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A tailor-made colorimetric and red-emitting fluorescent dual probe for G-quadruplex nucleic acids was developed by incorporating a coumarin–hemicyanine fluorophore into an isaindigotone framework. The significant and distinct changes in both the color and fluorescence of this probe enable the label-free and visual detection of G-quadruplex structures.

The development of highly sensitive and selective probes to detect nucleic acids is of profound importance in a wide range of investigations, such as biochemistry and clinical diagnosis. G-quadruplexes are unique four-stranded structures formed by guanine-rich nucleic acid sequences in many crucial genomic regions. During the past two decades, G-quadruplex structures have captivated considerable attention because of their biological significance and potential applications in supramolecular chemistry. The ever-increasing interest in G-quadruplexes has required the development of rapid and facile approaches for the sensitive and selective detection of these structures. Thus, visualizing G-quadruplexes with chemical sensors has been an extremely active area of research, and significant progress has been made towards the development of colorimetric or fluorescent probes for G-quadruplexes. Colorimetric detection with visible color changes is an attractive method because it is simple and even allows instrument-free and on-site detection. On the other hand, a fluorescence-based method is especially attractive, mainly due to its high sensitivity, remote operation and intracellular application.

Isaindigotone is a naturally occurring alkaloid. Its scaffold is an appropriate framework for the design of a specific G-quadruplex ligand. Recently, we developed a colorimetric probe ISTO (Fig. 1) for the universal detection of G-quadruplex structures by incorporating thiazole orange into the isaindigotone framework. However, the sensitivity and further application of ISTO in cellular detection was restricted due to the weak fluorescence emission. Notably, the direct intracellular detection of G-quadruplexes remains a formidable challenge because a limited number of fluorescent G-quadruplex probes can penetrate into living cells and because their actual targets in cells are not fully known. Besides, it is highly desirable to develop long wavelength (red to near infrared) G-quadruplex-responsive fluorescent probes for in cellulo and in vivo bioimaging. Such probes offer various advantages, including minimum photodamages to biological samples, diminishing Rayleigh-Tyndall scattering of light, deep tissue penetration and minimum autofluorescence. Additionally, recent studies have shown that most of the G-quadruplex probes exhibited only one type of output; thus, it would be highly valuable to develop multifunctional probes that combine different outputs. Such a probe will take advantage of different techniques for more reliable measurements and versatile applications. To this end, we considered the possibility of upgrading ISTO and constructing a new isaindigotone-based colorimetric and long wavelength fluorescent dual probe for G-quadruplexes.

![Fig. 1 Structures of the isaindigotone framework, the colorimetric probe ISTO and the upgraded colorimetric and fluorescent dual probe ISCH-1.](image-url)

Drawing from our experience with isaindigotone derivatives and ISTO, we attempted to screen and incorporate another appropriate fluorophore into the isaindigotone framework, aiming not only to modulate its fluorescence emission property but also to maintain its colorimetric performance. Herein, we present a novel colorimetric and red-emitting fluorescent dual probe ISCH-1 for the detection of G-quadruplexes. The isaindigotone-based probe ISCH-1 included a coumarin moiety rather than the intrinsic phenyl group. The introduction of a diethylamino group on the coumarin moiety and quaternization of the quinazolone moiety led this probe to emerge as a donor-π-acceptor hemicyanine-like dye. Further substitution of two electron-withdrawing fluorine atoms on the quinazolone moiety might also enhance the push-pull effects. Collectively, ISCH-1 exhibited a particular coumarin–hemicyanine hybrid pattern restricted in the isaindigotone framework. Coumarin–hemicyanine...
hybrids are attractive scaffolds that have been applied in the design of colorimetric and fluorescent probes for other bioanlytes. However, to the best of our knowledge, this is the first utilization of such a fluorophore for the direct sensing of G-quadruplexes.

The desired probe ISCH-1 was prepared through the condensation of coumarin aldehyde and an N-methylated quinazolone moiety (Scheme S1 and Fig. S1-S5, ESI†). To investigate the interactions of ISCH-1 with G-quadruplexes, UV-Vis spectroscopic titrations were firstly performed. Upon the addition of the G-quadruplex formed by oligonucleotide htg21 (Fig. S6, ESI†), the intensity of ISCH-1 at 535 nm gradually decreased, and a large red-shift of the maximum was observed with an isosbestic point at 570 nm, which eventually led to a new peak at approximately 600 nm accompanied by a marked and vivid color change from pink to blue in ambient light (Fig. 2a). As compared to ISTO, ISCH-1 exhibited significantly higher absorbance intensity and a more distinct color change at the same concentration, which is valuable for the naked-eye detection of targets. Furthermore, we investigated the interaction between ISCH-1 and other DNA oligomers, including other G-quadruplexes, single- and double-stranded DNA and BSA protein. As shown in Fig. 2c, G-quadruplexes, including pu22, htg21, rDNA-5701, dimer and HT-6, could clearly enhance the absorbance at 600 nm. However, much smaller changes were observed with single-stranded DNA (dA21 and dT21), double-stranded DNA (ds15, CT-DNA), and BSA protein. These results indicated that G-quadruplexes could be easily and specifically detected by using ISCH-1 as a colorimetric probe.

**Fig. 2** (a) UV-Vis spectroscopic and (b) fluorescence titration of 5 µM ISCH-1 with stepwise addition of G-quadruplex forming oligonucleotide (htg21, arrows: 0-3 mol equiv) in 10 mM Tris-HCl buffer, 60 mM KCl, pH 7.4. (c) The absorbance enhancement of 5 µM ISCH-1 at 600 nm against the ratio of [Sample]/[ISCH-1]. (d) The fluorescence intensity enhancement of 5 µM ISCH-1 at 653 nm against the ratio of [Sample]/[ISCH-1]. λ<sub>ex</sub> = 570 nm. (e) Distribution for the values of A<sub>600</sub>/A<sub>355</sub> and (f) (FI−FI<sub>0</sub>)/FI<sub>0</sub> at 653 nm for ISCH-1 with all the tested samples.

The fluorescence properties of ISCH-1 with G-quadruplexes were explored by using fluorescence titration. As shown in Fig. 2b, ISCH-1 alone in buffer displayed extremely weak emission at 640 nm with a fluorescence quantum yield (Φ<sub>F</sub>) of 0.0008. Upon titration of htg21, an emission peak at approximately 653 nm appeared and was significantly enhanced. The corresponding Φ<sub>F</sub> also increased to 0.186 at the saturated concentration of htg21. The turn-on fluorescence changes could be observed by the naked eye under UV light. This fluorescence enhancement might be caused by conformational changes in the excited state of ISCH-1, most likely by the rotation restriction around the methine-bridge that separates the coumarin and N-methylated quinazolone moieties upon its G-quadruplex binding, as clearly shown by the emission enhancement in viscous medium (Fig. S7, ESI†). Besides, ISCH-1 exhibited excellent selectivity for G-quadruplexes over single- and double-stranded DNA and BSA (Fig. 2d), which was consistent with the UV-Vis spectroscopic titration studies and further SPR results (Fig. S8, ESI†). Notably, the fluorescence of ISCH-1 was slightly changed with addition of just single- and double-stranded G-rich DNA, showing ISCH-1 actually bind specifically to G-quadruplex structures (Fig. S9, ESI†). It is also noteworthy that the sufficiently long excitation and red emission of ISCH-1 are very attractive for cellular imaging. Taking all of these results together, ISCH-1 displayed promising application prospects for the specific detection of G-quadruplexes with both colorimetric and fluorescent outputs.

On the basis of the dual outputs of ISCH-1 for G-quadruplexes, various G-quadruplex structures could be easily differentiated based on such visual signals (Fig. S10, ESI†), and we further developed a facile, versatile and reliable approach for dual-channel and large-scale identification of G-quadruplexes. The protocol was constructed to improve upon our previous method. The concentration of ISCH-1 and DNA used was reduced to 5 µM. The absorbances at 535 nm and 600 nm and the emission intensities at 653 nm for ISCH-1 without (FI<sub>0</sub>) and with (FI) for all of the tested samples were determined using a microplate reader. As shown in Figs. 2e and 2f, the values of A<sub>600/A<sub>355</sub></sub> and (FI−FI<sub>0</sub>)/FI<sub>0</sub> were greater than 1.1 and 15, respectively, for G-quadruplexes, whereas these values were less than 0.4 and 2 for non-quadruplexes. Thus, this dual-channel screening approach, with reduced consumption and improved accuracy and flexibility, should have widespread applications for the label-free and large-scale identification of G-quadruplexes.

Encouraged by the above findings, we further investigated the intracellular application of ISCH-1 in live and fixed cells by using confocal laser scanning microscopy. Co-staining experiments of Hela and A549 cell lines revealed that ISCH-1 gave emission signal in the nucleus (Fig. 3 and Fig. S11, ESI†). Notably, distinct strong
emission response to the nucleoli was found in the fixed cells stained by ISCH-1. Nucleoli are the organelles in the nucleus where rDNA undergoes transcription to rRNA. To further verify the sensing target of ISCH-1 in nucleoli, deoxyribonuclease (DNase) and ribonuclease (RNase) digest experiments were conducted (Fig. 3).1 The enhanced fluorescent signals of ISCH-1 in nucleoli clearly disappeared after DNase treatment but not after RNase treatment. These results indicated that ISCH-1 mainly bound to the rDNA regions in nucleoli accompanied by switching on its fluorescence because only DNAs in the cells could be hydrolyzed in DNase treatment. It has been reported that rDNA contain abundant putative G-quadruplex-forming sequences (PQSs).27 These sequences might adopt G-quadruplex structures. In the above tests, ISCH-1 also had a strong fluorescence turn-on response to three types of rDNA G-quadruplexes (Fig. 2). We were therefore interested if such significant emission in nucleoli might be attributed to the binding of ISCH-1 to rDNA G-quadruplexes.

Fig. 4 ChIP analysis of the effect of nucleolin associations with indicated sites in the rDNA of A549 cells (a. rDNA-2957, b. rDNA-2121, c. rDNA-5701, d. rDNA-4995, e. rDNA-13079, f. rDNA-12323), treated with 0.1% DMSO or 5 µM ISCH-1. 10% of lysate was used as the input control.

To test whether ISCH-1 could competitively displace nucleolin form rDNA in cells, we performed a chromatin immunoprecipitation (ChIP) assay according to the procedure employed for a phase II drug, CX-3543.27 CX-3543 is reported to be an rDNA G-quadruplex-targeting agent that selectively disrupts nucleolin/rDNA G-quadruplex complexes. On this basis, three regions containing PQSs in the non-template strand of rDNA and three corresponding regions that are flanking the three PQSs but they are themselves are not PQSs were selected for ChIP, and nucleolin was used as the binding protein of rDNA. As shown in Fig. 4, interrogation of three regions (rDNA-2957, rDNA-5701, and rDNA-13079) containing PQSs in the rDNA showed that treatment of cells with 5 µM ISCH-1 (Fig. S12, ESIF) caused significant dissociation of nucleolin from all of them, whereas there was little effect on the occupancy of nucleolin at their flanking rDNA regions (rDNA-2121, rDNA-4995, and rDNA-12323) that are not PQSs. These results were comparable with those of CX-3543, which might further support our hypothesis that ISCH-1 could bind to rDNA G-quadruplexes, thus turning on its fluorescence in nucleoli.

In summary, we have successfully developed a colorimetric and red-emitting fluorescent dual probe ISCH-1 for the detection of G-quadruplexes. This tailor-made probe contains a coumarin-hemicyanine fluorophore within an isaindigotone framework, which represents a novel and distinctive chemotype for a G-quadruplex probe. ISCH-1 displayed vivid color changes and significant fluorescence enhancement upon its incubation with G-quadruplexes, which enabled the facile naked-eye detection or rapid large-scale identification of G-quadruplexes using a microplate reader. Considering the remarkable properties of this versatile probe, one can easily appreciate its promising application prospects in vitro and in vivo. This work will shed light on the search for a new generation of probes with dual or even multiple outputs for the detection of G-quadruplexes. ISCH-1 also showed great potential in sensing intracellular rDNA G-quadruplexes, and further detailed investigations on the intracellular and in vivo applications of ISCH-1 are now underway.

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3 Supplementary Information (ESI) available: [Synthesis and characterization of ISCH-1, experimental procedures, and Supplemental spectra and graphs]. See DOI: 10.1039/c000000x/