Analytical Chemistry

Design and Synthesis of a Dimethylindole Red Trimer: A New Light-Up Red-Emitting Fluorescent Probe for G-Quadruplexes

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We present a zwitterionic fluorescent probe based on dimethylindole red (Dir) derivative (named Dir-Trimer), which was synthesized by organic amine covalently linking three red-emitting Dir units. The unique structure of Dir-Trimer with three zwitterionic units largely decreased its intrinsic fluorescence. Fluorescence experiments exhibit that Dir-Trimer selectively interacts with G-quadruplexes with large fluorescence enhancement and sensitivity. Further studies indicate that Dir-Trimer could visualize G-quadruplexes on cell level.

Introduction

G-rich sequences have a tendency to form planar G-quartets through Hoogsteen hydrogen bonds, which can further fold into G-quadruplexes.[1] G-quadruplex sequences are prevalent in many important genomic regions,[2] such as telomeres,[3] and gene promoters (c-myc,[4] c-kit,[5] kras,[6] and bcl-2[7]). Extensive biophysical evidences confirm that G-quadruplexes play vital roles in regulation of certain key biological processes, such as protecting the ends of linear chromosomes to control the cell proliferation or regulating gene expression in the gene promoter.[8] Ligands which stabilize G-quadruplex structures have great potential to become antitumor drugs by inhibiting telomere growing and gene expression.[9] Quarfloxine (CX-3543) is one case to enter phase II clinical trials as selective stabilizer of c-myc in neuro-endocrine carcinomas.[10] Besides, it is important to develop small-molecule ligands for G-quadruplex exploration. In the past decade, great efforts have been devoted to seek G-quadruplex ligands, as well as optical probes for G-quadruplex.[11]

Fluorescent G-quadruplex ligands are expected to be a useful tool to image telomeres and G-quadruplex formation sites within cells.[12] The development of “light-up” probes is a very hot research topic, and a number of G-quadruplex probes have been designed.[13] It is envisioned to develop the desired G-quadruplex probes by different G-quadruplex ligands. However, only a few probes through the conjugation of functional groups with fluorophores have been reported, such as Alexa Fluor-labeled Pyridostatin,[14] thiazole orange-labeled PDC derivatives,[15] and BODIPY-labeled macrocyclic heptaoxazole.[16] Another approach constructing G-quadruplex probes is modifying the structure of current fluorescent dyes.[17] Wong and Monchaud groups designed a multiphoton fluorescent probe NaphthoTASQ through connecting four guanine monomers to a naphthalene template for the direct visualization of RNA-quadruplexes in cells.[18] Ma and colleagues reported a G-quadruplex groove binder through the linking of benzo[d,e]isoquinoline to a cyclometallated Ir(III) complex.[19] Several other groups investigated the selective interaction between fluorescent ruthenium complexes and G-quadruplexes.[20] These studies show that such an approach is beneficial to develop G-quadruplex probes. Qu group reported zinc-finger-like chiral supramolecular complexes preferred binding to higher-order G-quadruplex over monomeric G-quadruplex and the selectivity was about 200-fold different.[21] Our group has found a cyanine dye-dimethylindole red (Dir) to behave as a high specific light-up G-quadruplex probe in red-emitting region above 650 nm.[22] On the basis of Dir, we further synthesized Dir-Trimer, which was expected to increase binding affinity towards G-quadruplex, even higher-order G-quadruplex. From the cell level, it is highly desirable to directly visualize G-quadruplex without antibodies.

Herein, we developed a zwitterionic and near infrared fluorescent probe Dir-Trimer. The special structure made it selectively interact with G-quadruplex, even higher-order G-quadruplex. Moreover, the interaction of Dir-Trimer with cells has also been investigated. Importantly, Dir-Trimer could be utilized to image G-quadruplexes on cell level.

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/slct.201601889
Results and Discussion

Synthesis of Dir-Trimer

The c3-symmetric Dir conjugate Dir-Trimer was prepared by condensation of carboxyl substituted Dir with tris(2-aminoethyl)amine by HATU. The synthesis route of Dir-Trimer is shown in Figure 1. Dir-Trimer was well characterized by 1H NMR, ESI-MS and HPLC (Figure S1-S3). As shown in Figure S1, the integration of the single peak in 3.21 ppm, which is ascribed to the characteristic peak of the terminal methylene of tris(2-aminoethyl)amine, is 6, indicating that three methylenes of tris(2-aminoethyl)amine are all conjugated with carboxyl Dir. Furthermore, the characteristics of molecular ion peaks are clearly shown, and the purity of Dir-Trimer reaches up to 99% (Figure S2-S3). Compared with Dir, the presence of multiple zwitterionic units in Dir-Trimer obviously reduced the background fluorescence, which may be useful to improve the sensitivity for G-quadruplexes.

Photophysical properties of Dir-Trimer

The photophysical properties of Dir-Trimer were investigated by fluorescence spectroscopy and absorption spectroscopy in different solvents. As shown in Figure 2, the fluorescence of Dir-Trimer in aqueous solution is weak and the maximum emission locates at ca. 653 nm. However, remarkable increases in fluorescence intensity were observed in other different organic solvents. In protic solvents, the fluorescence intensity is the lowest in aqueous solution, and evidently increases with decreasing solvent polarity (except for glycol). In high viscous glycol, Dir-Trimer emits much brighter fluorescence. In dipolar aprotic solvents, such phenomenon was also occurred in DMSO.

The photophysical properties of Dir-Trimer were also studied by absorption spectroscopy in Tris-HCl buffer and ethanol. In ethanol, Dir-Trimer mainly exhibits a sharp and intense absorption band with a maximum at 600 nm which resembles that of Dir in aqueous solution.[22] While in Tris-HCl buffer, Dir-Trimer shows a absorption peak around 553 nm together with a shoulder near 600 nm. It is well known that most cyanine dyes self-aggregate in aqueous solution by van der Waals forces and π-π stacking interaction.[23] The self-aggregation ability of cyanine dyes depends on multiple factors, such as the structural features, solvent polarity, temperature, pH, dye concentration and ionic strength.[24] As a cyanine dye, Dir-Trimer consists of three Dir chromophores. Besides, anionic and cationic substituents are both present in Dir. It is reasonable that Dir-Trimer self-assembles in aqueous solution through electrostatic, hydrophobic and other interactions, while not aggregates in ethanol. Therefore, the intense short wavelength absorption band of Dir-Trimer in aqueous solution could be assigned to the mainly existed aggregated form, and the weak long-wavelength band similar to that in ethanol could be assigned to the small amount of non-aggregated form.[24] The above mentioned low fluorescence of Dir-Trimer in aqueous solution maybe results from two reasons. The aggregated form leads to fluorescence quenching, while non-aggregated form is non-fluorescent as a result of non-radiative torsional relaxation.[24]

UV–Vis absorption spectra of Dir-Trimer in the presence of different DNA

The properties of Dir-Trimer binding to G-quadruplexes were then studied by absorption spectra. The parallel G-quadruplexes (c-myc, Kit1), antiparallel G-quadruplexes (Hras, TBA), and a mixed type G-quadruplex (HT22 in K+ buffer) were employed in the studies. All DNA sequences are shown in Table S1. To evaluate the binding selectivity of Dir-Trimer with G-quadruplexes, the absorption spectra of Dir-Trimer with single-stranded DNA (ssDNA1 and its complementary sequence, ssDNA2) and duplex DNA (dsDNA27 and ct-DNA) were also investigated.

The maximum absorption of Dir-Trimer in buffer solution locates at 553 nm, while the absorption profile and peak position dramatically changed with the addition of DNA. As shown in Figure 3, a hyperchromic effect associated with a red shift of absorption peak was observed in the presence of 5-fold excess of G-quadruplexes. Especially, the red shift in the presence of c-myc reached up to 31 nm (Table 1). For a mixed-type G-quadruplex, HT22 caused a 19 nm red shift. The antiparallel G-quadruplexes, Hras and TBA caused 17 nm and 6 nm red shifts, respectively. Additionally, upon Dir-Trimer binding to the parallel G-quadruplexes (c-myc, Kit1), this...
maximum absorption is stronger than that with antiparallel G-quadruplexes (TBA, Hras, and TBA). More importantly, ssDNA and dsDNA caused a very small red shift under the same condition. These results indicate that Dir-Trimer could selectively bind to parallel G-quadruplexes, while the interaction with ssDNA and dsDNA is very weak. Besides, the absorption band of the Dir-Trimer with G-quadruplexes resembles the absorption profile of the non-aggregated form in organic solvents, pointing to the transition of Dir-Trimer from the aggregated form to the non-aggregated form when binds to G-quadruplexes in aqueous solution. Then UV-Vis titrations were performed to further evaluate the interaction between Dir-Trimer and G-quadruplexes. With increasing the amount of c-myc, absorption spectra show a remarkable hyperchromic effect of the whole absorption band and a bathochromic shift of the absorption peak at 553 nm (Figure S4 A), which indicates the presence of increasing amount of the non-aggregated form in aqueous solution because of c-myc.\[17, 22\] Excited at other wavelength, such as 500 and 550 nm, the fluorescence enhancements were lower than that at 600 nm (Figure S4B). The reason is that fluorescence enhancements excited at 600 nm result from two parts: the increase in both molar absorption coefficient and quantum yield of Dir-Trimer upon binding to G-quadruplexes. As a G-quadruplex fluorescent probe, more fluorescence enhancements are highly desirable.

To gain more information about the interaction of Dir-Trimer with G-quadruplexes, fluorescence titration experiments were carried out. As shown in Figure 4 A, Dir-Trimer exhibits almost no fluorescence in buffer solution without DNA. In the presence of the parallel c-myc G-quadruplexes, the fluorescence intensity of Dir-Trimer was significantly increased with an emission peak at 653 nm. Upon interacting with 30 μM c-myc, the fluorescence enhancement is up to 138-fold (Table 1). The titration was also carried out in the presence of other G-quadruplexes (Kit1, HT22, Hras, and TBA), double-stranded DNA

Table 1. The oligonucleotides mainly used in this work, the characters of these oligonucleotides, the fluorescence enhancement factors (F/F0), the absorbance red shifts of Dir-Trimer when binding with DNA in Tris-HCl buffer containing KCl.

<table>
<thead>
<tr>
<th>Name</th>
<th>Character / in KCl</th>
<th>F/F0[a]</th>
<th>Red Shift /nm</th>
<th>K a/10^5</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-myc</td>
<td>parallel</td>
<td>138</td>
<td>31</td>
<td>1.25</td>
</tr>
<tr>
<td>Kit 1</td>
<td>parallel</td>
<td>91</td>
<td>24</td>
<td>1.08</td>
</tr>
<tr>
<td>HT22</td>
<td>hybrid</td>
<td>61</td>
<td>19</td>
<td>0.157</td>
</tr>
<tr>
<td>HT70</td>
<td>hybrid</td>
<td>49</td>
<td>/</td>
<td>3.38</td>
</tr>
<tr>
<td>Hras</td>
<td>antiparallel</td>
<td>26</td>
<td>17</td>
<td>/</td>
</tr>
<tr>
<td>TBA</td>
<td>antiparallel</td>
<td>18</td>
<td>6</td>
<td>0.45</td>
</tr>
<tr>
<td>ssDNA1</td>
<td>single-stranded</td>
<td>3.1</td>
<td>1</td>
<td>/</td>
</tr>
<tr>
<td>ssDNA2</td>
<td>single-stranded</td>
<td>4.3</td>
<td>1</td>
<td>/</td>
</tr>
<tr>
<td>dsDNA27</td>
<td>duplex</td>
<td>3.4</td>
<td>0</td>
<td>/</td>
</tr>
<tr>
<td>ct-DNA</td>
<td>duplex</td>
<td>1.7</td>
<td>0</td>
<td>/</td>
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</table>

[a] The concentration of Dir-Trimer and DNA are 1 μM and 30 μM, respectively.
(ct-DNA and dsDNA27) and single-stranded DNA (ssDNA1 and ssDNA2). The titration curves indicate that the fluorescence enhancements at 653 nm are increased to different extents and strongly depend on the types of DNA (Figure 4B). The presence of parallel Kit1 G-quadruplex (30 μM) caused ca. 91-fold fluorescence enhancement of Dir-Trimer. While antiparallel G-quadruplexes induced slight fluorescent enhancement (26-fold for Hras and 18-fold for TBA). Only very weak fluorescence enhancement of Dir-Trimer was observed upon binding to single-stranded and double-stranded DNA (Figure 4B). These results are also summarized in Table 1. Combining with the studies on Dir,[22, 27] the strong fluorescence enhancement of Dir-Trimer originates from the dissociation of the aggregates and the inhibition of non-radiative torsional relaxation of monomeric form upon binding to G-quadruplexes. [24] Besides, the presence of multiple anionic substituents suppressed nonspecific binding to polyanionic nucleic acids, which improved its selectivity. [28] Hence, these results indicate that Dir-Trimer could be used as a sensitive and selective fluorescent probe for G-quadruplexes, especially parallel G-quadruplexes.

The apparent binding equilibrium constants (K_a) were also determined by fitting the above fluorescence titration curves (Table 1). The values of K_a for Dir-Trimer with G-quadruplexes are in the order of 10^5-10^4. In light of the presence of three light-up Dir signal units in Dir-Trimer, we constructed a trimeric G-quadruplex (HT70) as the higher-order structure to compare its binding properties with monomeric G-quadruplex (HT22). The nonlinear curve fitting results reveal the binding constants are 3.38 x 10^5 for HT70 and just 1.68 x 10^4 for HT22, with ca. 20-fold higher. We suppose that three Dir subunits of Dir-Trimer were capable of binding with three quadruplex sites of HT70, respectively, therefore the binding affinity was remarkably enhanced. [21, 29]

To further ascertain the selectivity of Dir-Trimer for G-quadruplexes, we employed ct-DNA and dsDNA27 as competitors of G-quadruplexes for binding to Dir-Trimer. With excess amounts of ct-DNA or dsDNA27, a series of fluorescence spectra were performed. As shown in Figure S6-S7, compared with the fluorescence of Dir-Trimer with G-quadruplexes, there was little fluorescence change when excess amount of double stranded DNA were further added. These results also indicate that Dir-Trimer could selectively bind to G-quadruplexes, even in the existence of excess amount of double stranded DNA.

The fluorescence response of Dir-Trimer to G-quadruplexes was also compared with its monomeric homologue Dir-COOH. Dir-Trimer and Dir-COOH were employed at the same monomeric molar concentration. As shown in Figure S8, Dir-Trimer (1 μM) and Dir-COOH (3 μM) were respectively interacted with parallel c-myc G-quadruplexes. In the presence of 10 μM c-myc, Dir-Trimer exhibits ca. 96-fold fluorescent enhancement, while Dir-COOH only causes ca. 8-fold enhancement. Dir-Trimer displays much more fluorescence response than its Dir unit. This improvement benefits by the low fluorescence background of Dir-Trimer, which probably originates from the aggregation of Dir units within Dir-Trimer, and the reduction of electrostatic repulsion. These results also illustrate that the sensitivity of Dir-Trimer over c-myc has prominently increased.

Next we investigated the time response when Dir-Trimer interacted with c-myc G-quadruplexes. The fluorescence was recorded with time when Dir-Trimer was added (Figure S9). The fluorescence intensity shows a sharp peak once c-myc is added and reaches the maximum within 2 min, demonstrating that the probe shows an excellent performance in quick measurement.

Effect of Dir-Trimer on circular dichroism spectra of G-quadruplexes

We then studied the topology of the G-quadruplex upon the addition of Dir-Trimer using a circular dichroism (CD) spectrometer. In the absence of Dir-Trimer, the CD spectrum of the parallel G-quadruplex c-myc exhibits a characteristic positive peak at 265 nm and a negative peak at 240 nm. The antiparallel G-quadruplex Hras shows a characteristic positive peak at 295 nm and a negative peak at 260 nm. The hybrid-type G-quadruplex HT22 shows a positive peak at 290 nm and a characteristic shoulder peak at 265 nm. As shown in Figure S10, Dir-Trimer has a negligible influence on the characteristic peaks of c-myc. Similar results were also observed when Dir-Trimer interacted with antiparallel Hras or hybrid-type G-quadruplex HT22.

Cell imaging

To gain insights into the cellular location of Dir-Trimer, fixed Hela cells were stained with Dir-Trimer directly or after enzyme treatments (DNase I or RNase A). As shown in Figure 5, a strong fluorescence response of Dir-Trimer was induced in whole nucleus. The enhanced fluorescence signals of Dir-Trimer in nucleus clearly disappeared after DNase I treatment but not
after RNase treatment. After DNase I digestion, the DNAs were fully hydrolyzed. Therefore, the fluorescence signals of Hoechst 33258 disappeared since it binds to DNA in cell nucleus. The remarkable decrease in red light with DNase I predigestion demonstrates that the enhanced fluorescence signal of Dir-Trimer without DNase I digestion may come from the interaction of Dir-Trimer with DNA in cells. Although Dir-Trimer exhibits selectivity towards G-quadruplexes in the tested buffer conditions, the selectivity is still insufficient. The ratio of duplex DNA to single-stranded G-quadruplex sequences (and G-quadruplex structures) in cell nucleus is much higher than that in vitro studies here used. The presence of abundant duplex DNA in cell nucleus is expected to play very important roles in the fluorescence enhancement of Dir-Trimer. Therefore strong fluorescence of Dir-Trimer was induced within almost the whole nucleus. To confirm whether Dir-Trimer interacts with RNA, the fluorescence of Dir-Trimer with G-quadruplex RNA and arbitrary single-stranded RNA (Figure S11) was determined. The results indicate that the fluorescence responses of Dir-Trimer are large to G-quadruplex RNA but small to arbitrary ssRNA, which resembles those to DNA. Because the amount of RNA is much less than DNA in cells, the fluorescence signals mainly result from the interaction of Dir-Trimer with DNA. Thus, the fluorescence signals have almost no change after RNase treatment.

Although G-quadruplexes in human and mouse cells were visualized through G-quadruplex specific antibodies in recent reports,[1c, 14, 18a, 31] the life of G-quadruplex was transitory during gene transcription and duplication,[12] and it was hard to observe the fluorescence signal of probes upon linking to G-quadruplex in the live cells.[13] The results in aqueous solution show that Dir-Trimer has highly selective binding towards G-quadruplexes, especially trimeric G-quadruplex. As exogenous prefolded G-quadruplexes that were transferred into cells could remain their conformations,[34] we chose HT70 as an unlabeled G-quadruplex sequence and transfected it into cells by transfection reagent. As shown in Figure 6, the fluorescence signal of cells incubated with Dir-Trimer only is very weak. When HT70 was transfected into MCF-7 cells, then incubated with Dir-Trimer, remarkable red fluorescence signals were observed. The result suggests that Dir-Trimer has selectively bound to G-quadruplexes transfected into MCF-7 cells. Thus, transfection experiments in MCF-7 cells realized visualization of DNA G-quadruplexes on cell level. Dir-Trimer definitely represents a novel strategy in designing new fluorescence probes for G-quadruplexes.

Supporting Information Summary

General experimental methods and characterizations are available in the Supporting information.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (21575154, 21628502, 21507156) and the CAS/SSAFEA International Innovation Teams program.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: Cell imaging · DNA · Fluorescent probes · G-quadruplexes

References
