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Selective Fluorescent Detection of RNA in Living Cells by Using Imidazolium-Based Cyclophane

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S Supporting Information

ABSTRACT: A water-soluble imidazolium-based fluorescent chemosensor senses RNA selectively through fluorescence enhancement over other biologically relevant biomolecules in aqueous solution at physiological pH 7.4. Fluorescence image detection of RNA in living cells such as onion cells, HeLa cells, and animal model cells was successfully demonstrated which displays a chelation-enhanced fluorescence effect. These affinities can be attributed to the strong electrostatic $(C-H)^{+}\cdots A^{-}$ ionic H-bonding and the aromatic moiety driven π-stacking of imidazolium-based cyclophane with the size-complementary major groove of RNA.

Ribonucleic acids (RNA) are well-known to play vital roles in cell physiology.[1] Real-time monitoring RNA transport into living cells and the information about spatial-temporal distribution of RNA are crucial to understand the role of RNA dynamics in cellular functions. Several classes of molecular probes have been developed for RNA detection in living cells.[2] Using fluorescence in situ hybridization assays[3] has led to the identification of gradients of RNA in cells, embryos, and tissues.[4] However, this technique is conducted for fixed samples and therefore provides limited information. Other disadvantages are the laborious time-consuming nature of the assay and the difficulty in analyzing the images. The most adequate procedure to study RNA dynamics is using living cell assays. Under these conditions, the probes to be used must have high specificity and sensitivity for RNA. Here we report the fluorescence imaging of RNA in live cells by using stable, small chemical compounds which do not significantly disturb the RNA dynamics by involving only noncovalent binding to RNA due mainly to the electrostatic interactions and π-stacking.

Cyclophanes are used in molecular recognition, catalytic processes, molecular electronic devices, drug deliveries, and anion sensing for biologically important anionic moieties due to their important applications in biology, chemical processes, and environmental sciences.[5] The semirigid structure of cyclophanes with a defined cavity and fixed arrangement of atoms as compared to open chain systems, facilitates the formation of specific and stable supramolecular complexes with anionic moieties through noncovalent interactions.[6] Much work has been done in the field of biomolecular recognition in biological systems,[6,7] but still the potential to design water-soluble cyclophanes for the recognition of particular bioanions like RNA, DNA, and proteins at physiological pH remains to be of the utmost importance. Thus, our major focus in this communication is on fluorescence sensing and imaging of RNA in aqueous solution at physiological pH 7.4 in living cells by using a fluorescent cyclophane.

To find an RNA-selective probe for living cell imaging has proven to be difficult because nucleic acid binding to small molecules generally tends to have better affinity for double stranded DNA (dsDNA) than single stranded RNA (ssRNA). Furthermore, living cell systems are complex with proteins and membranes which may lead to nonspecific binding to imaging probes. Chang and co-workers reported small molecules showing a slightly higher fluorescence intensity for RNA than DNA.[8] Such molecules have positive charges which interact with the electronegative region of RNA/DNA.[9] Therefore, it would be useful to take advantage of noncovalent interactions for RNA fluorescence sensing.

In the case of DNA fluorescence sensing, noncovalent binding modes of receptors with DNA in solutions are broadly divided into two categories: intercalation and minor groove binding. Intercalation is the insertion of the planar aromatic part of receptors between base pairs of DNA, whereas for the minor groove binding a small receptor fits into the minor groove of DNA. In this regard, RNA recognition by small molecules (like cationic receptors) would be governed by the more electronegative potential on the surface of the major groove having multiple hydrogen-bond acceptors due to the presence of tandem GC base pairs of RNA,[10] as well as the shape complementarity of the RNA major groove to small molecules.

For noncovalent binding, receptor molecules commonly utilize amide, pyrrole, urea, ammonium, and guanidinium groups as binding sites to form $N-H\cdots A^{-}$ hydrogen bonds.[11] Pyridine-, benzene- and anthracene-based receptors with imidazolium units bind effectively with anionic species through $(C-H)^{+}\cdots A^{-}$ ionic hydrogen-bonding interactions.[6,12] This makes it possible to differentiate simple bioanions under physiological pH conditions due to competitive hydrogen bonding against solvent molecules. Attractive features of

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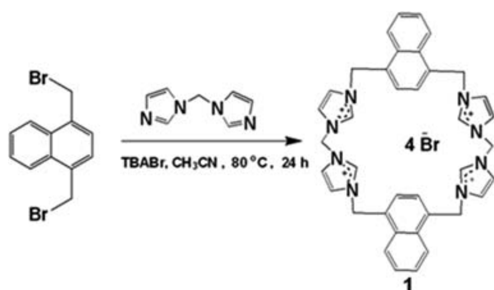
sensors based on anion-induced fluorescence intensity changes are simplicity and high sensitivity. For the recognition of RNA, different strategies[10] have been adopted, but it remains a challenging task to find new approaches that could improve the simplicity, selectivity, and sensitivity.

While varieties of techniques have been introduced for sensing anions earlier, fluorescent imidazolium-based receptors have also been extensively investigated in recent years.[6,12] It is quite difficult to design scaffolds for biomolecular recognition, especially cyclophanes that are soluble in aqueous solution at physiological pH. A water-soluble imidazolium-based cyclophane would be an excellent choice for the development of receptors for biologically relevant targets as highly negatively charged, stable species.[6] Thus, we report a new water-soluble imidazolium naphthalene derivative, which gives specific turn-on fluorescence sensing for RNA in 100% aqueous solution (pH = 7.4, 10 mM phosphate buffer). This new fluorescent chemosensor senses RNA by the chelation-enhanced fluorescence (CHEF) effect. It displays no particular response to other bioanions. This work demonstrates that the imidazolium receptor is a good candidate for sensing with selective interactions toward RNA in buffer. The imidazolium protons $(C-H)^+$ would favor ionic hydrogen bonding with phosphate groups probably in the major groove of RNA. The $\pi-\pi$ or π–H interaction[13] between nucleic base and fluorophore might cause the unique selectivity for RNA through turn-on emission phenomena.

Cyclophane **1** was synthesized by the reaction of 1,4-bis(bromomethyl)naphthalene with 1-(1*H*-imidazol-1-ylmethyl)-1*H*-imidazole in anhydrous CH_3CN followed by recrystallization (ethanol) in 78% yield (Scheme 1). Fluorescence

Scheme 1



titrations occurred in 100% aqueous solution at pH 7.4 (10 mM phosphate buffer). The receptor **1** displays a structureless fluorescence emissions (λ_{max} = 421 nm) when irradiated at 350 nm in aqueous solution (Figure 1b). Owing to the presence of imidazolium units in receptor **1**, very negligible fluorescence emission was observed at 449 nm. To gain support for the optimized geometry of receptor **1**, density functional theory was used (Supporting Information, Figure S5). The most stable form of **1** is shown in Supporting Information, Figure S6. The distance between the nearest carbons in naphthalene on each side is 5.2 Å, which is too far to have dispersion interactions. Hence there is no $\pi-\pi$ stacking between the two naphthalene rings. On the basis of the literature,[14] the formation of an excimer in a cyclophane is unexpected because such a process is reported to be feasible only in systems having an interplanar distance of less than 4 Å.

Fluorescence titrations were done in 100% aqueous solution at pH 7.4 (10 mM phosphate buffer). Visual features are shown

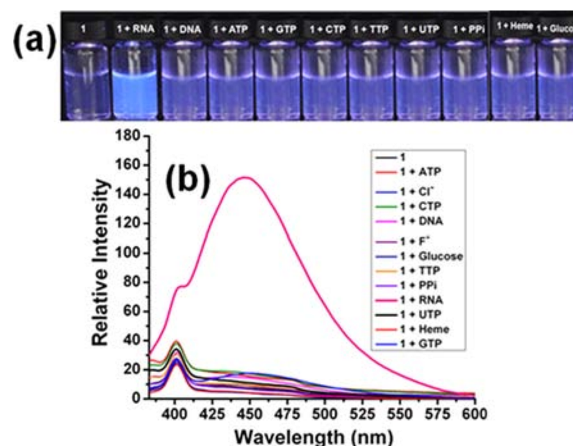


Figure 1. (a) Visual fluorescence features of **1** (10 μM) in the presence of RNA, DNA, heme, glucose and sodium salts of ATP,GTP, CTP, TTP, UTP, PPi (1 equiv each) at pH 7.4 (10 mM phosphate buffer). (b) Fluorescence of **1** (10 μM) in the presence of RNA, DNA, heme, glucose along with sodium salts of ATP, GTP, CTP, TTP, UTP, PPi and TBA salts of F^- and Cl^- (1 equiv each) at pH 7.4 (10 mM phosphate buffer) (slit width = 5 nm; excitation at 350 nm).

in Figure 1a and the spectra in Figure 1b. There were only minor changes (quenching) upon the addition of 1 equiv of sodium salts of phosphate anions (ATP, GTP, CTP, UTP, TTP, pyrophosphate (PPi)), glucose, heme, and TBA salts of F^- and Cl^-. Similarly minor change (quenching) was observed upon the addition of DNA. On the other hand, turn-on fluorescence was observed in the emission spectrum with RNA, which is apparent from the blue color enhancement of the **1**–RNA solution (Figure 1a). Supporting Information, Figure S4 clearly shows the fluorescence enhancement at 449 nm with increasing concentration of RNA (0–5 equiv). Such excimer-like fluorescence enhancement at 449 nm results from the π-stacking of aromatic part of cyclophane **1** in the GC base pairs major groove region of RNA.[15] From the fluorescence titrations, the binding constant for **1** with RNA is determined to be 4.9×10^6 M^{-1}.[12,16]

While the binding stoichiometry for RNA and **1** is 1:1 (Supporting Information, Figure S4), the characteristic fluorescence response for their interaction indicates different types of interactions. The main driving force for the formation of such interaction in the presence of RNA could be attributed to the characteristic of electrostatic interactions between the cationic cyclophane and the phosphate backbone at RNA in the major groove and the other is π-stacking of the aromatic part of cyclophane **1** in the major groove region of RNA, which allows selective detection of RNA. Receptor **1** binds RNA through the interaction of the imidazolium protons with the oxygen atoms of phosphate groups. The putative involvement of electrostatic interactions is hypothesized based upon observation of the significant influence of RNA on the fluorescence enhancement at 449 nm in buffer solution.

^{1}H NMR titration experiment of RNA binding with **1** was performed to assess the binding pattern. The ^{1}H NMR spectra for **1**–4Br^- and **1**–RNA are shown in Supporting Information, Figure S3. The addition of 1 equiv of RNA to a 2 mM solution of **1** in DMSO-d_6 causes quenching and shows an upfield shift (<0.3 ppm) of the imidazolium proton $(C-\mathbf{H})^+$ and also upfield shifts (<0.15 ppm) for the remaining imidazolium protons. This arises presumably from the strong binding of the phosphate group of RNA with the imidazolium protons.

Upfield shifts (<0.25 ppm) of the aromatic protons of the naphthalene may be attributed to the $\pi-\pi$ interactions between naphthalene and the nucleobase of RNA in the major groove region. In addition, the peaks of H_b and H_c, which belong to the ring and probably directly interact with the major groove, are merged. Peak broadening and splitting of the H_g and H_h protons of the methylene groups accompanied by an upfield shift (<0.25 ppm) were also observed as a result of the interaction with the phosphate oxygen atoms of RNA.

For the detection of RNA with **1** in gel, the samples of **1** treated with RNA and DNA were subjected to electrophoresis in agarose gel. The fluorescence was observed in the RNA treated sample, but not in the DNA treated sample (Supporting Information, Figure S7C,D). The fluorescence was observed from both RNA and DNA when treated with ethidium bromide (Figures S7A,B). These results strongly suggest that **1** is capable of RNA sensing.

Cyclophane **1** was also used as a probe to target RNA in live onion cells. Prior to the addition of **1**, these cells showed no autofluorescence (Figure 2A), but after incubation with probe

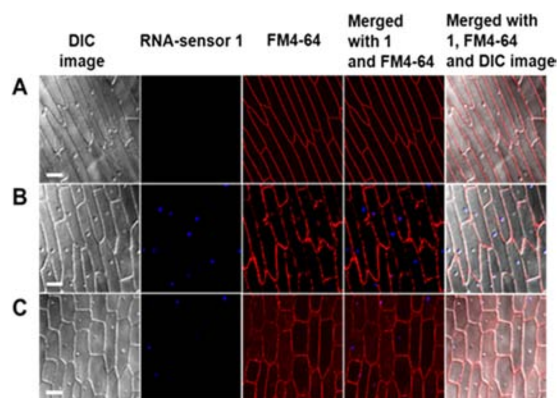


Figure 2. Visualization of endogenous mRNA localization in onion cells. Optical images of onion cells incubated without probe **1** (A), with probe **1** (10 μM) (B), and with probe **1** after cordyceptin treatment for 1 h (C). The cell membrane was stained with the fluorescent dye FM4−64 (red) for the identification of individual cells. Scale bars represent 100 μm.

1, the cells showed bright blue fluorescence at the nucleus as compared with untreated cells (Figure 2B). For further studies we treated one group of cells with 150 μg/mL cordyceptin (inhibitor of RNA synthesis) for 2 h. Then the cells were exposed to the 10 μM RNA sensor **1** for 1 h. Microscopic observation was assessed in the confocal microscope (images were recorded at the excitation wavelength of 405 nm and the emission wavelength of 425−475 nm for blue fluorescence). As shown in Figure 2C, cordyceptin is known to decrease cellular RNA levels, and hence this addition induced the fluorescence quenching.

The RNA-binding property of **1** was also evaluated with HeLa cell lines using confocal laser scanning microscopy (CLSM). mRNA is transcribed from DNA which is localized in the nucleus, and transferred to the cytosol for translation. Other RNAs such as tRNA and rRNA are localized in the cytosol. Therefore, RNA is localized in both the nucleus and the cytosol. Fluorescent images of individual fixed cells treated with **1** clearly showed that it was capable of entering into the cell and binding to the RNA in both cytosol and nucleus, giving rise to a strong blue fluorescence signal coming from the nucleus and cytosol (Figure 3A). 7-Aminoactinomycin D (7-AAD) is a fluorescent chemical compound with a strong affinity for DNA

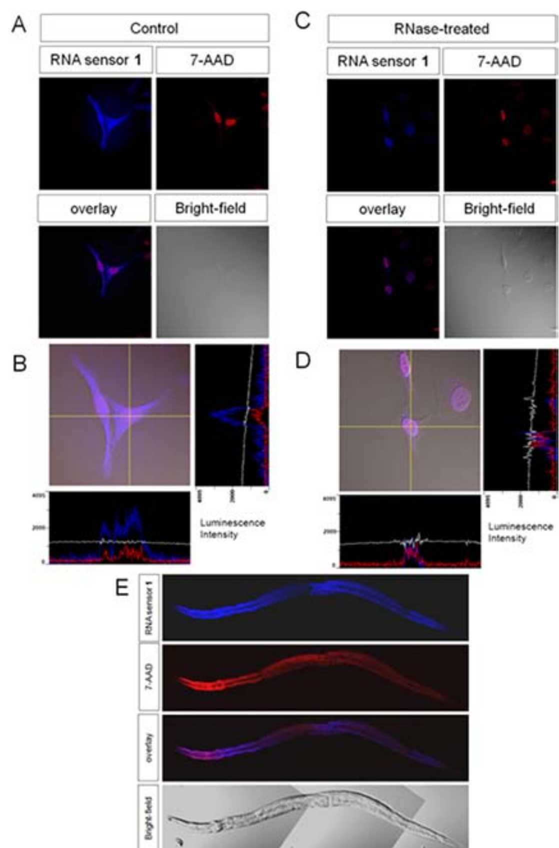


Figure 3. Confocal laser scanning microscopy (CLSM) images of Hela cells (A−D) and *C. elegans* (E) incubated with RNA sensor in PBS for 1 h at room temperature. (A,C) RNA sensor **1**, 7-ADD, overlay, and bright-field image of control or RNase treated Hela cells. (B,D) Luminescence stems exclusively from the HeLa cells at the amplified overlay image of panels A and C. Blue lines indicate the intensity of RNA sensor; red lines indicate the intensity of 7-ADD. (E) *C. elegans* stained with RNA sensor **1** and 7-ADD at the young adult stage.

which emits red fluorescence. The luminescence intensity plot also showed that the total amount of RNA is larger in the nucleus than the cytosol (Figure 3B). To investigate whether **1** specifically binds to RNA, we examined the effect of RNase-treatment. When RNase was treated, 7-AAD showed similar intensity compared with control cells, however **1** was only weakly detected in the nucleus and totally disappeared in the cytosol (Figure 3C,D). These results indicated that RNA-**1** complexation was decreased by RNase-mediated degradation of RNA.

We investigated the biological application of **1** in an animal model system. As *Caenorhabditis elegans* (*C. elegans*) is transparent, it should be one of the most efficient animal model systems to detect emitting fluorescence from an **1**-RNA complex in an individual cell. **1** efficiently binds to RNA and emits blue fluorescence from each cell which is composed of the whole embryo (Figure 3E). From this result, we can investigate the RNA expression map of the whole embryo at a specific stage. For instance, the total amount of RNA is lower in the middle region of *C. elegans* than the anterior and posterior regions of embryo. This demonstrates the utility of **1** for biological applications.

In conclusion, we designed a novel fluorescent chemosensor, which senses RNA selectively through fluorescence enhancement over other biologically relevant biomolecules in aqueous solution of physiological pH 7.4. These affinities can be attributed to the strong complementary $(C-H)^+\cdots A^-$ ionic H-bonding with the phosphate group of RNA and the π-stacking of the aromatic part of cyclophane **1** with RNA, which result in selective detection of RNA. Fluorescence imaging of RNA in living cells including onion cells, HeLa cells, and animal model cells was also successfully demonstrated. We anticipate that the present fluorescent probe could serve as a new tool in biological studies.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, NMR spectral analysis, fluorometric analysis, theoretical calculations and methods for biological study. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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