Biosensors Hot Paper

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Photocaged DNAzymes as a General Method for Sensing Metal Ions in Living Cells**

*Kevin Hwang, Peiwen Wu, Taejin Kim, Lei Lei, Shiliang Tian, Yingxiao Wang, and Yi Lu**

Abstract: *DNAzymes, which are sequences of DNA with catalytic activity, have been demonstrated as a potential platform for sensing a wide range of metal ions. Despite their significant promise, cellular sensing using DNAzymes has however been difficult, mainly because of the "always-on" mode of first-generation DNAzyme sensors. To overcome this limitation, a photoactivatable (or photocaged) DNAzyme was designed and synthesized, and its application in sensing Zn^{II} in living cells was demonstrated. In this design, the adenosine ribonucleotide at the scissile position of the 8–17 DNAzyme was replaced by 2′-O-nitrobenzyl adenosine, rendering the DNAzyme inactive and thus allowing its delivery into cells intact, protected from nonspecific degradation within cells. Irradiation at 365 nm restored DNAzyme activity, thus allowing the temporal control over the sensing activity of the DNAzyme for metal ions. The same strategy was also applied to the GR-5 DNAzyme for the detection of Pb^{II}, thus demonstrating the possible scope of the method.*

Metal ions are involved in many critical functions in biology, for example, by providing structural stability and catalytic activity to proteins, and alone as second messengers.[1] The wide variety of functions held in whole or in part by metal ions has led to significant interest in developing sensors to probe the location and distribution of metal ions in cells.[2] Toward this goal, a number of fluorescent sensors, most notably those based on small organic molecules or proteins, have been developed, allowing the detection of changes in cellular metal-ion concentrations.[2b,3] While these reports have demonstrated that cellular metal sensors can add to our knowledge on the biological functions of metal ions, the current scope of metal-ion sensors has been limited to relatively few metal ions, mostly Ca^{2+}, Zn^{2+}, and $Cu^{2+/+}$. Further advances in understanding the role of metal ions in biology will require the development of new sensors for many more metal ions. However, most methods rely on rational design, but success in designing one metal sensor may not be readily translated the design of another metal sensor, because the difference between metal ions can be very subtle, and designing sensors with high selectivity and little or no interference is thus very difficult. A complementary approach to the rational design is combinatorial selection, which does not rely on prior knowledge of metal-binding, and in which the selectivity and affinity of a sensor can be improved by adjusting the selection conditions.[4]

DNAzymes are a class of functional DNA that offers great promise in the development of metal-ion sensors. Discovered in 1994, DNAzymes are sequences of DNA that have catalytic activity, for example in RNA hydrolysis, porphyrin metalation, or Diels–Alder reactions.[4c,5] Frequently these DNAzymes make use of a metal-ion cofactor in order to carry out their functions, and based on this requirement, our group and others have developed corresponding metal-ion sensors.[4a,b,6] Unlike other types of sensors, DNAzymes can be obtained through a combinatorial selection process, called in vitro selection, from a large DNA library of up to 10^{15} different sequences.[5] This process does not require the immobilization of the metal ion on a solid support and instead relies on DNAzyme cleavage as a measure of metal binding and activity. The selection process allows DNAzymes with specific binding affinity, selectivity, and sensitivity to be obtained.[4c,d,5,6g,h] As the DNAzyme can be readily modified by different signaling agents, such as fluorophores and gold nanoparticles, we can transform metal-dependent catalytic activity into sensor readouts.[7] In this way, our group and others have developed DNAzyme-based metal-ion sensors for a wide range of metal ions, including Zn^{2+}, Pb^{2+}, Cu^{2+}, UO_2^{2+}, Mg^{2+}, and Hg^{2+}.[4b,c,5,6f–h]

Even though the use of DNAzymes for the sensing of metal ions has been established for some time, the majority of previously published methods is limited to the sensing of metal ions in environmental samples, such as water and soil,

[*] K. Hwang, S. Tian, Prof. Y. Lu
Department of Chemistry
University of Illinois at Urbana-Champaign
Urbana, IL, 61801 (USA)
E-mail: yi-lu@illinois.edu
P. Wu, Prof. Y. Lu
Department of Biochemistry
University of Illinois at Urbana-Champaign
Urbana, IL, 61801 (USA)
T. Kim
Department of Chemical and Biomolecular Engineering
University of Illinois at Urbana-Champaign
Urbana, IL, 61801 (USA)
L. Lei, Prof. Y. Wang
Department of Bioengineering and
Institute of Engineering in Medicine
University of California
San Diego, La Jolla, CA, 92093 (USA)

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with very few demonstrating the detection inside cells.[4b,6a] In 2013, we have reported the uptake of a gold nanoparticle/DNAzyme conjugate by cells, thus enabling the detection of endosomal uranyl.[8] Recently, a dendritic polymer was used to deliver DNAzymes into cells for the detection of lead ions.[9] While these results are encouraging, a significant unaddressed issue is the activity of a DNAzyme in the presence of its metal cofactor during cellular delivery and uptake. Depending on the presence of metal cofactors inside and outside of the cells, a DNAzyme may not be able to reach its cellular destination before it is cleaved. Furthermore, most DNAzymes require an internal RNA base at the cleavage site of the substrate strand. Although chimeric DNA/RNA substrates are relatively stable compared to all-RNA substrates, the RNA site makes the sensor vulnerable to endogenous-nuclease activity. Both metal-catalyzed cleavage and nuclease-induced degradation result in loss of dynamic range, thus negatively affecting the signal-to-background ratio and sensor performance. It is thus necessary to develop a method that allows both the reversible protection of the RNA cleavage site of a DNAzyme from enzymatic degradation and its controlled activation.

To overcome this major limitation, we here report the design and synthesis of DNAzymes whose activity can be controlled by a photolabile group (called photocaged DNAzymes), and their applications for the sensing of metal ions in cells. While the addition of photolabile or photoswitchable groups has been used previously to control the activity of DNAzymes,[10] the control of both the activity of the DNAzyme and the stability and cleavage of the substrate strand has not been reported thus far. As a result, despite the frequent use of photolabile groups as a means to develop photoactivatable proteins,[11] small molecules,[2d,11c,d,12] and oligonucleotides,[11c,d,13] no strategy has yet been reported that enables the use of DNAzymes for sensing metal ions in living cells. In addition to showing the intracellular activation of a DNAzyme-based metal-ion sensor, we also demonstrate that this strategy is applicable to all members of the broader class of RNA-cleaving DNAzymes.

The sensor design and photocaging strategy is shown in Figure 1a, using the 8–17 DNAzyme as an example. The DNAzyme contains an enzyme strand and a substrate strand, both of which are all-DNA except for a single adenosine ribonucleotide (rA) in the substrate strand at the cleavage site. The substrate strand is also functionalized at the 5′ end with a fluorophore (F), such as fluorescein, and at the 3′ end with a quencher (Q), such as Black Hole Quencher-1 (BHQ-1); the enzyme strand is functionalized with another quencher (Dabcyl) at the 5′ end. At ambient conditions, the enzyme and substrate strands can hybridize, as the pair has a melting temperature of 57.5 °C. This hybridization places the quenchers in close proximity to the fluorophore, resulting in a low background fluorescence signal prior to sensing.[6g] In the presence of its metal-ion cofactor, the substrate strand is cleaved at the scissile bond, resulting in two fragments with a reduced melting temperature (14.7 °C) to the enzyme strand. This allows the fluorophore to be separated from the quenchers, resulting in a dramatic increase in the fluorescent signal.

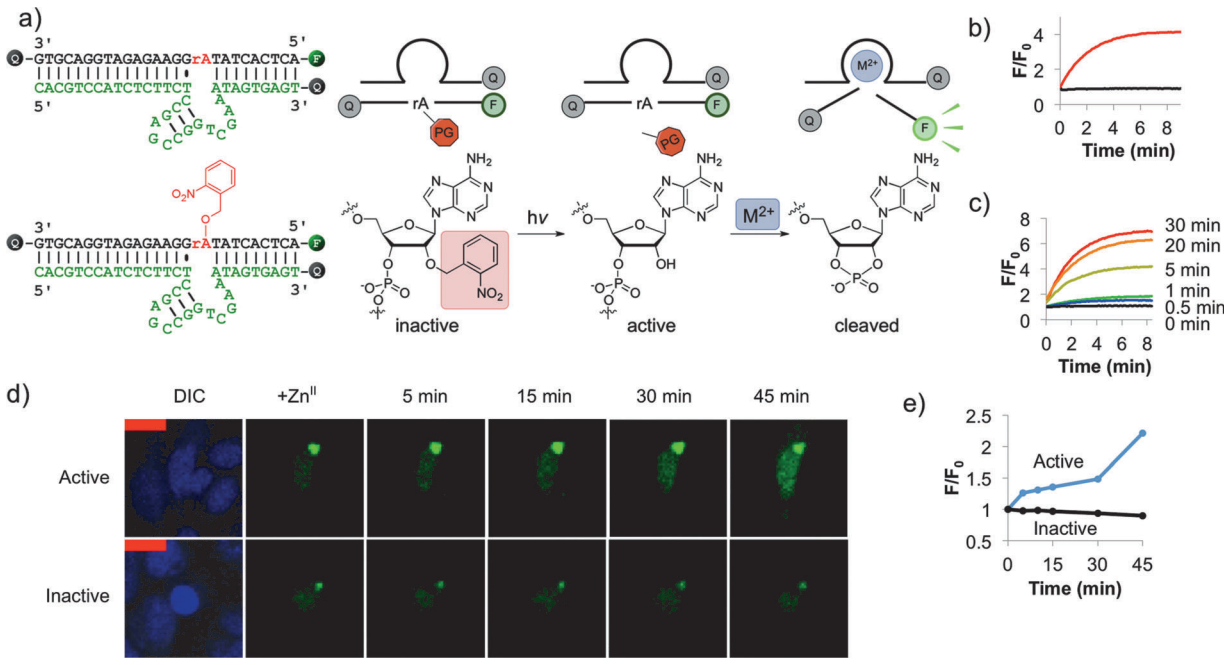

Figure 1. a) Design of the catalytic beacon DNAzyme sensor, and caging strategy for DNAzyme protection and activation. F = Fluorophore (fluorescein), Q = Quencher (Dabcyl, BHQ-1), PG = protecting group (o-nitrobenzyl). b) Fluorescence of caged (black) and unmodified (red) 8–17 DNAzyme in response to 50 μM Zn^{2+}. c) Fluorescence of caged DNAzyme uncaged by increasing doses (in minutes) of 365 nm light, in response to 50 μM Zn^{2+}. d) HeLa cells transfected with caged active or caged inactive 8–17 DNAzyme (0.5 μM) for 11 h, then irradiated for 30 min at 365 nm, followed by the addition of 50 μM Zn^{2+}-citrate. Scale bar: 20 μm. e) Normalized fluorescence intensity of cells in part (d).

To prevent the substrate strand from being cleaved, a 2′-O-nitrobenzyl adenosine (called caged or photocaged adenosine) is used in place of the normal adenosine at the cleavage site. As the 2′-OH of the adenosine plays a critical role in the activity of the DNAzyme, adding the 2′-O-nitrobenzyl protection group (PG) to this location can render the DNAzyme inactive. In this way, the DNAzymes is able to enter into cells and distribute in different compartments without being cleaved prematurely. In order to restore the active DNAzyme for sensing, we exploited the photolabile characteristic of the 2′-O-nitrobenzyl group. Under irradiation by 365 nm light, the PG is removed from the caged adenosine, uncovering the native adenosine with the 2′-OH group. The reactivated (uncaged) DNAzyme will then cleave the substrate strand, leading to fluorescent signal increase. Because the DNAzyme is highly specific to the metal ion that is used, this photoactivation strategy allows the detection of metal ions in cells. As the deprotection is achieved with light, it is orthogonal to cellular delivery and function, and thus allows the temporal control over the uncaging and activation of the DNAzyme sensor.

The performance of the photocaged DNAzyme was first assessed in a buffer under physiological conditions. The substrate strands containing either caged adenosine or native adenosine were annealed to the enzyme strand. The DNAzyme reaction was initiated by the addition of Zn^{2+}. In the absence of 365 nm light, the fluorescence increased rapidly only in the case of the unmodified substrate that contained the native adenosine (Figure 1b), similar to those observed previously. In contrast, when the substrate strand that contained the caged adenosine was used, no increase in fluorescence was observed, thus indicating complete inhibition of the DNAzyme.

In order to test whether the protective effect of the 2′-O-nitrobenzyl PG could be reversed through activation with light, we used both a portable hand lamp (Spectroline, 365 nm) and a Blak-Ray B100 lamp (365 nm) to irradiate samples of the caged 8–17 DNAzyme for different lengths of time. While no increase in the fluorescence was observed in the absence of light, the fluorescence increased with time after the addition of metal ions (Figure 1c). Longer exposure to 365 nm light led to a greater increase in the fluorescence. (Figure 1b). These results strongly suggest that the activity of the DNAzyme can be restored through irradiation with light: the longer the exposure to light, the more active DNAzyme was present and the higher the fluorescence. The same conclusion can be drawn from the results of polyacrylamide gel electrophoresis (PAGE, Figure S4 in SI)

For biological applications, the stability of the 2′-protecting group is paramount. To test the stability of caged DNAzymes, substrate strands containing either caged or native adenosine were annealed to the enzyme strand and incubated in either a buffer containing 50 μM Zn^{2+} or in human serum (80% serum, 20% Tris-buffered saline, pH 7.4) for extended periods of time. PAGE analysis showed that the substrate strand containing the native adenosine was cleaved in less than one hour under both conditions, but little cleavage was observed even at times up to seven days in the presence of 50 μM Zn^{2+} or two days in the presence of human serum (see Figures S5 and S6 in the Supporting Information).

Having demonstrated that the caged DNAzymes are stable, we then proceeded to their use for sensing metal ions within cultured HeLa cells, employing the Zn^{2+}-responsive 8–17 DNAzyme as our model system. As zinc is present in both cells and growth media, the cell-delivery process itself poses a major challenge, because the presence of endogenous Zn^{2+} can promote DNAzyme-based cleavage of the substrate strand before the DNAzyme reaches the interior of the cells, if no protection strategy is used. The 8–17 DNAzyme containing the caged adenosine was delivered to HeLa cells by Lipofectamine 2000 following a modification of manufacturer's protocols. Briefly, the enzyme and caged substrate strands were heated to 80°C and allowed to anneal. Lipofectamine 2000 (5 μL) and the annealed DNAzymes (1 nmol) were incubated separately in Opti-MEM media (Invitrogen) for 5 min, then combined and incubated for additional 25 min. The prepared DNAzyme–Lipofectamine mixture was added to HeLa cells and incubated for several hours. Confocal microscopy images of the DNAzyme (Figure 1d) showed the delivery of the fluorescent DNAzyme inside the cells in a diffuse staining pattern mainly localized in the nucleus (determined by co-localization with Hoechst stain). This distribution pattern is in agreement with previous reports that demonstrated the nuclear accumulation of DNA delivered by cationic liposomes (Lipofectamine PLUS).[14] In order to introduce the DNAzyme probe into other organelles, alternative delivery methods can be used, such as gold nanoparticles for lysosomal distribution.[8]

Upon irradiation with a 365 nm lamp, followed by the addition of Zn^{2+}-citrate, an increase in fluorescence intensity was observed over time (Figure 1e). To confirm that the observed increase in fluorescence was caused by the metal-ion sensing by the DNAzyme and not by nonspecific cleavage by other cellular components, we used an enzyme sequence in which two critical bases in the catalytic loop were substituted (Supporting Information, Table S1).[15] Using this inactive 8–17 DNAzyme, no increase in the fluorescence was observed in the buffer (Figure S7), thus confirming that it was unable to cleave the native adenosine-containing substrate strand, even in the presence of Zn^{2+}. Furthermore, the inactive DNAzyme showed no significant increase in fluorescence over 45 min in the HeLa cells under the same condition (Figure 1d,e). Together, these results strongly indicate that the caged DNAzyme can be used to detect and image metal ions in living cells.

The 8–17 DNAzyme described in this manuscript displayed a linear response to $[Zn^{2+}]$ in the range of 0–500 μM in buffer.[6f] Similarly, the GR-5 DNAzyme described in this manuscript showed a linear response to $[Pb^{2+}]$ in the range of 0–1 μM.[16] As many other reported metal-ion sensors,[3d] the current intensity-based sensor does not allow accurate determination of metal-ion concentration within cells, thus making it difficult to show linearity of the sensor response to intracellular metal ions. To overcome this limitation, we are currently investigating the design of new ratiometric sensors that may allow the quantification of metal ions within cells.

After demonstrating the use of the 8–17 DNAzyme for cellular sensing and imaging of Zn^{2+}, we investigated the applicability of this method to other DNAzymes for the detection of their respective target metal ions. Since the first discovery of DNAzymes in 1994 using in vitro selection, many DNAzymes have been obtained using similar selection methods. As a result, the majority of currently identified DNAzymes share a similar secondary structure consisting of two double-stranded DNA binding arms flanking the cleavage site. More interestingly, the sequence identity of the two binding arms is not conserved, as long as they can form Watson–Crick base pairs with the chosen substrate. The metal-ion selectivity of DNAzymes results from the sequence identity of the loop in the enzyme strand. As a result, the exact substrate sequence that can be recognized by a DNAzyme can be arbitrarily chosen. This feature also allows multiple DNAzymes to recognize the same substrate sequence. An attractive advantage of our photocaging strategy is that we can use the same caged substrate strand to sense different metal ions by using different enzyme strands. As a proof-of-concept, we synthesized a DNAzyme sequence that can hybridize to the same caged substrate strand as the 8–17 DNAzyme, but contains the catalytic loop of the GR-5 DNAzyme, (the first DNAzyme, obtained in 1994),[5a] which shows significant activity in the presence of Pb^{2+} but not with any other metal ions.[16] This caged GR-5 DNAzyme showed little cleavage activity in the absence of light, but increased dose-dependent DNAzyme activity upon photoactivation (Figure 2).

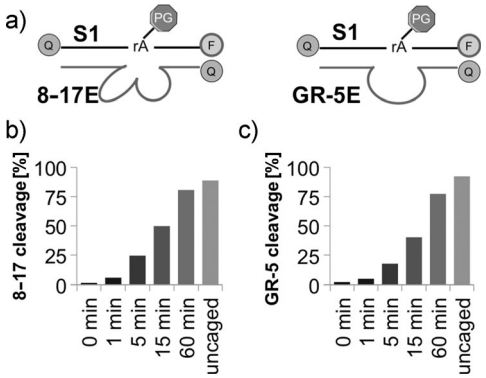

Figure 2. Scope of the caging strategy. a) Hybridization of different DNAzymes to the same substrate strand (S1). Cleavage of caged and uncaged b) 8–17 and c) GR-5 in the presence of Zn^{2+} (8–17, 500 μM) and Pb^{2+} (GR-5, 2 μM), respectively.

In conclusion, we have demonstrated a general and effective strategy for protecting the substrate strand of a DNAzyme, thus enabling its delivery into cells without being cleaved during the process, and showing its use as a cellular metal-ion sensor upon photoactivation. This strategy provides enhanced DNAzyme stability (up to multiple days in serum) and allows temporal control over its activity. As the only modification to the original DNAzyme is located on the substrate strand, we can replace the enzyme strand without the need for re-optimization for each DNAzyme. Furthermore, as a result of the enhanced stability of the caged DNAzyme, its delivery into cells does not require the use of nanomaterials. This method will thus greatly expand the applicability of DNAzymes as biosensors and advance the field of metal-ion sensing.

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