A molecular fluorescent dye for specific staining and imaging of RNA in live cells: a novel ligand integration from classical thiazole orange and styryl compounds†

Yu-Jing Lu,*a Qiang Deng,a Dong-Ping Hu,a Zheng-Ya Wang,a Bao-Hua Huang,a Zhi-Yun Du,a Yan-Xiong Fang,a Wing-Leung Wong,a Kun Zhang,*a,b and Cheuk-Fai Chowb

A new RNA-selective fluorescent dye integrated with a thiazole orange and a p-(methylthio)styryl moiety shows better nucleolus RNA staining and imaging performance in live cells than the commercial stains. It also exhibits excellent photostability, cell tolerance, and counterstain compatibility with 4',6-diamidino-2-phenylindole for specific RNA–DNA colocalization in bioassays.

Live cell imaging or staining using target-specific molecular fluorescent probes is a very important and useful technology in medical diagnosis and biomedical research because it allows the investigation of the distribution, migration and transcriptional dynamics of the cell nucleus.1 RNA molecules in living cells are known to be responsible for a wide variety of functions including physical transportation, interpretation of genetic information, regulation of gene expression, and some essential bio-catalytic roles.2 However, information on distribution dynamics and transcriptional activities of RNA in cell nucleus and the relationship with special secondary structures of DNAs, such as G-quadruplex and temporal/spatial processing of RNA, is still limited to date.1 It is probably because of the limited RNA-selective technology that has been developed during the past decades.

In recent years, a considerable amount of effort has been devoted to the development of small-molecule-based fluorescent dyes for RNA imaging in live cells.4 Some fluorescent molecular ligands like crescent-shape and V-shape probes,5a,b styryl and E36 dyes,5c,d and a near-infrared probe5e were examined for RNA detection. In comparison with DNA dyes, a common difficulty encountered is the low specificity of small molecular dyes for RNA, especially the nucleus RNA dyes for live cell imaging, because small molecules usually have a better affinity for DNA. The poor nuclear membrane permeability of the RNA dyes is also a big problem to be resolved.6 SYTO RNASelect is the only commercially available dye for RNA imaging in live cells, but its molecular structure is not known and thus cannot be further modified to fit for different purpose. The molecular design of new target-specific fluorescent compounds is the key to address these problems. Styryl-based molecular dyes have been recently reported and proved to undergo selective binding with RNA in nucleoli and cytoplasm of live cells.5d The findings indicate that the molecular structure of styryl is crucial for RNA-specific binding.7α-c In addition, thiazole orange (TO, Scheme 1) has been used as a good structural platform for designing novel fluorescent probes for the targeting of different types of nucleic acids.8 However, TO has never been explored as a RNA dye because of its poor selectivity.

[a] Institute of Natural Medicine and Green Chemistry, School of Chemical Engineering and Light Industry, Guangdong University of Technology, Guangzhou 510006, P. R. China. E-mail: luwj@gdut.edu.cn; kzh@gdut.edu.cn; Tel: +86-20-39322235
[b] Department of Science and Environmental Studies, Centre for Education in Environmental Sustainability, The Hong Kong Institute of Education, 10 Lo Ping Road, Tai Po, Hong Kong SAR, P. R. China. E-mail: wingleung@ied.edu.hk; Tel: +852-2948-8401
[c] School of Chemical and Environment Engineering, Wuyi University, Jiangmen 529020, P. R. China
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Scheme 1 Molecular design of Styryl-TO through ligand integration of thiazole orange TO and DNA dye C61.
1-Methyl-2,6-bis(4-(methylthio)-styryl)-pyridinium iodide C61 is an example of a styryl compound, which is also known as a promising green fluorescent DNA probe. The most attractive property of C61 is that it provides a broader colour option for biological imaging and the p-(methylthio)styryl moiety has a unique functionality for discrimination of dsDNA and RNA. We therefore attempt to design a novel RNA-specific fluorescent dye that possesses both of the advantages of TO and C61 by integrating their special scaffolds into a single molecule. The concept of ligand integration to merge with two or more unique characteristics and functionality of small organic molecules could be a promising approach for the development of RNA-specific dyes. Herein, we report a new RNA-specific switch-on fluorescent dye (Styryl-TO†), which is designed by the integration of a TO structure and a p-(methylthio)styryl moiety of C61, and demonstrates its performance for nuclear RNA staining and imaging in live cells.

Styryl-TO was prepared by the integration of a TO and a p-(methylthio)styryl moiety of C61 through a multi-step synthesis process (Scheme S1, ESI†). To prove the concept, the functionality, binding specificity, and fluorescent properties of the new dye interacting with RNA and various DNAs were investigated by fluorescence titration. The fluorescence intensity increases by approximately 152 ± 23 fold upon Styryl-TO binding with RNA as shown in Fig. 1a. Nevertheless, under similar conditions, the treatment with a wide variety of DNA substrates including duplex DNA (salmon testes stDNA, ds26: self-complementary duplex DNA), single-stranded DNA (da21), and G-quadruplex DNA (htg21), it induced a much smaller fluorescence signal enhancement (Fig. 1b). This new molecular scaffold surprisingly changes the binding preference of the TO moiety to RNA rather than DNA; it also retains the merit fluorescence-signalling property of TO dyes. Styryl-TO shows only a very weak background fluorescent signal in blank solutions without RNA. In order to further explain the selectivity of the dye towards RNA, the equilibrium binding constants ($K_a$) of the compound and different nucleic acids were investigated and calculated (Table 1). Obviously, the RNA shows a much larger binding affinity (from 3- to 7-fold in terms of $K_a$ values) than other DNA substrates, which indicates that Styryl-TO has higher specificity towards RNA. The good specificity is attributed to the new integrated p-(methylthio)styryl moiety which is proved to bind RNA with high selectivity and its analogues are known to be unable to differentiate RNA from DNA.

The photophysical properties of Styryl-TO in both aqueous and organic media were studied and the results (Table S3 and Fig. S7, ESI†) showed that the fluorescence quantum yield ($\Phi_f$) of the dye in an aqueous solution is much lower ($\Phi_f = 1.6 \times 10^{-4}$) that in dichloromethane ($\Phi_f = 5.3 \times 10^{-3}$). Interestingly, the fluorescence quantum yield of the dye upon binding with RNA in an aqueous solution is significantly increased by about 35 times ($\Phi_f = 5.6 \times 10^{-2}$). The RNA induced fluorescence signal (orange) is strong enough for observation by the naked eye under UV-illumination as shown in Fig. 2.

To study the higher-order nuclear organization of RNA molecules, cell staining with a fluorescent RNA-specific dye is needed to be counter-labelled with DAPI, which is a DNA-selective dye. Fig. 3a shows the fluorescence imaging for the treatment of PC3 cells (human prostate cancer cell line) with Styryl-TO that produces a strong and bright fluorescence response signal mainly confined to certain regions of the nucleus. Some green spots with weak brightness are also observed in the cytoplasm. A reported RNA probe E36 was also employed for performance comparison (Fig. 3d–f). E36 stains the nucleus but most regions of the cell are also stained (Fig. 3d). The comparison study indicates that Styryl-TO shows much better RNA specific staining performance in cells. The cellular localization of Styryl-TO in PC3 cells is similar to that of E36 and other RNA fluorescent probes. In addition, the fluorescent intensity of Styryl-TO in the nucleoli where RNA undergoes transcription is found to be much higher than that in the nucleus; however, no change in cell morphology or viability was observed during the imaging experiments. Fig. 3c further demonstrates the cell imaging performance of Styryl-TO.

**Table 1** Fluorescence characteristics of Styryl-TO and its binding constants with different nucleic acids

<table>
<thead>
<tr>
<th>Nucleic acid</th>
<th>B shift$^{a}$ (nm)</th>
<th>Concentration of nucleic acid$^{b}$ (µM)</th>
<th>$K_a$’ (×10² M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>5</td>
<td>15</td>
<td>12.32</td>
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<tr>
<td>ds26</td>
<td>12</td>
<td>12</td>
<td>4.55</td>
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<tr>
<td>htg21</td>
<td>10</td>
<td>16</td>
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<tr>
<td>st-DNA</td>
<td>11</td>
<td>8</td>
<td>1.78</td>
</tr>
<tr>
<td>da21</td>
<td>15</td>
<td>8</td>
<td>2.17</td>
</tr>
</tbody>
</table>

$^{a}$E36, Single DNA da21, duplex DNA ds26 and St-DNA in Tris-HCl buffer.

Fig. 1 (a) Fluorescence titration of Styryl-TO with an increasing concentration of RNA ($\lambda_{ex} = 476$ nm); (b) the plots of fluorescence intensity ($\lambda_{em} = 535$ nm) versus various nucleic acids at different concentrations.

Fig. 2 The fluorescence changes of Styryl-TO under UV-illumination ($\lambda_{ex} = 302$ nm) with the presence of various nucleic acids: G-quadruplex htg21, Oxy28, Single DNA da21, duplex DNA ds26 and St-DNA in Tris–HCl buffer.
by co-staining with the blue nucleus dye DAPI in the FITC or Cy3 channel. **Styryl-TO** exhibits a very low fluorescence response to DNA and good counter-labelling properties with the DNA-selective dye DAPI in the living PC3 cells. The images clearly reveal different patterns of RNA–DNA co-localization in the live cells. This observation further supports that **Styryl-TO** has a very good counterstaining compatibility with the DNA-selective dye (DAPI). In addition, the utility of the dye was also demonstrated in other cell lines (HUVEC, NIH-3T3, L929) and gave very good RNA specificity and imaging performance (Fig. S10B, ESI†).

To confirm the specificity of **Styryl-TO** towards RNA in cells, deoxyribonuclease (DNase) and ribonuclease (RNase) digest tests were performed. In the DNase digest test, only DNA substrates are hydrolysed in the cells while in the RNase digest test, only RNA substrates are hydrolyzed. Fixed-permeabilized PC3 cells were used in the experiment and E36 and SYTO RNASelect were selected as the control. As expected, in the DNase digest test (Fig. 4 DNase), no obvious diminishing of fluorescence in the nucleoli stained with **Styryl-TO** was found; in contrast, for the RNase digest test, the originally intensive fluorescence signal of the nucleoli stained with **Styryl-TO** in cells was dramatically disappeared (Fig. 4 RNase). Similar photo-behaviour was also observed in the control study with E36 and SYTO RNASelect. These results evidently indicate that the enhanced fluorescence signal is originated from the interaction of **Styryl-TO** with RNA in the nucleoli of PC cells.

Photostability has always been a key factor to determine the usefulness of a dye for cell imaging. **Styryl-TO** is evaluated in live PC3 cells using an inverted fluorescence microscope. Fig. 5 shows the photo-stability comparison of SYTO RNASelect and **Styryl-TO**. The fluorescence intensity of SYTO RNASelect was found to be decreased by more than 80% after 1 h irradiation while the intensity of **Styryl-TO** showed almost no changes. After 3 h, the fluorescence signal of SYTO RNASelect was completely disappeared; nonetheless, the fluorescence signal of **Styryl-TO** in the nucleoli of the PC cells was still retained obviously (approximately 90% of the intensity in beginning). Moreover, the photo-stability of **Styryl-TO** in solution conditions was examined and it also exhibited better stability than SYTO RNASelect (Fig. S11, ESI†).

In conclusion, a new and robust RNA-specific switch-on fluorescent dye was developed from the ligand integration of...
a classical nucleic acid fluorescent probe and a DNA dye moiety. The newly developed molecular scaffold retains both the merit properties of its parent compounds. The study demonstrated the concept of ligand integration and proved that the dye is able to offer excellent RNA specificity, photo-stability, and cell tolerance. The dye shows very good counterstain compatibility with DAPI for specific RNA–DNA colocalization investigations in live cells. Also, the dye was successfully utilized in staining of nucleolus RNA for cell imaging and its performance is found to be much better than the commercial SYTO RNASelect dye.

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Notes and references


