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Caged molecular beacons: controlling nucleic acid hybridization with light†

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We have constructed a novel class of light-activatable caged molecular beacons (cMBs) that are caged by locking two stems with a photo-labile biomolecular interaction or covalent bond. With the cMBs, the nucleic acid hybridization process can be easily controlled with light, which offers the possibility for a high spatiotemporal resolution study of intracellular mRNAs.

Biological events, such as gene expression, protein synthesis and cell signaling, are precisely controlled with high temporal and spatial resolutions. Therefore, developing methods to study these biological events at both high temporal and spatial resolution would prove valuable in elucidating the activity of genes, proteins and other biological molecules, including their interaction and regulation. Recently, photocaging strategy has emerged as an innovative solution for the spatiotemporal study of dynamic cellular events,[1] such as gene expression,[2] drug release,[3] enzymatic activity,[4] and signal transduction.[5] Essentially, molecular activity is temporally inactivated by a light-responsive caging group, then quickly and simply restored by illumination at a given time and location with a specific light. Building on this technology, we have constructed a new class of light-activatable caged molecular beacons (cMBs). Utilizing these cMBs to monitor RNA both *in vivo* and in real-time should bring greater insight into the mechanisms underlying RNA synthesis, processing, transportation, and subcellular localization.[6]

In the normal state, cMBs are inactive, and their stems cannot be opened, even in the presence of complementary sequences (Fig. 1). However, they can be activated rapidly by a pulse of light at a specific wavelength and immediately respond to the binding of a target molecule (Fig. 1). As such, this type of photocaged MB allows us to determine when and where to execute the function of the probe. More specifically, a "photo-removable molecular lock" can be constructed between the two arms of the molecular beacon stem, either by incorporating strong biomolecular interactions or by forming a covalent bond. In our design, biotin–avidin interaction or triazole linkage locks the MBs' stems *via* a photocleavable linker (PC-linker) bearing an *o*-nitrobenzyl moiety. Compounds bearing *o*-nitrobenzyl moieties have been used most extensively as caging agents based on their fast photolysis kinetics, as well as clean and efficient uncaging capacities. Following this strategy, a molecular beacon sequence, PC1, was designed as follows: 5′-Biotin-PC-linker-PEG-FAM-CCT AGC TCT AAA TCA CTA TCG CGC TAG G-DABCYL-PEG-Biotin-3′, where FAM stands for fluorescein, DABCYL is a quencher molecule and PEG is the polyethylene glycol linker with 6 repeat units. Under physiological conditions, PC1 can fold into a hairpin structure, extending two biotins at the 5′ and 3′ termini, which bind strongly to NeutrAvidin (NeutrAvidin–Biotin, $K_d = 10^{-15}$ M). Strong binding of 5′ and 3′ biotin

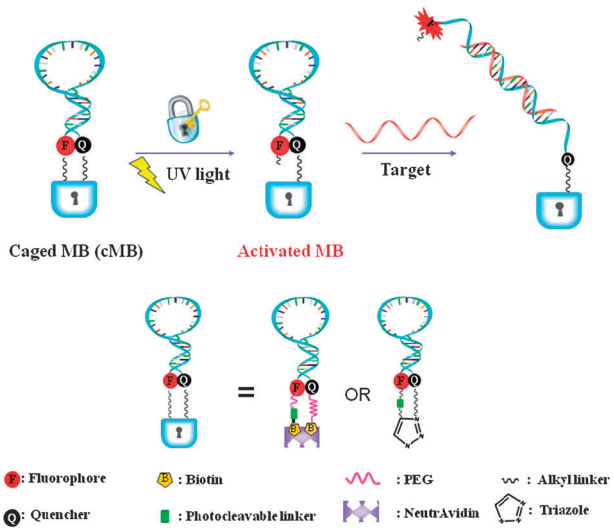

Fig. 1 The working principle of caged molecular beacons (cMBs). cMB is insensitive to the presence of its cDNA and stays in an "inert" state unless it is activated by the UV irradiation.

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termini to a NeutrAvidin molecule locks the hairpin structure (Fig. 1). Once locked, the caged MB will stay in an inactive state, giving out low fluorescence even in the presence of cDNA. However, after a brief pulse of UV light activation, the PC-linker is cut into two pieces, unlocking the hairpin, and the probe then functions as a normal molecular beacon. In our design, a PEG moiety was inserted in between the fluorophore/quencher and biotin to minimize steric hindrance so that two terminal biotins can freely bind to the same NeutrAvidin molecule while allowing the MB stem to remain closed.

To study photolysis kinetics and efficiency of the photocleavable probe, we first synthesized the probe with a biotin attached to its 5′ end and used HPLC to monitor the reaction progress. Under these conditions, 5′ biotin will be removed if the probe is cut by UV light, producing, in turn, an activated probe with the following sequence: PEG-FAM-CCT AGC TCT AAA TCA CTA TGG TCG CGC TAG G-DABCYL-PEG-Biotin. The activated probe is less hydrophobic and possesses a shorter retention time on the reverse phase C18 column compared to an intact probe using the same elution gradient. As shown in Fig. 2, the probe without UV illumination showed a unique peak in the HPLC chromatograph (Fig. 2), while an additional peak showed up after the sample was exposed to a short pulse of UV illumination (200 mW/cm^2). Moreover, the ratio of the two peaks varied quickly with different UV exposure times. After a simple linear fitting, we were able to find that the half-life of the photocleavage reaction was about 0.6 s. MALDI-MS analysis was also performed and confirmed that a photocleavage reaction took place as expected (Fig. S1, ESI†). These data establish that the photolysis reaction is rapid and very efficient.

To confirm that PC1 can be completely caged by avidin binding and activated by UV illumination, non-denaturing PAGE was used to analyze the free, caged and activated forms of the probe and other control probes (Fig. S2, ESI†). The results demonstrated that these MBs could be fully caged by interacting with avidin and completely activated by UV light illumination.

To prove that the cMB could work as we expected, fluorescence measurements of the probe were performed. Compared to the uncaged probe, background fluorescence from the caged probe was slightly weaker (Fig. 3a). Such a low background fluorescence can be explained in two ways. First, NeutrAvidin has a quenching effect on the fluorescence of FAM and can thus lower the background fluorescence of the probe.[7] Second, addition of the molecular lock to the MB

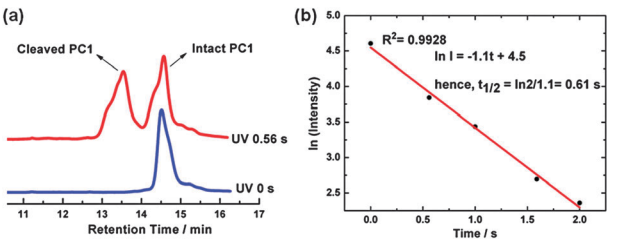

Fig. 2 Kinetics study of the photocleavage reaction. (a) HPLC analysis of PC1 before and after exposure to UV light. (b) Linear fitting of the natural logarithm of PC1 intensity (peak area) *versus* irradiation time.

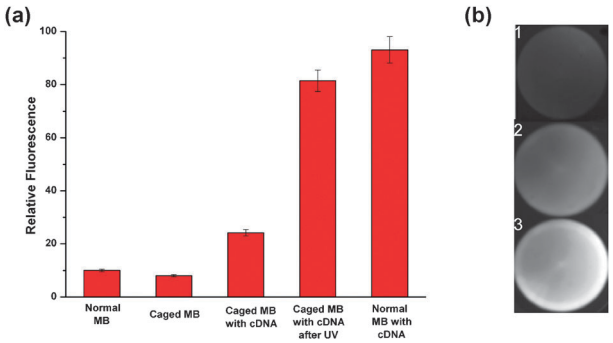

Fig. 3 (a) The fluorescence test of cMBs under different conditions. 100 nM of MBs in 20 mM tris-HCl buffer (140 mM NaCl, pH 7.4), cDNA concentration = 500 nM. (b) Fluorescence imaging of cMBs: 1. cMB; 2. cMB with cDNA; 3. cMB with cDNA after UV irradiation. 500 nM of MBs in 20 mM tris-HCl buffer (140 mM NaCl, pH 7.4), cDNA concentration = 2.5 μM.

stem further stabilizes the loop–stem conformation, leading to a higher fluorescence resonance energy transfer efficiency. After adding cDNA, the caged probe showed little fluorescence enhancement (Fig. 3a). This slight change of fluorescence intensity was most likely a result of partial binding of cDNA to the cMB, which slightly destabilized or distorted the MB's stem–loop structure and as a consequence, elongated the distance between the fluorophore and the quencher. In contrast, the UV-illuminated caged sample showed a significant fluorescence increase of about 3-fold (Fig. 3a), indicating that the liberation of cMB occurred after exposure to UV light. Similar results were achieved from a fluorescence imaging assay using an inverted fluorescence microscope (Fig. 3b).

Additional thermal analysis indicated that cMBs were thermally stable and only showed a little fluorescence enhancement, even when they were heated to 95 °C (see Fig. S3, ESI†). However, after the probes were activated by UV light, they functioned just like normal loop–stem-structured MBs and showed significant fluorescence enhancement upon heat treatment.

To investigate whether the hybridization kinetics of cMBs would be affected by the presence of a large molecule like NeutrAvidin, we compared the hybridization properties of uncaged MBs (which contain only the oligonucleotide moiety

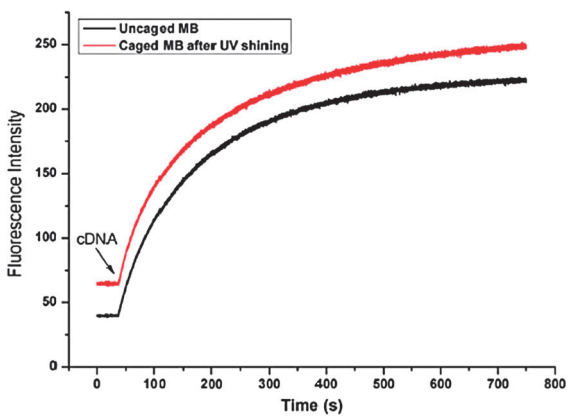

Fig. 4 The hybridization kinetics comparison of cMB with normal MB. 100 nM of MBs in 20 mM tris-HCl buffer (140 mM NaCl, pH 7.4).

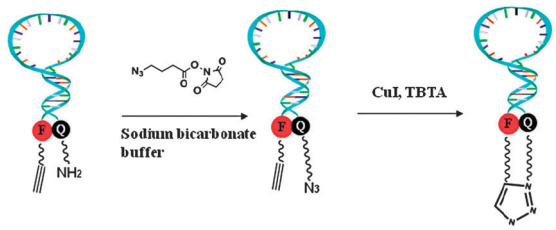

Fig. 5 The synthesis of click reaction-based caged molecular beacons.

without the addition of NeutrAvidin) and cMBs (Fig. 4). Time-course fluorescence scan curves showed almost identical probe hybridization times, strongly suggesting that the presence of large biomolecules would not affect the intrinsic hybridization properties of MBs.

After succeeding in constructing cMBs by incorporation of biomolecular interaction, we next tried to make cMBs by creating a photolabile covalent bond linkage between the two MB stems. Herein, a click reaction was employed because of its high yield and the high reaction specificity between alkyne and azide. Similarly, a click MB was designed with the following sequence: 5′-alkynyl-FAM-CCT AGC TCT AAA TCACTATCG CGC TAG G-DABCYL-PC-linker-azido-3′. A caged MB could be obtained after a one-step click reaction using similar procedures from the literature[8] (Fig. 5). We believed that the cMB constituted in this way should behave just like the one terminated with biotin. Indeed, this type of cMBs also responded to the presence of cDNA and showed a significant fluorescence enhancement to UV light irradiation (Fig. S4 and S5, ESI†). They possessed similar hybridization kinetics with biotin-terminated cMBs, which again demonstrated that the incorporation of protein did not compromise the hybridization properties of cMBs.

In conclusion, we have demonstrated the feasibility of regulating the hybridization activity of MBs with light. Normally, the photolabile caged MBs are biologically inert and give out only a weak fluorescence signal, even in the presence of cDNA. However, by irradiation with a pulse of light, the photocleavable moieties are cut, thus activating the MBs. This photoactivated strategy allows fast, remote, and, above all, spatiotemporal control of the molecular probe function. Because of the advantage of detection-without-separation, molecular beacons are ideal probes to monitor mRNA in living cells.[6,9] The capability of caging a molecular beacon with photocleavable locks enables us to glean important biological information that is otherwise difficult to obtain with a regular molecular beacon. For example, with the capability to control time zero with cMB, dynamic mRNA events following any biological events can be easily obtained.[10] In addition, cMB offers the flexibility for site-specific photo-activation, allowing visualization of RNA transportation with an otherwise unobtainable signal-to-background ratio. Efforts on using cMBs for high spatiotemporal resolution intracellular mRNA analysis are currently undertaken.

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