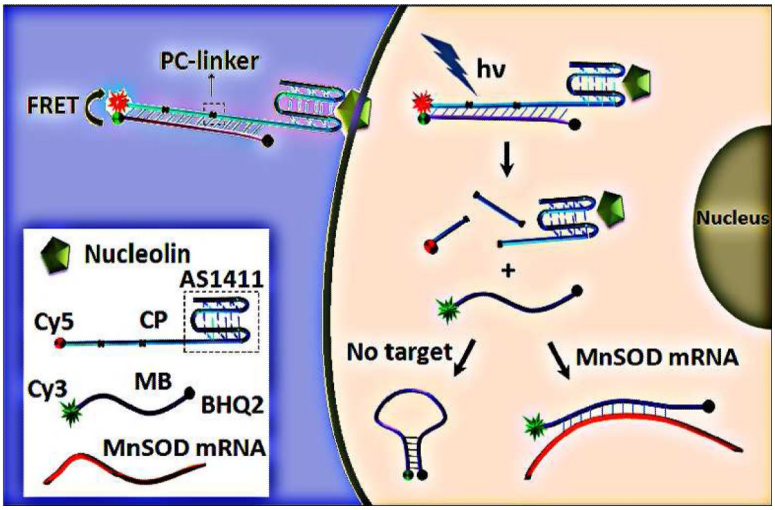

Scheme 1. Schematic illustration of the CP/MB probe for spatiotemporal MnSOD mRNA detection in living cells

The CP/MB probes enter cells via specific binding of aptamer AS1411 to nucleolin on the cell membrane. Because of the more stable hybridization with CP, MB is inert. However, upon light illumination, MB is released, and its ability to signal the target is restored. Two signals from the dye-labeled aptamer are used to monitor cellular entry and function of the CP/MB probe. First, the amount internalized and the intracellular distribution of MBs can be monitored in real time by the Cy5 signal through FRET from Cy3 before photoinitiation. Second, probe detection of mRNA by MB is monitored by the Cy3 signal through direct excitation. Moreover, the Cy5 signal through direct excitation can serve as an internal reference for mRNA ratiometric analysis.

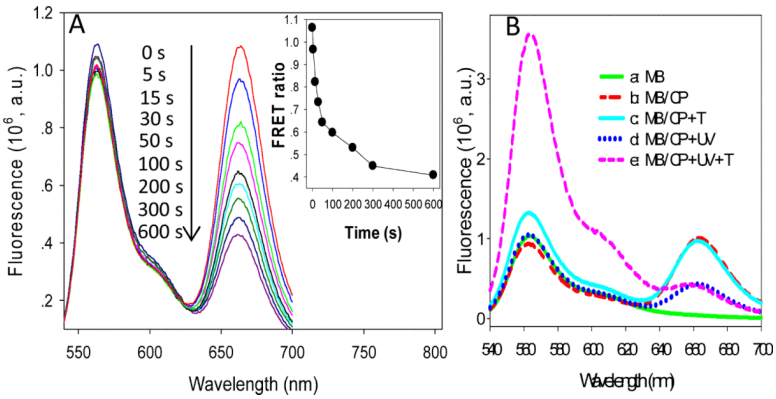

Figure 1.

Photoregulation of the CP/MB probe in buffer solution. A) Fluorescence spectra of the probe after exposure to UV light for different lengths of time; Inset: FRET ratio of the probe versus light irradiation time. B) Fluorescence spectra of the sensing system under different conditions.

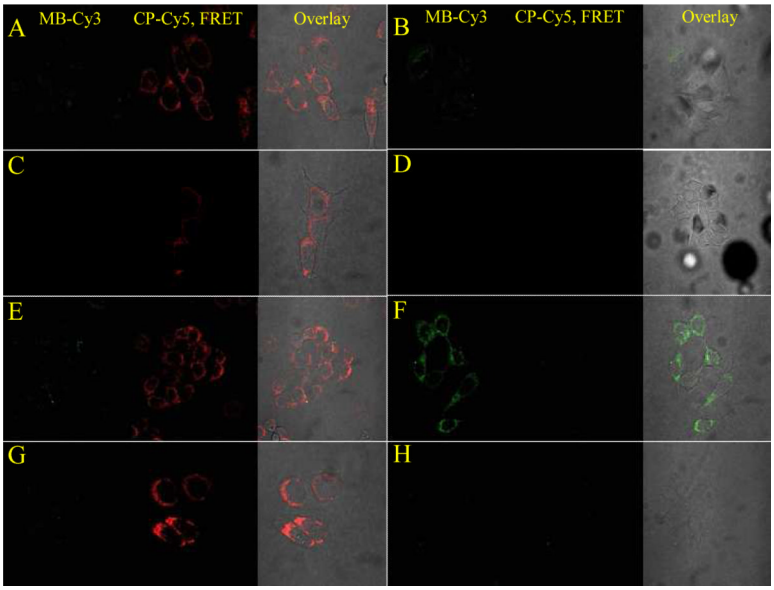

Figure 2.

Confocal microscopy images of MCF-7 cells under different conditions. Cells were treated with PBS (A, B), 150 μg/mL cordycepin (C, D), or 10 μg/mL LPS (E, F, G, H) for 2 hr, respectively, followed by a 2-hr incubation with the CP/MB probe (A, B, C, D, E, F) or the control probe (G, H), and then imaged before (A, C, E, G) and after (B, D, F, H) light irradiation. In each group, from left to right: fluorescence image for Cy3, FRET image for Cy5 under Cy3 excitation, and overlay of the fluorescence channels and bright field channel.

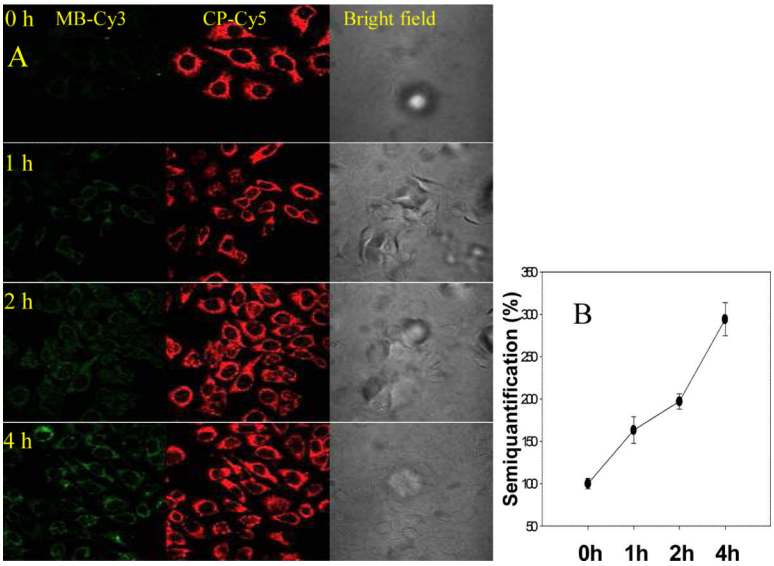

Figure 3.

Ratiometric detection of MnSOD mRNA in MCF-7 cells. A) Confocal microscopy images of MCF-7 cells with LPS stimulation for different lengths of time. From left to right: fluorescence images of MB-Cy3, CP-Cy5, and bright field. B) Relative fluorescence intensity of the corresponding samples.