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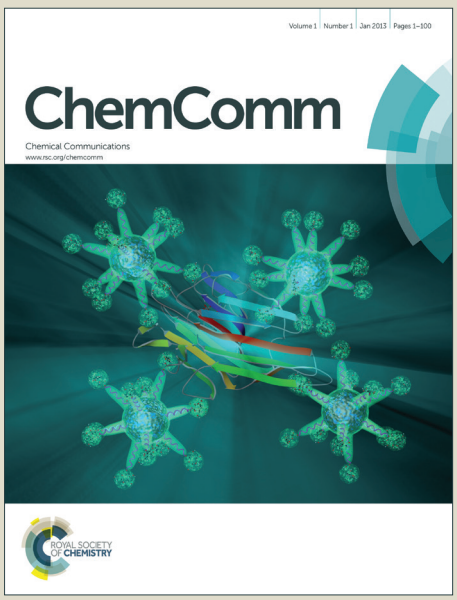

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Discovery of a New Fluorescent Light-Up Probe Specific to Parallel G-Quadruplexes

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A novel 2,4,5-triaryl-substituted imidazole (IZCM-1) has been found to display distinct and specific fluorescence enhancement upon binding to parallel G-quadruplexes. Such a sensitive and topology-specific probe is able to light up without affecting the topology or thermal stability of the G-quadruplex sample. Thus, these advantages distinguish IZCM-1 from other G-quadruplex probes.

The development of highly selective and sensitive probes to detect nucleic acids is of profound importance in a wide range of fields, such as biology and clinical diagnostics.[1-3] G-quadruplexes refer to four-stranded structures formed by guanine-rich nucleic acid sequences that are present in many crucial genomic regions.[4, 5] During the past two decades, G-quadruplex structures have attracted extensive attention because of their biological significance and potential applications in supramolecular chemistry.[6-8] The ever-increasing interest in G-quadruplexes has promoted the development of rapid and simple approaches for the selective and sensitive detection of these structures. Thus, the design of fluorescent probes for G-quadruplexes has become an extremely active area of research.[9-11]

Several fluorescent probes capable of discriminating G-quadruplexes from single- and double-stranded nucleic acids have previously been described.[12-15] Nevertheless, it is also noteworthy that G-quadruplex structures exhibit a high degree of polymorphism that depends on the characteristics of the sequences, loop geometry, and the local environment. G-quadruplexes can be basically divided into three main topologies: parallel, antiparallel, and hybrid-type structures.[16, 17] The design of fluorescent probes for G-quadruplexes when only considering their ability to discriminate single- and double-stranded nucleic acids would hamper their further application. In addition to the selectivity in topology, an ideal fluorescent probe should not affect the topology or thermal stability of the targeted G-quadruplex because the fluorescence response to a G-quadruplex with an alterative structure or enhanced/impaired thermal stability would not reflect its true features. However, among the reported fluorescent probes for G-quadruplexes, few molecules were able to distinguish different G-quadruplex topologies and even fewer molecules did not affect the topology or thermal stability of G-quadruplex samples simultaneously.[18-21]

Herein, we present a novel fluorescent probe, **IZCM-1**, for parallel G-quadruplexes that does not affect their topology or thermal stability (Fig. 1). To the best of our knowledge, **IZCM-1** is the first G-quadruplex fluorescent probe bearing these features. **IZCM-1** belongs to the 2,4,5-triarylimidazole-derived chromophore family, which have been applied in the design of fluorescent probes for other bioanalytes.[22, 23] The idea of using this scaffold as a template for G-quadruplex fluorescent probes is based on our previous virtual screening study showing that triarylimidazole **TSIZ01** is a selective G-quadruplex ligand (Fig. 1).[24] In addition, compound **IZCM-1** includes a coumarin fluorophore and a fluorobenzene moiety rather than the 1-methyl-4-phenylpiperazine groups of **TSIZ01**.

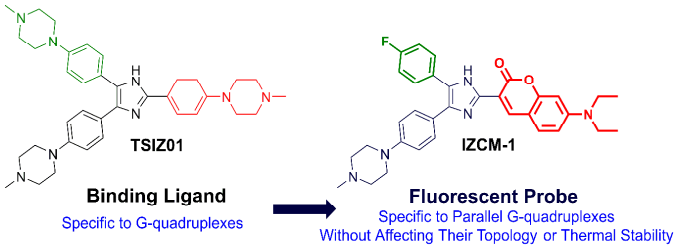

Fig. 1 Structures of triarylimidazole **TSIZ01** and **IZCM-1**.

The desired probe **IZCM-1** was prepared through the condensation of coumarin aldehyde and a diphenyl diketone moiety according to our previous studies (Scheme S1 and Fig. S1-S4, ESI†).[24] The optical properties of **IZCM-1** were investigated by absorption and fluorescence spectroscopy in different solvents. As shown in Table 1, **IZCM-1** exhibited a slight difference in the absorption maximum in different solvents and showed negligible emission in most low-viscosity liquids. Such fluorescence properties of **IZCM-1** were quite different from the fluorophore 7-diethylamino-4-methylcoumarin. It is also noteworthy that remarkable fluorescence enhancement of **IZCM-1** took place in the high viscosity glycerol solvent, showing that the fluorescence intensity of **IZCM-1** could be tunable.

Table 1 Optical data of 7-diethylamino-4-methylcoumarin and **IZCM-1** in different solvents.

Solvents	η^a (Cp)	7-diethylamino-4-methylcoumarin			**IZCM-1**		
		λ_{abs} (nm)	λ_{fl} (nm)	Φ_F	λ_{abs} (nm)	λ_{fl} (nm)	Φ_F
Toluene	0.6	363	411	0.951	445	525	0.018
Chloroform	0.6	369	427	1.053	445	521	0.017
DCM	0.4	371	427	0.933	450	527	0.009
Acetone	0.3	369	429	0.947	440	525	0.006
Ethanol	1.2	374	446	0.730	445	521	0.006
Methanol	0.6	378	453	0.424	445	525	0.007
DMSO	2.2	375	440	0.833	447	520	0.023
90% Glycerol	219.0	384	462	0.300	463	520	0.123
Water	1.0	382	469	0.051	450	560	0.009

[a] Viscosity at 20 °C

The fluorescence properties of **IZCM-1** with G-quadruplexes and other nucleic acids were first explored by using a fluorescence titration assay. As shown in Fig. 2A, **IZCM-1** alone in buffer displayed extremely weak emission. Upon gradual addition of parallel *c-MYC* G-quadruplex DNA formed by the oligonucleotide Pu22, an emission peak at approximately 525 nm appeared and was significantly enhanced. This significant light-up fluorescence was also observed when **IZCM-1** was treated with the G-quadruplexes Kras, bcl-2 and c-kit2, which have all been determined to form a parallel structure (Fig. 2B). In contrast, we observed much weaker fluorescence enhancement for the three hybrid-type G-quadruplex structures htg22, UspBQ1 and UspBQ2 in K^+ solution and for antiparallel G-quadruplexes including Hras, G3T3 and TBA under experimental conditions. In addition, negligible fluorescence enhancement was observed when titrating **IZCM-1** with single-stranded DNA (mutPu22, T21 and Py22), double-stranded DNA (ds26, hairpin and ctDNA), and triplex DNA (TAA). Meanwhile, such a trend in fluorescence intensities could be observed by the naked eye under UV light (Fig. S5, ESI†) or by using **IZCM-1** as a staining reagent in electrophoresis gels (Fig. S6, ESI†).

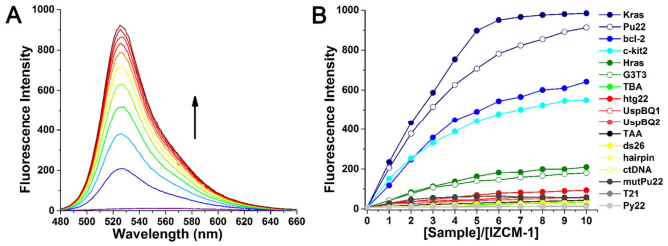

Fig. 2 (A) Fluorescence titration of 1 μM **IZCM-1** with stepwise addition of the G-quadruplex-forming oligonucleotide (Pu22, arrows: 0-10 mol equiv) in 10 mM Tris-HCl buffer, 100 mM KCl, pH 7.2. (B) The fluorescence intensity enhancement of 1 μM **IZCM-1** at 525 nm against the ratio of [Sample]/[**IZCM-1**], λ_{ex} = 450 nm.

The molar extinction coefficient, fluorescence quantum yield and relative brightness values of **IZCM-1** for different nucleic acids are summarized in Table 2. Notably, the quantum yield and relative brightness values are in agreement with the results of a titration experiment showing that fluorescence enhancement was always more pronounced for parallel G-quadruplexes. The quantum yield of **IZCM-1** for the parallel *c-MYC* G-quadruplex Pu22 reached 0.404. This value is approximately 18 times higher than the value for hybrid-type telomeric G-quadruplex htg22, which was approximately 6 times higher than that for the antiparallel G-quadruplex Hras, approximately 56 times higher than that for the double-stranded hairpin, and approximately 30 times higher than that for the single-stranded mutPu22.

Table 2 Molar extinction coefficients, fluorescence quantum yields (Φ_F) and fluorescence brightness of **IZCM-1**.

Sample	Extinction Coefficient ($M^{-1}\cdot cm^{-1}$)	Φ_F	Relative Φ_F	Relative Brightness[a]
IZCM-1$_{Tris}$	33758	0.004	1.0	1.0
IZCM-1$_{Kras}$	28484	0.402	100.5	84.8
IZCM-1$_{Pu22}$	26022	0.404	101.0	77.8
IZCM-1$_{bcl-2}$	28484	0.228	57.0	48.1
IZCM-1$_{c-kit2}$	27160	0.244	61.0	49.0
IZCM-1$_{Hras}$	22710	0.068	17.1	11.5
IZCM-1$_{G3T3}$	27077	0.068	16.9	13.6
IZCM-1$_{TBA}$	29011	0.014	3.5	3.0
IZCM-1$_{htg22(hybrid-type)}$	18963	0.022	5.6	3.1
IZCM-1$_{UspBQ1}$	26022	0.033	8.3	6.4
IZCM-1$_{UspBQ2}$	22857	0.036	9.1	6.1
IZCM-1$_{TAA}$	29187	0.016	4.0	3.5
IZCM-1$_{ds26}$	31824	0.013	3.2	3.0
IZCM-1$_{hairpin}$	16000	0.007	1.8	0.8
IZCM-1$_{mutPu22}$	32000	0.014	3.4	3.3
IZCM-1$_{T21}$	31121	0.007	1.8	1.6
IZCM-1$_{Py22}$	27253	0.007	1.7	1.4

[a] Brightness (molar extinction coefficients × Φ_F / 1000) is reported relative to **IZCM-1** alone.

To further ascertain the selectivity of **IZCM-1** for parallel G-quadruplexes, we employed telomeric G-quadruplex-forming oligonucleotide, htg22, in different molecular crowding solutions and therefore controlled its G-quadruplex conformation for fluorescence titration studies. In a K^+ solution containing the crowding agent Ficoll 70, htg22 folded into the ordinary hybrid-type G-quadruplex structure.[25] Using the crowding agent PEG 200 instead of Ficoll 70, htg22 folded into the parallel G-quadruplex structure.[26] In agreement with the selectivity of **IZCM-1** for the parallel G-quadruplex structure, the fluorescence enhancement was obviously stronger when **IZCM-1** was treated with htg22 in the crowding solution containing PEG 200 (Fig. S7, ESI†). Taken together, these results reveal that **IZCM-1** has promise for use in the sensitive and selective detection of parallel G-quadruplexes.

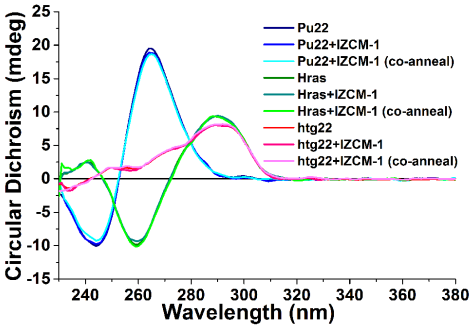

Fig. 3 CD spectra of 3 μM G-quadruplex-forming oligonucleotides Pu22, Hras and htg22 in 10 mM Tris-HCl buffer, 100 mM KCl, pH 7.2, with and without 15 μM **IZCM-1** under different experimental treatments.

We then investigated the topology and thermal stability of the G-quadruplex upon the addition of **IZCM-1** by using circular dichroism (CD) spectroscopy, as an ideal G-quadruplex fluorescent probe should not affect these two characters.[27] In the absence of

IZCM-1, the CD spectra of the parallel G-quadruplex Pu22 exhibited a characteristic positive peak at 265 nm and a negative peak at 240 nm. The antiparallel G-quadruplex Hras showed a characteristic positive peak at 295 nm and a negative peak at 260 nm. The hybrid-type G-quadruplex htg22 showed a positive peak at 290 nm and a characteristic shoulder peak at 265 nm. As shown in Fig. 3, **IZCM-1** had a negligible impact on the characteristic peaks of these three G-quadruplexes. Although the mixtures of G-quadruplex and **IZCM-1** were subjected to extreme heat-denaturation and co-annealing, the characteristic peaks of the G-quadruplexes were slightly changed. Similar results could be found when other G-quadruplexes were treated with **IZCM-1** (Fig. S8, ESI†). In addition, further CD melting experiments demonstrated that **IZCM-1** had a negligible effect on the stabilization of G-quadruplex structures (Table S2, ESI†). Collectively, **IZCM-1** can exercise its topology-specific sensing function without affecting the topology or thermal stability of the G-quadruplex sample.

In addition to the performance of **IZCM-1**, we were also interested in its sensing mechanism. **IZCM-1** did not exhibit fluorescence when quenched in water or buffer solutions but was able to light up in viscous glycerol or after treatment with parallel G-quadruplexes. **IZCM-1** had little propensity to aggregate in buffer according to its concentration-dependent absorbance spectrum (Fig. S9, ESI†). Thus, we hypothesized that such variations in fluorescence emission may be caused by conformational changes in the excited state of **IZCM-1**, most likely based on the rotational state of the single bonds around the imidazole moiety. This assumption was further supported by the correlation between fluorescence quantum yield and solvent viscosity, showing that the quantum yield of **IZCM-1** increased significantly with increasing glycerol content in the glycerol-water mixed solution (Fig. S10, ESI†).[28] The double-logarithmic plot of the quantum yield vs. the viscosity was linear (r^2 = 0.987).

Similar to the effect of viscous glycerol, parallel G-quadruplexes could also bind and lock **IZCM-1** in its fluorescent active conformation and activate the fluorescence accordingly. To further understand this process, surface plasmon resonance (SPR) experiments were first carried out to quantitatively determine the dissociation constants of **IZCM-1** binding to two typical G-quadruplexes, Pu22 and htg22. It was found that **IZCM-1** effectively bound to the parallel G-quadruplex Pu22 with a K_D value of 2.8 μM. In contrast, the binding of **IZCM-1** to the hybrid-type G-quadruplex htg22 was much weaker, with a K_D value of 15.1 μM. These results were in agreement with the correlation between fluorescence titration data and fluorescence quantum yield values, suggesting that the much more significant fluorescence enhancement of **IZCM-1** in the presence of parallel G-quadruplexes could be due to strong and specific interactions.

In addition to the SPR experiments, we also employed an absorption titration assay to validate the selectivity of **IZCM-1** for parallel G-quadruplexes (Fig. S11, ESI†). The binding constants of **IZCM-1** for the G-quadruplexes Pu22 and htg22 calculated from the absorption titration assay were similar to those from SPR experiments, showing that **IZCM-1** selectively bound to parallel G-quadruplexes with strong discrimination against hybrid-type G-quadruplexes. It was also noteworthy that **IZCM-1** bound to Pu22 exhibited distinct red shifts and isosbestic points in the absorbance spectra.[29] Nevertheless, insignificant red shifts and no clear isosbestic point were observed while it was bound to htg22. The clear isosbestic points observed for **IZCM-1** binding to the parallel G-quadruplex Pu22 indicated that there were at least two spectroscopically distinct chromophores (free and bound) in the solution. The lack of an isosbestic point could suggest that its chromophore environment was not altered following interaction with hybrid-type G-quadruplex htg22. The binding modes of **IZCM-1** for these two G-quadruplexes were evidently different.

To gain more details on the interactions of **IZCM-1** with G-quadruplexes, modeling studies were performed. The parallel NMR G-quadruplex structure for Pu22 and a hybrid-type NMR G-quadruplex structure for htg22 were used as the templates.[30, 31] Molecular models of **IZCM-1** with Pu22 and htg22 were first generated by docking studies and then examined by 10 ns molecular dynamics runs. The examined models and the estimated free energies of the binding of **IZCM-1** in molecular mechanics Poisson-Boltzmann surface area (MM/PBSA) calculations were shown in Fig. 4. It was found that **IZCM-1** perfectly stacked on the G-quartet plane of Pu22. Optimization of π-π interactions restricted **IZCM-1** to the planar conformation. By contrast, in the complex formed by **IZCM-1** with htg22, **IZCM-1** bound closely to the 5′-terminus of htg22 and stacked over the adenine base in the flanking and loop sequences. These results were consistent with the absorption titration data showing **IZCM-1** bound to these two G-quadruplexes in two different modes. In addition, **IZCM-1** exhibited stronger binding with Pu22 (-25.7 kcal/mol) than with htg22 (-10.0 kcal/mol). These findings were also in agreement with the trends observed in SPR, absorption titration data, fluorescence titration data and fluorescence quantum yield values, which may further strengthen the reliability of the models.

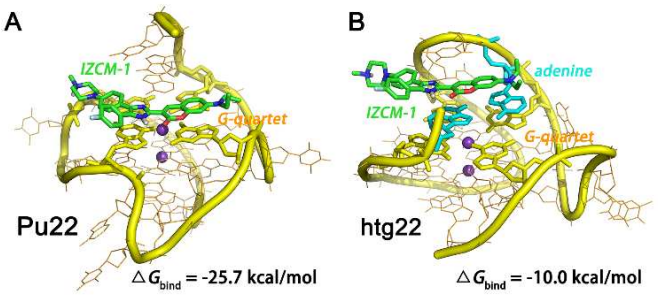

Fig. 4 Averaged structures obtained through MD simulations. (A) Complex model of **IZCM-1** with Pu22. (B) Complex model of **IZCM-1** with htg22. (**IZCM-1** is shown using green sticks; the 5′-end G-quartet is shown using yellow sticks; and adenine stacked by **IZCM-1** is shown using cyan sticks.)

Modification of 2-aminopurine (2-Ap) in different loops has been widely used to estimate the binding mode of small molecules with G-quadruplexes.[32] To further validate the modeling results, we performed fluorescence experiments using the parallel G-quadruplex Pu22 with 2-Ap substitutions at adenine residue positions 3, 7, 12, and 16. Hybrid-type G-quadruplex htg22 with 2-Ap substitutions at adenine residue positions 1, 7, 13, and 19 were used as well (Fig. S12, ESI†). It was found that the fluorescence intensities of Ap3 in Pu22 and Ap13 in htg22 were most affected upon the addition of **IZCM-1**, indicating that **IZCM-1** had closer contacts with these bases. The Ap3 in Pu22 was a flanking base that was positioned closely to the 5′-end G-quartet, but the Ap13 in htg22 was positioned in the loop region. Such results were consistent with the binding models showing that **IZCM-1** stacked on the G-quartet plane of Pu22 but bound to the adenine base in the loop region of htg22. Taking the spectroscopic and modeling results together, the sensing mechanism of **IZCM-1** was clear. The significant and selective fluorescence response of **IZCM-1** may arise from its special rotation-restricted state arising from tight binding to the exposed G-quartet of parallel G-quadruplexes via end-stacking. It was also understandable that the lateral loop of hybrid-type G-quadruplexes would hinder **IZCM-1** from stacking on the corresponding G-quartet, thus leading to their loose interaction and the subsequent

weak fluorescence response of **IZCM-1** in the presence of hybrid-type G-quadruplexes.

In summary, we have successfully developed a sensitive and topology-specific probe, **IZCM-1**, for the detection of parallel G-quadruplexes. This probe contains a coumarin fluorophore within the triarylimidazole framework, which represents a novel and distinctive chemotype for a G-quadruplex probe. Most importantly, **IZCM-1** exercises its specific light-up function without affecting the topology or thermal stability of the G-quadruplex sample. These advantages distinguish it from other G-quadruplex probes. The use of **IZCM-1** in the detection of G-quadruplexes could more accurately determine the true feature of the samples. Furthermore, detailed mechanistic studies suggest that the selective fluorescence response of **IZCM-1** arises from its rotation-restricted state arising from tight binding to the more exposed G-quartet of the parallel G-quadruplexes via end-stacking. These collective results will shed light on the search for a new generation of sensitive, accurate and topology-specific probes for different types of G-quadruplexes. Further investigations on the intracellular applications of **IZCM-1** are now underway.

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Notes and references

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