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Review

Recent Developments in G-Quadruplex Probes

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Accumulating evidence has linked G-quadruplex structures to a number of biological processes in vivo, including DNA replication, transcription, and genomic maintenance. However, the precise function and mechanism of G-quadruplex formation in mammalian cells remains poorly defined. Therefore, the development of small-molecule G-quadruplex probes has attracted significant attention in recent years. This review highlights examples of G-quadruplex probes that have been reported over the last few years, some of which have been employed for cellular imaging or for use in the construction of G-quadruplex-based analytical sensing platforms.

Introduction

Nucleic acids play essential roles in the storage and transfer of genetic information that is essential for living organisms. In cells, genomic DNA typically exists as a double helix that is stabilized by hydrogen bonding interactions between Watson-Crick base pairs. Over the past several decades, however, the ability of guanine-rich oligonucleotide sequences to form non-canonical four-stranded structures known as G-quadruplexes has attracted intense scrutiny (Gellert et al., 1962). The basic unit of a G-quadruplex is the guanine tetrad, which is composed of a planar arrangement of four guanine bases stabilized by Hoogsteen hydrogen bonding. Two or more guanine tetrads can associate via π-π stacking interactions to form a G-quadruplex motif, which can be further stabilized by the presence of metal cations in the central ionic channel (Georgiades et al., 2010; Monchaud and Teulade-Fichou, 2008).

G-Quadruplexes show a rich structural polymorphism that can be highly dependent on the underlying oligonucleotide sequences (Figure 1). For example, G-quadruplexes can be not only unimolecular, formed by a single oligonucleotide chain, but bimolecular, trimolecular, or tetramolecular, formed by two, three, or four separate oligonucleotide chains, respectively. In addition, G-quadruplexes can vary in the number of guanine tetrads, the relative orientation of the strands, the lengths and positions of loop regions, and the nature of the four grooves (Guédin et al., 2010; Parkinson, 2006). Moreover, G-quadruplex conformations can also be sensitive to the identity of the stabilizing cation as well as molecular crowding effects (Miyoshi et al., 2002, 2006; Xue et al., 2007). G-quadruplexes can have a parallel or antiparallel arrangement of strands, or even a so-called (3 + 1) or mixed configuration (Du et al., 2013; Nakayama and Sintim, 2009; Zhang et al., 2013a). It should also be stressed that oligonucleotides are highly dynamic, and that it is possible for a particular guanine-rich sequence to exist as several G-quadruplex topologies that are in dynamic equilibrium with each other (Ambrus et al., 2006).

Analysis of the human DNA has revealed an abundance of putative G-quadruplex-forming sequences in the human genome. G-quadruplex motifs are known to be found in telomeres, which are protein-DNA complexes at the ends of chromosomes. Strikingly, more than 80% of human cancers have enhanced telomerase activity, which acts to maintain the lengths of telomeric DNA after cell division, and delay senescence. Consequently, the stabilization of telomeric G-quadruplex DNA by endogenous ligands to block telomerase activity has been proposed as a potential anticancer strategy (Neidle, 2010; Xu, 2011). G-Quadruplex formation has also been proposed to play a role in telomere stability, with implications in the induction of telomeric defects and human disease (Mergny et al., 2002; Neidle and Parkinson, 2002; Zahler et al., 1991).

In addition, bioinformatics analysis has suggested that G-quadruplex-forming elements may be found in up to 43% of human genes (Todd et al., 2005). A number of human oncogenes, including c-*myc* (Gonzalez and Hurley, 2010), *bcl*-2 (Dai et al., 2006), k-*ras* (Cogoi and Xodo, 2006), and c-*kit* (Rankin et al., 2005), contain promoter regions with G-quadruplex-forming sequences. These promoter G-quadruplexes have been proposed to be involved with the regulation of transcription (Eddy and Maizels, 2008), and have attracted attention as therapeutic targets (Balasubramanian et al., 2011).

In recent years, a multitude of G-quadruplex structures have been extensively characterized ex vivo using biophysical techniques such as nuclear magnetic resonance (NMR) spectroscopy, circular dichroism (CD) spectroscopy, and X-ray crystallography (Chung et al., 2015; Clark et al., 2012; Martadinata and Phan, 2013; Mukundan and Phan, 2013). The diverse structural polymorphism of G-quadruplex structures have also made them versatile scaffolds for the construction of oligonucleotide-based analytical sensing platforms (Li et al., 2009; Wang et al., 2014a; Zhang and Li, 2011; Zhang et al., 2011). Despite all this, the existence and function of G-quadruplex structures in mammalian cells remains controversial. Therefore, tools for detecting and monitoring G-quadruplex structures in living cells are required to further our understanding of the roles played by G-quadruplex structures in biology.

In this review, we highlight recent developments in small-molecule probes for G-quadruplex DNA. We first discuss recent examples of organic molecules and metal complexes that have been developed as probes for G-quadruplex DNA. We then describe recent examples of the application of G-quadruplex

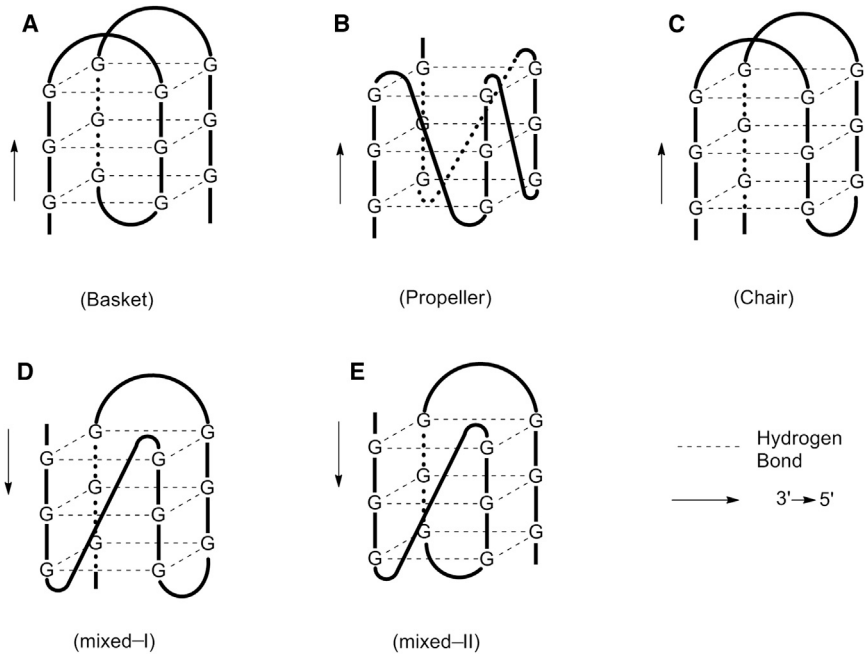


Figure 1. Schematic Representations of Common G-Quadruplex Topologies
(A) Antiparallel basket topology.
(B) Parallel propeller topology.
(C) Antiparallel chair topology.
(D) Type I mixed topology.
(E) Type II mixed topology.

probes as signal-transducing elements in G-quadruplex-based sensing assays. Finally, we highlight recent examples of G-quadruplex small-molecule probes that have been used as G-quadruplex DNA imaging agents in living cells. Here, we focus mainly on examples that have been described in the last few years, as previous reviews on G-quadruplex probes describing earlier developments have been published by our group (Ma et al., 2012) and others (Neo et al., 2012; Largy et al., 2013; Ren et al., 2015; Ruttkay-Nedecky et al., 2013; Vummidi et al., 2013). For ease of access, a summary of the photophysical properties of the G-quadruplex probes highlighted in this review can be found in Table 1.

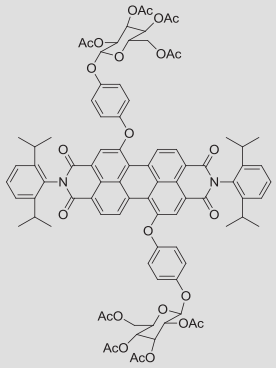
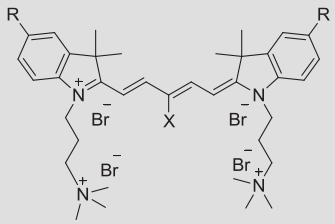
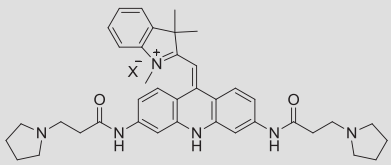
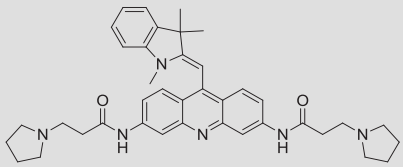
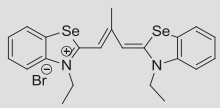
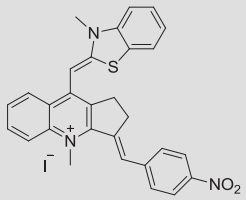
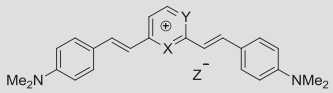
Organic Molecules as G-Quadruplex Probes

As reviewed by Teulade-Fichou and co-workers, G-quadruplex probes can be classified into three types (Largy et al., 2013). The first are "light-up" (also known as "switch-on" or "turn-on") probes, which are molecules that show enhanced fluorescence upon binding to G-quadruplexes. The second class comprises "light-off" probes, whose fluorescence are quenched by binding. The final class of G-quadruplex probes are permanent probes, which are molecules whose fluorescence intensity are unaffected by G-quadruplex binding. Furthermore, while both organic molecules and metal complexes can act as G-quadruplex probes, the former class has received the lion's share of attention in this field. Various classes of organic G-quadruplex probes have been described, with the most widely studied dyes being based on cyanine, thiazole, triphenylmethane, or carbazole. Moreover, natural products have also been reported to target G-quadruplexes (Shan et al., 2013).

An ideal G-quadruplex probe should display several key features, including high luminescence quantum yield, an intense emission enhancement in the presence of G-quadruplexes, strong photostability and chemical stability, excellent discrimination for G-quadruplex DNA over other forms of DNA such as single-stranded or duplex DNA, and good solubility. Additional features are desirable for probes applied for the cellular imaging of putative G-quadruplex structures (see below). While there currently is no universally accepted gold standard for G-quadruplex probes, molecules with impressive G-quadruplex-binding properties have been reported. For example, thiazole orange (TO) has been reported to display emission enhancements of 1,000-fold upon binding to G-quadruplexes (Nygren et al., 1998); however, it displays little selectivity for G-quadruplex DNA over duplex DNA. Meanwhile, a cationic manganese porphyrin showed 10,000-fold selectivity for G-quadruplex DNA over duplex DNA, but was non-fluorescent (Dixon et al., 2007). The following sections highlight examples of organic molecules that have been developed as G-quadruplex probes over the past few years. For ease of access, the dyes are grouped according to their structural class.

Perylene Dyes. Perylene dyes have attracted interest in a range of sensing applications, such as pH (Zhang et al., 2009), organic amine vapors (Jiang et al., 2010), and pyrophosphate ions (Feng et al., 2012). Perlyene diimide dyes are known to possess high photochemical stabilities and fluorescence quantum yields. In the context of G-quadruplex sensing, Dinçalp and co-workers have reported a perylene diimide dye **1** containing glucopyranosyl groups (PDI-Ph-β-GluOAc) as a visible and near-infrared absorbing photosensitizer (Dinçalp et al., 2012). **1** showed absorption bands localized at 350–500 nm and 600–850 nm in benzonitrile, giving a green color. Interestingly, the absorption wavelengths of the dye could also be modified by changing the solvent and/or molar concentration of dye. **1** was emissive in toluene (λ_{em} = 565 nm, Φ = 0.57) and chloroform (λ_{em} = 583 nm, Φ = 0.39), but was only very weakly fluorescent in PBS (λ_{em} = 573 nm, Φ = 0.003). The authors also investigated the absorption behavior of the dye in the presence of intermolecular G-quadruplex DNA, intramolecular G-quadruplex DNA, dimeric hairpin DNA, double-stranded DNA (dsDNA), or single-stranded DNA (ssDNA). **1** showed bathochromic shifts from 569 to 591 nm for every DNA sequence, which was accompanied by hypochromic changes of 13%–27%. The singlet oxygen quantum yield of **1** was determined to be Φ = 0.06 (compared with Φ = 0.13 for fluorescein). Overall, **1** may be useful as a general probe for nucleic acids due to its pronounced bathochromic shifts upon binding to various nucleic acid motifs. However, because the dye did not show selectivity for G-quadruplex DNA over the

Table 1. Summary of the G-Quadruplex Probes Described in this Review

Compound	Structure	Excitation (nm)	Emission (nm) (Bound to G-Quadruplex)	Quantum Yield	Lifetime (μs)
1		350–500	565 (toluene) 583 (chloroform)	0.57 (toluene) 0.39 (chloroform)	NA
2–10	 **2**: R = H; X = H **3**: R = H; X = Cl **4**: R = H; X = Br **5**: R = Cl; X = H **6**: R = Br; X = Cl **7**: R = Cl; X = Br **8**: R = Br; X = Cl **9**: R = Cl; X = Cl **10**: R = Bt; X = Br	NA	NA	NA	NA
11		400	488	NA	NA
12		400	488	NA	NA
13		NA	535, 600	NA	NA
14		600 (absorbance)	NA	NA	NA
15–17	 **15**: X = CH, Y = NMe, Z = I **16**: X = CH, Y = $N(CH_2)_4N^+Et_3$ Br^-, Z = Br **17**: X = NMe, Y = CH, Z = I	532	623–629	NA	NA

(*Continued on next page*)

Table 1. ***Continued***

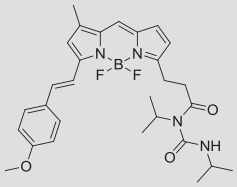
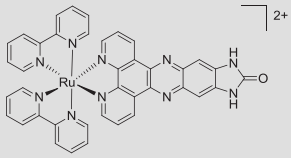
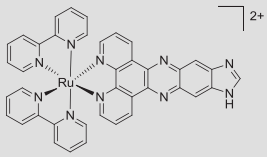
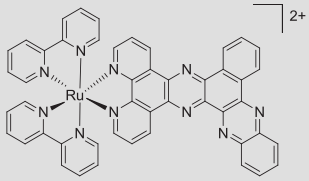
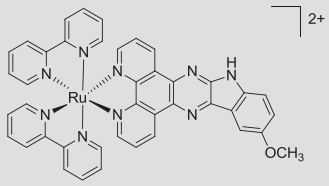
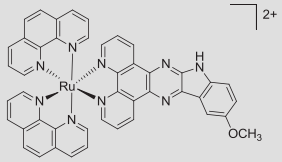
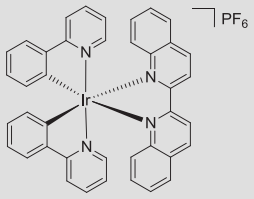
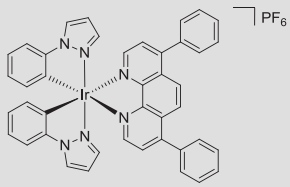
Compound	Structure	Excitation (nm)	Emission (nm) (Bound to G-Quadruplex)	Quantum Yield	Lifetime (μs)
18		360	597	NA	NA
19		450	605	NA	NA
21		450	605	NA	NA
22		NA	NA	NA	NA
23		450	595	NA	NA
24		450	595	NA	NA
25		390	638	NA	NA
26		335	571	0.067	4.437

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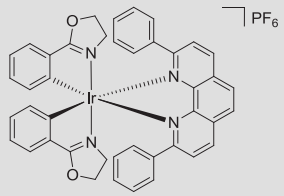
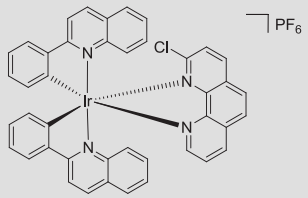
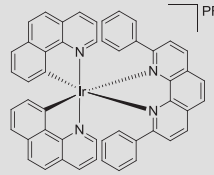
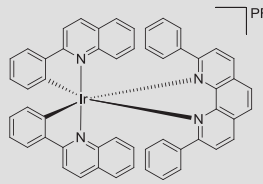
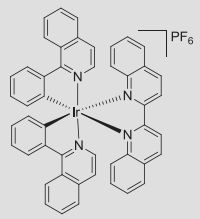
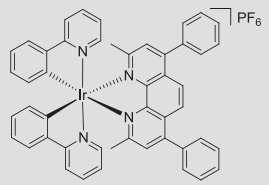
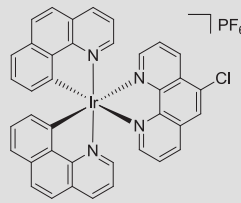
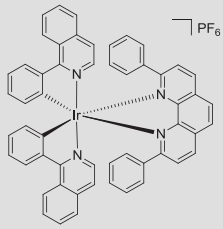
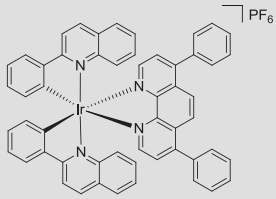
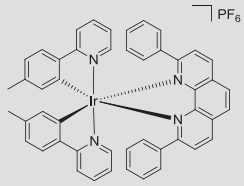
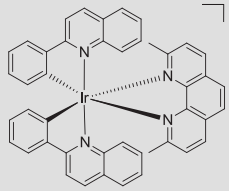
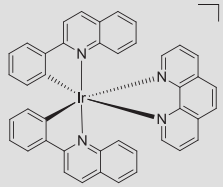
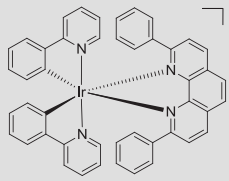
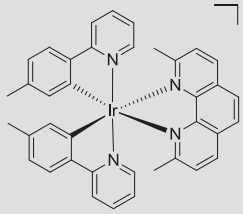
Table 1. ***Continued***

Compound	Structure	Excitation (nm)	Emission (nm) (Bound to G-Quadruplex)	Quantum Yield	Lifetime (μs)
27		290	604	0.031	NA
28		336	560	0.1363	4.80
29		379, 434	580	0.13	4.843
30		429	583	0.27	4.31
31		360	624	NA	NA
32		360	568	0.2152	3.692
33		320	580	0.054	4.492

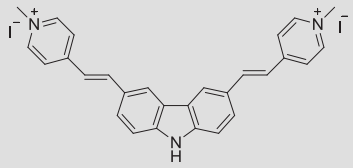
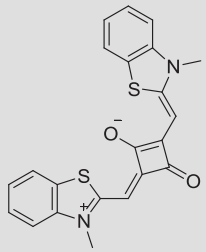
(*Continued on next page*)

Table 1. ***Continued***

Compound	Structure	Excitation (nm)	Emission (nm) (Bound to G-Quadruplex)	Quantum Yield	Lifetime (μs)
34		273, 451	615	0.042	4.6
35		360	560	NA	2.39
36		278	580	0.079	4.452
37		429	583	0.27	4.31
38		333	570	0.12	8.13
39		320	585	0.16	3.73
40		267, 320	547	0.662	3.311

(*Continued on next page*)

Table 1. ***Continued***

Compound	Structure	Excitation (nm)	Emission (nm) (Bound to G-Quadruplex)	Quantum Yield	Lifetime (μs)
41		470	580	NA	NA
42		610	668	NA	NA

NA, not reported.

other kinds of DNA tested, it would probably be unsuitable for use as a G-quadruplex-selective probe.

Cyanine Dyes. The structure of a cyanine dye is based on two aromatic nitrogen-containing heterocycles linked together by a polymethine bridge. Cyanine dyes have been applied as probes for live cell imaging (Carreon et al., 2007) or for biomolecular labeling (Gonçalves, 2009). In 2012, Wilson and co-workers reported a series of dimethylindoline cyanine dyes (**2**–**10**) with varying halogen substitutions as G-quadruplex-targeting ligands, and explored their interactions with G-quadruplex DNA using a variety of biophysical techniques (Nanjunda et al., 2012). UV thermal melting experiments showed that the compounds stabilized telomeric G-quadruplex DNA, but had little effect on duplex stability. Interestingly, the pentamethine cyanines with multiple halogen substituents (**7**–**10**) showed significantly improved telomeric G-quadruplex stabilization compared with the unsubstituted parent molecules. The binding of the molecules to G-quadruplex DNA was confirmed using surface plasmon resonance (SPR) spectroscopy experiments, which further showed the compounds bound to the c-*myc* G-quadruplex with even greater (ca. 10- to 25-fold) affinities than for telomeric DNA. Interestingly, the compounds all showed an initial strong binding followed by a weaker secondary binding (ca. 5- to 50-fold) for both quadruplexes. The most avid G-quadruplex-binding ligand **4** showed binding association constants of 1.7×10^6 and 13.0×10^6 M for telomeric and c-*myc* G-quadruplex DNA, respectively. In addition, the lack of induced CD signals in CD spectroscopy experiments suggested that the molecules interacted with telomeric or c-*myc* G-quadruplex DNA primarily through end-stacking at one or both ends of the terminal G-quartets. Mass spectrometry analysis suggested that the molecules interacted with G-quadruplex DNA via a two-site non-cooperative interaction, consistent with the SPR analysis. However, the ability of the dyes to act as probes for G-quadruplex DNA was not investigated in this work.

Ladame and co-workers have developed an acridine-containing cyanine dye **11** as a pH-responsive colorimetric indicator and fluorescent probe for G-quadruplex DNA (Percivalle et al., 2013). The absorption and emission spectra of dye **11** in potassium phosphate buffers at pH values ranging from 4 to 9 were recorded. Compound **11** was dark blue at acidic pH, but turned orange at pH values higher than 7.4 due to conversion to deprotonated form **12**, a change that could be observed by the naked eye. In the UV-Vis spectra, the absorbance peaks of the dye centered at 578 and 396 nm decreased as the pH was increased, while a new, lower-intensity maximum at around 476 nm appeared. Notably, no aggregation was ever observed over the pH value range tested. Moreover, the pH-responsive nature of **11** could also be monitored by fluorescence spectroscopy. The emission of **11** at 488 nm at an excitation wavelength of 400 nm decreased in a sigmoidal fashion as the pH was increased, and a pK_a value of 6.6 was estimated. In addition, the interaction of **11** with various G-quadruplex structures was studied by fluorescence spectroscopy. The fluorescence of compound **11** was strongly quenched by duplex DNA but was only moderately quenched by G-quadruplex DNA, indicating that **11** shows modest selectivity for G-quadruplex DNA over duplex DNA. Furthermore, compound **11** bound tighter to the c-*myc*, c-*kit*2, and K-*ras* quadruplexes than to the c-*kit*1 quadruplex. In SPR experiments, **11** was determined to bind to the c-*myc* and c-*kit*2 G-quadruplexes with binding dissociation constants of 670 and 2.75 μM, respectively, while unspecific binding to a dsDNA control was detected at concentrations above 1 μM. The G-quadruplex-stabilizing ability of compound **11** was further evaluated by CD experiments.

Tang and co-workers have recently reported a selenium-containing cyanine-based dye **13** as a fluorescent G-quadruplex probe (Yu et al., 2015). Based on UV-Vis titration results, the authors suggested that **13** could bind to the TG4T telomeric sequence via two different binding modes. In the end-stacking mode, **13** binds to the G-quadruplex as a monomer, whereas **13** binds as a dimer in the groove-binding mode. Interestingly, **13** showed preferential binding to parallel G-quadruplexes over antiparallel, hybrid, or mixed G-quadruplexes, indicating that

only the parallel topology could provide the appropriate groove dimensions for binding of **13**. However, the fluorescence behavior of the probe in the presence of G-quadruplex DNA was not studied. Interestingly, **13** also binds to i-motif DNA, a feature that was exploited for the construction of an Ag^+-sensing assay (Shi et al., 2015).

Further work is required to evaluate the performances of these cyanine dyes as G-quadruplex probes. While dyes **4** and **13** were shown to bind to G-quadruplex DNA through SPR, mass spectrometry, or UV-Vis absorption spectroscopy, their fluorescence response to G-quadruplexes was not studied. Moreover, although dye **11** showed greater fluorescence quenching in the presence of G-quadruplex DNA compared with duplex DNA, its switch-off mode of detection may limit its usefulness in biological applications.

Thiazole Dyes. TO has been used as a luminescent probe for various nucleic acid structures, and has been used in the G-quadruplex fluorescent intercalator displacement assay to study the interaction between small molecules and G-quadruplex DNA (Monchaud et al., 2006). However, TO itself shows only modest selectivity for G-quadruplex DNA. In 2012, Huang and co-workers reported a colorimetric probe for a variety of G-quadruplex structures by fusion of the TO and isaindigotone skeleton (Yan et al., 2012). Isaindigotone derivatives have shown potent binding affinity and selectivity for G-quadruplex DNA (Hou et al., 2011; Tan et al., 2009). These authors envisioned that the TO and isaindigotone scaffolds could be combined to generate fusion molecules with an expanded π-conjugate aromatic system as G-quadruplex probes. The interaction of compound **14** with the Pu27 G-quadruplex was studied using UV-Vis spectroscopy. Compound **14** exhibited a predominant H-aggregation band at around 535 nm in buffer, but this peak gradually decreased with the addition of Pu27 G-quadruplex DNA. Additionally a new peak at around 600 nm appeared, which was assigned to the monomeric state of the molecule. Interestingly, the disassembly of the aggregates of **14** to its monomers was accompanied by a red-shift in its absorption maximum, which led to a visible color change of the solution from purplish red to blue. Other parallel or antiparallel, intramolecular or intermolecular G-quadruplex structures could also be detected by compound **14**. On the other hand, ssDNA and dsDNA did not induce the dissociation of **14** aggregates, and only slight decreases in absorbance were observed. In addition, compound **14** could also differentiate G-quadruplex RNA from ssRNA, dsRNA and total RNA in cell lysate. The binding affinity and selectivity for **14** for various G-quadruplex structures over ssDNA and dsDNA was further confirmed by fluorescence resonance energy transfer melting (FRET melting), SPR, and NMR assays. It was postulated that **14** bound to G-quadruplex DNA via an end-stacking interaction with a higher affinity than the intermolecular affinity of H-aggregates of **14**, causing **14** to dissociate in the presence of G-quadruplex DNA. Overall, this study is interesting as it provides proof-of-concept that the G-quadruplex-binding specificity of isaindigotone can be combined with a fluorescent dye to generate a hybrid molecule that retains G-quadruplex-binding selectivity. In this respect, it would have been interesting for the authors to study whether the luminescent behavior of **14** could also have been used for G-quadruplex sensing, similar to the bisquinolinium/TO conjugates (Yang et al., 2009) reported some years before.

Distyryl Dyes. Distyryl dyes have found use for the construction of sensors for a wide range of analytes, including amyloid protein (Li et al., 2004), mercury ions (Tulyakova et al., 2007), and hydrogen peroxide (Zhan et al., 2010). The styryl scaffold can be easily prepared via the condensation reaction between aldehydes and pyridinium salts. Teulade-Fichou and co-workers studied the interactions of three cationic distyryl dyes, 2,4-bis(4-dimethylaminostyryl)-1-methylpyridinium (**15**), its quaternary aminoalkyl derivative (**16**), and the symmetric 2,6-bis(4-dimethylaminostyryl)-1-methylpyridinium (**17**) (Xie et al., 2013). Spectrophotometric and fluorimetric titrations and thermal denaturation experiments revealed that compounds **15** and **16** bound to various G-quadruplex structures with micromolar affinity, and with 2.5- to 8.4-fold selectivity over dsDNA. Compounds **15** and **16** showed high luminescence responses in the presence of human telomeric 22AG G-quadruplexes (ca. 110-fold and 80-fold enhancements at saturation, respectively), but weaker enhancements in the presence of oncogenic G-quadruplexes and the thrombin binding aptamer (TBA) G-quadruplex. Notably, the dyes could detect the presence of G-quadruplex DNA even in the presence of excess dsDNA. Intriguingly, the emission maxima of both **15** and **16** in the fluorescence spectra were blue-shifted by 15–20 nM in the presence of the G-quadruplexes (except TBA) compared with dsDNA and the TBA G-quadruplex. The authors also demonstrated the application of dye **15** for the selective staining of G-quadruplex DNA in a polyacrylamide gel. Overall, this distyryl dye shows significant promise as a G-quadruplex probe due to its high fluorescence enhancement in the presence of G-quadruplex DNA. Moreover, the dye showed excellent discrimination of G-quadruplex DNA over duplex DNA, and could even distinguish between various G-quadruplex topologies.

BODIPY Dyes. BODIPY compounds are an extremely widely studied class of dyes (Loudet and Burgess, 2007). As a class, BODIPY dyes possess sharp absorption bands, large fluorescence quantum yields and molar absorption coefficients, relatively long excited singlet-state lifetimes (these being around 1–10 ns), and robust chemical and photochemical stability (Ulrich et al., 2008). Recently, Chang and co-workers screened a library of 5,000 fluorescent sensors to identify hit molecules binding to G-quadruplex DNA (Zhang et al., 2014). Additional modifications furnished the BODIPY-based dye **18**, which showed a 30-fold luminescent enhancement at 597 nm and a 12-nm bathochromic shift in the presence of 93del, an interlocked, dimeric parallel-stranded G-quadruplex with four medium grooves. Interestingly, the luminescence behavior of **18** was highly sensitive to the structural elements of the G-quadruplex motif. **18** showed the highest luminescence enhancements to parallel-stranded G-quadruplexes containing exposed ends and four medium grooves, but only negligible enhancements in the presence of c-*kit*1 and Pu24T, which are also parallel G-quadruplexes but which possess additional snapback motifs that cap the G-quartet. Furthermore, **18** showed minimal fluorescence in the presence of non-parallel G-quadruplexes, dsDNA, or ssDNA. Molecular modeling analysis suggested that **18** bound to the groove regions of 93del rather than via end-stacking. Given that the groove regions of G-quadruplexes are generally more heterogeneous than the terminal G-tetrad faces, **18** could represent an efficient scaffold for the development of

structure-specific G-quadruplex probes. Overall, **18** shows excellent potential as a structure-sensitive G-quadruplex probe, given its ability to distinguish parallel-stranded G-quadruplexes containing exposed termini and four medium grooves over other parallel or non-parallel G-quadruplexes. Given the versatility of BODIPY dyes, we anticipate that future probe molecules based on this dye class can be synthesized to target the groove regions of G-quadruplexes in a similar fashion to **18**.

Metal Complexes as G-Quadruplex Probes

Luminescent metal complexes possess notable advantages that may be advantageous for sensing applications, such as their (1) high luminescence quantum yield, (2) long phosphorescence lifetime that allows their emission to be distinguished from a fluorescent background, (3) large Stokes shift for the effective discrimination of excitation and emission wavelengths, (4) sensitivity of their emissive properties to subtle changes in the local environment, and (5) modular synthesis that allows facile synthesis of analogues for fine-tuning of their chemical and/or photophysical properties. Therefore, metal complexes have also attracted significant attention as luminescent (and also colorimetric) probes for G-quadruplex DNA.

Yao and co-workers have reported the ruthenium(II) complex $[Ru(bpy)_2dppz\text{-}idzo]^{2+}$ (bpy = 2,2′-bipyridine, dppz-idzo = dipyrido-[3,2-a:2′,3′-c] phenazine-imidazolone) **19** as a luminescent G-quadruplex probe (Yao et al., 2013). In the absence of DNA, complex **19** was almost non-emissive. However, the luminescence of complex **19** rose sharply by about 300-fold enhancements in emission intensity ($I/I_0 \approx 300$, $\varphi = 0.067$) in the presence of 22AG G-quadruplex DNA ([DNA] = [**19**] = 2.5 μM). From the emission data, the binding constants of **19** for the 22AG G-quadruplex was determined to be approximately 4.8×10^6 M, while Job plot analysis indicated that two molecules of **19** bound to each molecule of DNA. Furthermore, complex **19** was selective for 22AG G-quadruplex DNA over duplex DNA. In comparison, the well-known "molecular light switch" complex $[Ru(bpy)_2dppz]^{2+}$ (**20**) bound less avidly to 22AG DNA ($I/I_0 \approx 80$, $\varphi = 0.02$, $K = 2.3 \times 10^6$), and also bound to G-quadruplex DNA in a 1:1 ratio. In UV-Vis spectroscopy experiments, both complexes exhibited hypochromism in the absorption band in the presence of G-quadruplex DNA. In addition, CD analysis showed that complex **19** was capable of inducing the formation of the human telomeric intramolecular G-quadruplex structure independently in the absence of other metal ions. Molecular modeling experiments suggested that one molecule of **19** was bound at each terminus of the G-quadruplex, potentially explaining its strong affinity for G-quadruplex DNA. The same group have also reported a ruthenium(II) complex $[Ru(bpy)_2(dppzi)]^{2+}$ **21** as a luminescent G-quadruplex probe (Shi et al., 2012). Although this complex exhibited a lower emission enhancement in the presence of G-quadruplex DNA, the authors showed that its luminescence could be switched on and off by successive addition of G-quadruplex DNA and $[Fe(CN)_6]^{4-}$. Molecular docking experiments suggested that each G-quadruplex DNA molecule bound to one ruthenium(II) complex through an end-stacking binding mode.

To improve the selectivity of the ruthenium(II) scaffold for G-quadruplex DNA, Chao and co-workers synthesized $Ru[(bpy)_2(bqdppz)]^{2+}$ (**22**) (bpy = 2,2′-bipyridine, bqdppz = benzo[j]quinoxalino[2,3-h]dipyrido[3,2-a:2′,3′-c]-phenazine) (Liao et al., 2012). The expanded aromatic bqdppz N^N ligand was chosen to reduce the ability of the complex to intercalate into other DNA structures. In luminescence measurements, complex **22** showed a much higher affinity for G-quadruplex DNA compared with duplex DNA. Interestingly, CD and FRET melting results showed that complex **22** interacted more strongly with the (3 + 1) hybrid G-quadruplex formed by $AG_3(T_2AG_3)_3$ in the presence of K^+ ions compared with the antiparallel G-quadruplex formed by the same sequence with Na^+ ions. The lower affinity of complex **22** to the antiparallel G-quadruplex was attributed to the presence of the linking diagonal loop, which hinders end-stacking interactions with the large ruthenium(II) complex.

Yu and co-workers have recently described the interaction of two ruthenium complexes $[Ru(bpy)_2(mitatp)]^{2+}$ **23** and $[Ru(phen)_2(mitatp)]^{2+}$ **24** (bpy = 2,2′-bipyridine, phen = 1,10-phenanthroline, mitatp = 5-methoxy-isatino[1,2-b]-1,4,8,9-tetraazatriphenylene) containing the indoloquinoline moiety with human telomeric G-quadruplex DNA (Telo22) (Yu et al., 2014). The luminescence emission of complexes **23** and **24** were increased by 6.5- and 2.4-fold, respectively, at saturating concentrations of Telo22. Interestingly, CD experiments showed that **23** could convert Telo22 into an antiparallel G-quadruplex while **24** promoted the formation of the parallel topology. However, the selectivity of the complexes for G-quadruplex DNA over other nucleic acid conformations was not reported.

Use of G-Quadruplex Probes in DNA-Based Sensing Assays

Over the past several decades, tremendous advances in chemistry and biology have unveiled the potential of nucleic acids to function as DNAzymes (or RNAzymes) and as aptamers (Navani and Li, 2006; Willner et al., 2008), which are capable of recognizing specific analytes with affinity and selectivity comparable with those of protein enzymes and antibodies. These developments have stimulated the development of a large variety of DNA-based probes. Compared with protein enzymes or antibodies, oligonucleotides are attractive scaffolds for the development of sensing platforms due to their relatively small size, low cost, ease of synthesis and modification, stability under a wider range of temperature and pH values, reusability, and low immunogenicity (Liu et al., 2009).

An oligonucleotide-based probe generally consists of a functional oligonucleotide that is able to interact specifically with its target accompanied by a conformational transition, and a signal transducer that transforms the recognition event into a measurable output signal. Early studies in the field of DNA-based sensing utilized fluorescently labeled oligonucleotides, in which the functional oligonucleotide is covalently linked to a signal transducer. However, the use of covalently modified nucleic acids entails certain disadvantages. Firstly, the covalent attachment of the signal transducer to the functional oligonucleotide may influence the binding affinity and/or selectivity of the oligonucleotide for the target analyte. Additionally, labeled DNA oligonucleotides are generally more expensive and time-consuming to prepare compared with unmodified oligonucleotides. In contrast, the "label-free" concept uses signal transducers that are not covalently attached to the nucleic acid backbone, but instead interact reversibly with DNA through non-covalent

interactions such as intercalation, groove-binding, end-stacking, or electrostatic interactions.

The probes used for the construction of label-free DNA probes are generally non-emissive or only weakly emissive in aqueous solution as a result of solvent-mediated quenching interactions, but show enhanced luminescence upon binding to defined DNA structures due to the protection of their excited states within the hydrophobic interior of the oligonucleotide (Joseph et al., 1996). Consequently, the conformational transition of the functional oligonucleotide in response to its cognate target may be transduced into an optical output using a suitable probe molecule. In this context, the use of G-quadruplex-forming functional oligonucleotides and G-quadruplex-specific probes has attracted particular attention for the construction of label-free DNA-based assays due to the rich structural diversity of the G-quadruplex motif. In addition, such assays may be less prone to interference from contamination from ssDNA and dsDNA, the most common forms of DNA found in biological samples.

In the following sections, we describe recent examples of the use of G-quadruplex probes for the development of oligonucleotide-based sensing platforms. For the sake of brevity, only the application of recently developed G-quadruplex probes are highlighted, and those interested in earlier work in the field of G-quadruplex-based sensing assays, or those that employ previously reported G-quadruplex probes, can consult several excellent reviews on the topic (He et al., 2013a; Neo et al., 2012; Ma et al., 2012; Ruttkay-Nedecky et al., 2013).

G-Quadruplex Probes for Sensing Metal Ions

A variety of monovalent and divalent metal ions can stabilize the G-quadruplex motif. Intriguingly, certain guanine-rich oligonucleotide sequences can be highly selective for a particular metal ion. Meanwhile, certain non-canonical DNA base pairs can be selectively stabilized by metal ions, such as the cytosine-Ag^{+}-cytosine and thymine-Hg^{2+}-thymine mismatched interactions (Miyake et al., 2006; Ono et al., 2011). These phenomena have been exploited for the development of G-quadruplex-based metal ion-sensing platforms.

Our group has developed several G-quadruplex-based assays for metal ions based on the general mechanism shown in Figure 2A. In the absence of the target metal ion, the oligonucleotide exists as a random conformation and the emission of the G-quadruplex-specific probe is weak. However, the addition of the metal ion induces the oligonucleotide into a G-quadruplex conformation, which is detected by the G-quadruplex probe with an enhanced luminescence response. For example, the G-quadruplex-selective luminescent iridium(III) complex **25** has been used in conjunction with the *Tetrahymena* telomeric sequence to selectively detect Sr^{2+} ions (Leung et al., 2012), and in concert with the PS2.M sequence to selectively detect Pb^{2+} ions (He et al., 2013c).

In a later study, we synthesized the iridium(III) complex $[Ir(ppyr)_2(BPhen)]PF_6$ (where ppyr = phenylpyrazole and BPhen = bathophenanthroline) **26** as a G-quadruplex probe for the detection of Ca^{2+} ions (He et al., 2013d). Initial luminescence titration experiments showed that complex **26** was selective for G-quadruplex DNA over ssDNA or dsDNA. Intriguingly, the emissive behavior of complex **26** toward G-quadruplex DNA was found to be structure dependent. Complex **26** showed a strong luminescence enhancement in the presence of c-*myc*, c-*kit*1, PS2.M, G_3T_4TT, and G_3T_2 DNA, which adopt parallel G-quadruplex conformations in the presence of K^{+} ions. However, complex **26** showed a reduced luminescence enhancement to the TBA and the 22AG human telomeric sequence, which are antiparallel G-quadruplex structures. Therefore, we envisioned that complex **26** could be used as a luminescent probe for the detection of Ca^{2+} ions in conjunction with the tetramolecular G-quadruplex G4 (5′-$G_4T_4G_4$-3′), which converts from an antiparallel conformation in Na^{+} to a parallel conformation when Ca^{2+} is added. We postulated that the exterior loops present in the antiparallel G-quadruplex structure may hinder complex **26** from interacting with the antiparallel G-quadruplex structure. On the other hand, the lack of loops near the terminus of the parallel G-quadruplex structure may allow complex **26** to form more effective end-stacking interactions with the terminal G-quartet.

We have also utilized the iridium(III) complex **27** for developing a G-quadruplex-based assay for Hg^{2+} ions (Lin et al., 2014). Using luminescence titration, UV-Vis titration, FRET melting, and G-quadruplex fluorescent intercalator displacement, we showed that complex **27** was selective for G-quadruplex DNA over dsDNA and ssDNA. Interestingly, complex **27** showed strong luminescence enhancement with PS2.M and c-*kit*87up G-quadruplexes, but only weak luminescence with the TBA G-quadruplex. TBA has previously been shown to readily accommodate planar aromatic ligands, but not ribbon-like molecules (Monchaud et al., 2008). This result suggests that complex **27** may bind outside the G-tetrad for the other G-quadruplexes. Furthermore, the ability of complex **27** to stabilize the G-quadruplex motif was found to depend on the size of the loop region. In the Hg^{2+} sensing assay, the addition of Hg^{2+} ions causes the dissociation of a duplex DNA substrate due to the formation of T-Hg^{2+}-T base pairs, which causes a guanine-rich oligonucleotide to be released into solution. This oligonucleotide then folds into a G-quadruplex structure that is detected by complex **27** with an enhanced luminescence response. We also adapted the assay to a microplate well format that allowed for the monitoring and removing Hg^{2+} ions in a real water sample. Later, we developed the iridium(III) complex **28** for the construction of a G-quadruplex-based assay for Ag^{+} ions employing exonuclease III-mediated digestion of C-Ag^{+}-C DNA (Wang et al., 2014b).

Tang and co-workers have recently reported a clip-like cyanine dye TC-P4 as a colorimetric G-quadruplex probe for Pb^{2+} detection (Sun et al., 2015). The principle of the assay is based on the preferential binding of TC-P4 to the K^{+}-stabilized PS2.M G-quadruplex compared with the Pb^{2+}-stabilized PS2.M G-quadruplex. In the presence of K^{+} ions, the absorption spectrum of TC-P4 and PS2.M shows strong absorption peaks at 541 and 585 nm. However, both absorption peaks decrease in intensity upon increasing concentration of Pb^{2+} ions.

Besides the novel G-quadruplex probe molecules highlighted above, DNA-based assays for metal ions that utilize previously discovered G-quadruplex probes have also been reported in the recent literature. For example, the porphyrin dyes *N*-methylmesoporphyrin IX (NMM) and protoporphyrin IX (PPIX) have been utilized for the detection of silver nanoparticles (Xu et al., 2015) and copper ions (Zhang et al., 2013b), respectively.

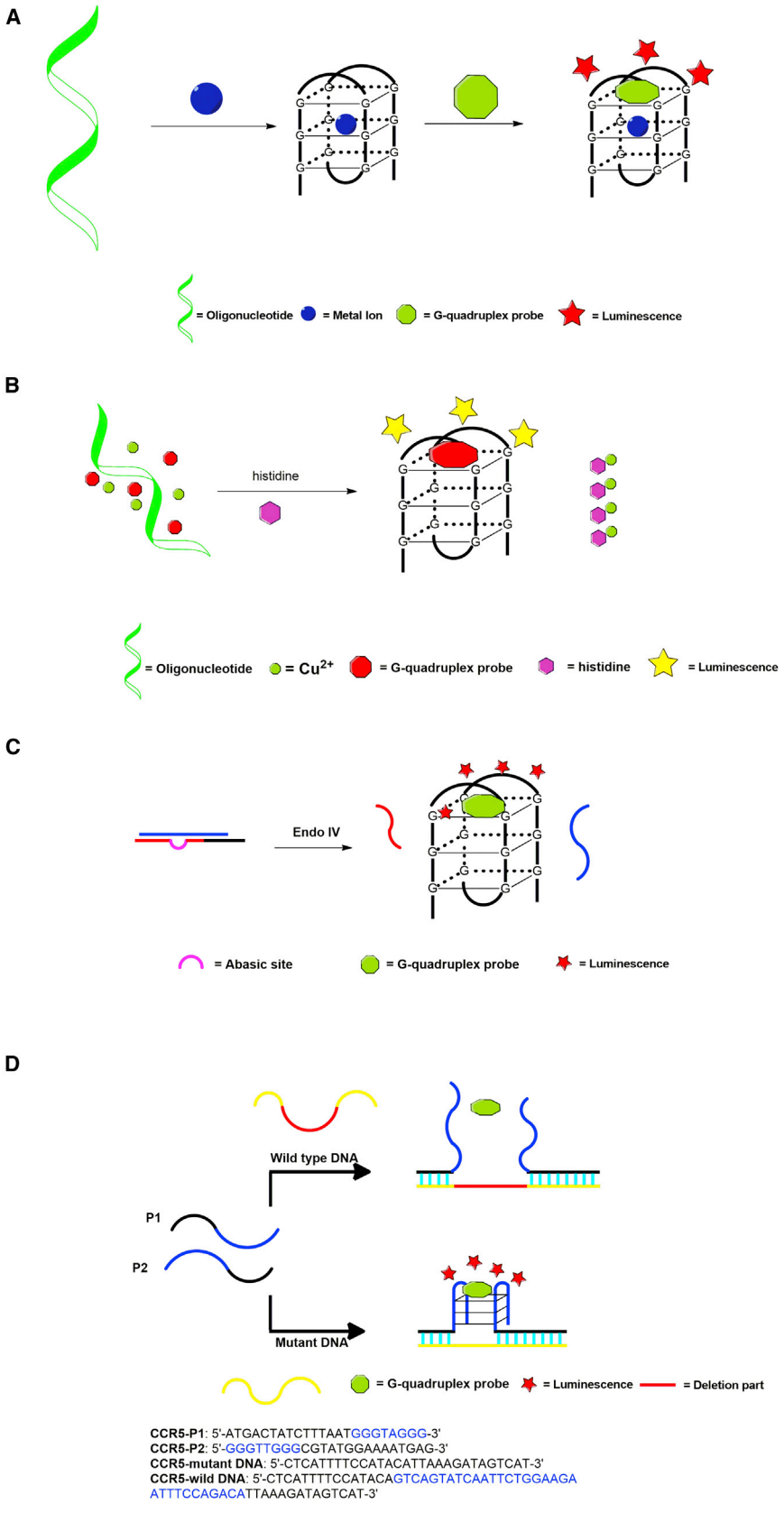


Figure 2. G-Quadruplex-Based Assays
Schematic representations of (A) G-quadruplex-based assay for the detection of metal ions, (B) G-quadruplex-based assay for histidine, (C) G-quadruplex-based assay for the detection of EndoIV activity, and (D) G-quadruplex-based assay for the gene deletion.

G-Quadruplex Probes for Sensing Small Molecules

Our group has also developed the iridium(III) complex $[Ir(bzq)_2(dpp)]^+$ (where bzq = benzo[h]quinoline and dpp = 2,9-diphenyl-1,10-phenanthroline) **29** as a G-quadruplex probe for the detection of histidine (He et al., 2013e). Using luminescence titration experiments, this complex was found to be selective for G-quadruplex DNA over dsDNA or ssDNA. The mechanism of the assay is based on the destabilization of the G-quadruplex motif by Cu^{2+} ions (Figure 2B). This causes the oligonucleotide to exist in a random coil conformation, resulting in a low luminescence signal of complex **29**. However, the addition of histidine can sequester Cu^{2+}, preserving the G-quadruplex motif and therefore restoring the emission of complex **29**. A similar strategy was employed for the detection of cysteine and glutathione using the novel G-quadruplex-selective complex $[Ir(phq)_2(dpp)]^+$ (where phq = 2-phenylquinoline and dpp = 2,9-diphenyl-1,10-phenanthroline) **30** (Leung et al., 2013a). The assay was based on the quenching of luminescence of the **30**-G-quadruplex ensemble by Hg^{2+} ions. The addition of cysteine or glutathione can sequester Hg^{2+} ions via the formation of strong Hg(II)-S bonds, restoring the luminescence of complex **30**.

We have also reported the use of the iridium(III) complex $[Ir(piq)_2(biq)]^+$ (where piq = 1-phenylisoquinoline) **31** as a G-quadruplex-selective for ATP detection (Leung et al., 2013e). The assay employed the ATP aptamer, which folds into a G-quadruplex motif in the presence of ATP molecules. Interestingly, the parent complex $[Ir(bpq)_2(biq)]^+$ showed inferior performance as a signal transducer in this assay, which we attributed to the presumably weaker binding affinity between $[Ir(piq)_2(biq)]^+$ and the ATP aptamer G-quadruplex.

In principle, it should be possible to develop a G-quadruplex probe for any small molecule as long as a DNA aptamer exists that folds into a G-quadruplex motif upon binding to that small molecule, as is the case for ATP. Alternatively, the specific interaction between a small molecule and G-quadruplex-destabilizing species, such as thiols with Cu^{2+}, can be exploited, as described in a G-quadruplex-based assay for cysteine using the previously reported porphyrin dye NMM (Li et al., 2013). These limitations therefore restrict the range of small molecules that can be monitored using a G-quadruplex-based assay.

G-Quadruplex Probes for Sensing Proteins and Enzyme Activity

Over the past few years, our group has developed a number of G-quadruplex-selective iridium(III) complexes for monitoring various DNA-modifying enzymatic activities. In these assays, the action of the DNA-modifying enzyme generates a G-quadruplex motif that is recognized by the G-quadruplex-selective iridium(III) complexes with an enhanced luminescent response. A representative example of a G-quadruplex-based enzymatic assay for detecting EndoIV activity is shown in Figure 2C (Leung et al., 2013d). A duplex DNA substrate containing an abasic site is cleaved in the presence of EndoIV, causing the dissociation of a G-quadruplex-forming fragment that can be recognized by the iridium(III) complex **32**. Similarly, duplex DNA substrate containing uracil bases or a nicking endonuclease cleavage site was used as substrate for uracil-DNA glycosylase (UDG) or nicking endonuclease, respectively, and the released G-quadruplex was detected by iridium(III) complexes **33** or **34** (Leung et al., 2013c; Lu et al., 2014).

Complexes **35** (He et al., 2013b) and **36** (He et al., 2014) were used for the detection of exonuclease III and T4 polynucleotide kinase activities, respectively. In both assays, the addition of the enzyme triggers digestion of one half of a duplex DNA substrate, causing the releasing of a guanine-rich oligonucleotide sequence that folds into a G-quadruplex motif. A similar approach was devised for the detection of mung bean nuclease activity using complex **37**, except that a G-triplex structure was formed in the assay instead of a G-quadruplex motif (Ma et al., 2015). Alternatively, the duplex DNA substrate could be unwound by hepatitis C virus NS3 helicase, generating a G-quadruplex structure that is recognized by complex **38** (Leung et al., 2015).

Finally, we have developed an iridium(III) complex $[Ir(ppy)_2(dpp)]^+$ (where ppy = 2-phenylpyridine and dpp = 2,9-diphenyl-1,10-phenanthroline) **39** for the G-quadruplex-based detection of human neutrophil elastase (HNE) (Leung et al., 2013b). Similar to the ATP assay described above, the HNE aptamer folds into a G-quadruplex structure in the presence of HNE, which is recognized by complex **39** with a luminescence response.

One aspect that deserves to be further explored in the future is the application of these G-quadruplex assays for the monitoring of protein or enzyme activities in living systems. While several of the G-quadruplex probes described here were able to function in spiked and diluted cellular extracts, the levels of endogenous protein or enzyme activity in native cells are probably below the threshold of detection of such assays. Further modifications in terms of enhancing the sensitivity and selectivity of the assays, possibly through the use of more specific substrates or the incorporation of some form of amplification mechanism, may be required to realize the in vivo detection of protein or enzyme activities. Furthermore, besides the novel G-quadruplex probes described, other G-quadruplex-based assays utilizing previously reported G-quadruplex probes such as NMM have also been developed for the detection of UDG and reverse transcriptase (Hu et al., 2010, 2011).

G-Quadruplex Probes for the Detection of Nucleic Acids

A vast amount of effort has been devoted to the development of probes for nucleic acids. The classic "molecular beacon" strategy reported two decades ago used a doubly labeled hairpin oligonucleotide that opens up in the presence of the target DNA sequence (Tyagi and Kramer, 1996). In recent years, a number of label-free G-quadruplex-based assays for nucleic acids detection utilizing well-known G-quadruplex probes such as NMM and PPIX have also been reported (Hu et al., 2011; Zhao et al., 2011; Zhu et al., 2012).

In terms of more recently discovered G-quadruplex probes, the luminescent iridium(III) complex $[Ir(ppy)_2(biq)]^+$ (**25**) has been employed as a signal transducer for the detection of gene deletion (He et al., 2012). In this assay, the presence of the target gene sequence brings two guanine-rich sequences into close proximity, promoting the formation of an intermolecular or "split" G-quadruplex (Figure 2D). Significantly, selectivity for a target sequence of any length could be realized by the judicious design of the split G-quadruplex oligonucleotides. Using a similar split G-quadruplex-based strategy, we have developed the iridium(III) complex $[Ir(ptpy)_2(dmph)]PF_6$ (where ptpy = 2-(p-tolyl)pyridine; dmph = 2,9-dimethyl-1,10-phenanthroline) (**40**) as a G-quadruplex probe for the detection of RNA sequences

(Ma et al., 2014). Importantly, both of these assays could discriminate between DNA or RNA sequences differing by only a single base. However, a drawback of this method is that only single strands of DNA are detected. Moreover, prior knowledge of the target sequence is required for analyte detection. Finally, as with the protein and enzyme assays described herein, further improvements in sensitivity and specificity will be required for these assays to detect endogenous DNA sequences in vivo.

G-Quadruplex Probes for Cellular Imaging

The putative roles of G-quadruplex DNA in gene expression and disease pathogenesis has stimulated the search of G-quadruplex probes capable of visualizing quadruplex structures in living cells. However, to be suitable as a cellular imaging agent of G-quadruplex DNA, the probe should preferably have the following properties: (1) high selectivity for G-quadruplex DNA over other nucleic acid conformations, (2) low propensity to bind to proteins and other biomolecules, (3) a strong "switch-on" luminescence response to G-quadruplex DNA with minimal luminescence background emission, (4) suitable hydrophobicity to pass through plasma and nuclear membranes, and (5) low cytotoxicity. "Switch-on" G-quadruplex probes are preferred for cellular imaging to "switch-off" probes, as the latter may be subjected to false-positive signals due to the presence of non-specific quenchers in the cellular environment.

At present, however, the direct visualization of G-quadruplex DNA by small-molecule probes has yet to be demonstrated. Encouragingly, several recently reported G-quadruplex probes have shown effective cellular uptake and distinctive subcellular localization in cells, which may be a first step toward developing cellular G-quadruplex probes.

In 2013, Chang and co-workers utilized 3,6-bis(1-methyl-4-vinylpyridinium)carbazole diiodide (BMVC) (**41**), a fluorescent carbazole derivative first described as a G-quadruplex ligand in 2003, to monitor the cellular uptake and localization of guanine-rich oligonucleotides (Tseng et al., 2013). Oligonucleotides that form parallel G4 structures, such as Pu22, T40214, and AS1411, were detected mainly in the lysosome of CL1-0 lung cancer cells. On the other hand, oligonucleotides that form non-parallel G-quadruplex structures, such as human telomeres (HT23) and TBA, were localized in the mitochondria. Moreover, by using fluorescently labeled oligonucleotides, the authors were able to show that parallel, but not non-parallel, G-quadruplex structures were able to retain their secondary structure after uptake into cells. Interestingly, treatment of living cells with the G-quadruplex-stabilizing ligand BMVC-8C3O could refold the disrupted non-parallel HT23 G-quadruplex into a probable parallel topology (Tseng et al., 2013).

Zhou and co-workers have recently described the G-quadruplex-binding properties of the squarine dye STQ1 (**42**) (Chen et al., 2014). STQ1 showed strong luminescence enhancements (up to 70-fold) in the presence of various G-quadruplex DNA structures, but not with ssDNA or dsDNA. CD experiments showed that **42** could not induce unfolded oligonucleotides into G-quadruplex structures in the absence of Na^+ or K^+ ions. This was construed to be a potential advantage for in vivo imaging, as it would mean that the product would be less likely to induce and thereby detect non-physiologically relevant G-quadruplex structures. In addition, the long-wavelength excitation (610 nm) and emission (668 nm) of STQ1 was advantageous for cellular imaging. Confocal microscopy showed that certain regions of the nucleus, particularly the nucleoli, showed red fluorescence when lung carcinoma A549 cells were treated with **42**.

In 2013, Xodo and co-workers reported a series of guanidino anthrathiophenediones as G-quadruplex ligands and investigated their cellular uptake, intracellular localization, and biological activity in cancer cells (Cogoi et al., 2013). The compounds were found to bind strongly to both DNA and RNA G-quadruplexes with sub-micromolar K_d values as determined by FRET-melting experiments, even in the presence of excess duplex DNA. Given the ability of the compounds to stabilize the *hras*-1 and *hras*-2 promoter G-quadruplexes, the authors next determined whether the ligands could inhibit HRAS-driven luciferase expression in living cells. A dual Renilla/firefly luciferase assay revealed that pretreatment of malignant T24 bladder cells with the compounds led to a dose-dependent reduction of HRAS-directed transcription. However, the compounds had little effect on cells harboring mutant promoter sequences that are unable to fold into a G-quadruplex, suggesting that the ligands are able to inhibit HRAS promoter activity by binding to the *hras*-1 and *hras*-2 G-quadruplexes. In addition, the compounds inhibited the growth of bladder cancer cells. Analysis of cellular uptake revealed that the compounds were taken up through endocytosis and/or passive diffusion. Confocal microscopy experiments showed that most of the compounds localized in the nucleoli.

Concluding Remarks

Accumulating evidence in recent years has suggested that G-quadruplexes may play important roles in gene expression and genomic stability in vivo. A recent review by the group of Balasubramanian summarizes recent data that support the existence of G-quadruplexes and discusses the possible consequences that G-quadruplex formation may have in biological systems (Murat and Balasubramanian, 2014). To further elucidate the putative functions of G-quadruplexes in DNA replication, transcription, and genome integrity, new tools are needed for visualizing and monitoring G-quadruplexes in a biologically relevant environment.

This review highlights recent examples of G-quadruplex probes that have been described in the literature in the past few years. Despite the extensive research that has been conducted on the study and detection on human G-quadruplexes in vitro, the visualization of G-quadruplex structures in cellulo has remained a demanding challenge. It has been posited by Teulade-Fichou and co-workers that only regions with a high density of G-quadruplexes will be able to be detected by microscopy, but that the existence and conditions for the formation of such domains under physiological conditions still remain unclear (Largy et al., 2013). Compounding this, the great abundance of duplex and random coil DNA or RNA molecules in living organisms makes the development of probes capable of selectively recognizing G-quadruplex structures in cells particularly difficult. Therefore, achieving discrimination for G-quadruplex DNA over duplex DNA or other biomolecules likely to be present in the cellular environment is of utmost importance for cellular probes. Moreover, cellular G-quadruplex probes must display suitable physiochemical properties that allow them to traverse effectively through cellular and organellar membranes. Another issue that

must be addressed by probes that achieve sufficiently high affinity for G-quadruplex DNA is the uncertainty over whether the G-quadruplex structures detected in cells were extant beforehand or were artificially induced by the presence of the ligand itself. To bring this field to the next level, it will be important to develop small-molecule ''switch-on'' luminescent G-quadruplex probes that are capable of not only showing uptake and subcellular localization within cells, but also specifically recognizing G-quadruplex structures in vivo without induction of spurious DNA secondary structures. Most G-quadruplex probes that have been investigated for cellular imaging have been found to localize in the nucleus, or more specifically the nucleolus, but whether they are actually binding to DNA or RNA G-quadruplex structures in cellulo, rather than to other nucleic acid topologies that are likely to be present in the nucleus, has not yet been definitively established (Largy et al., 2013). Moreover, G-quadruplex probes that show effective activities in vitro often can display variable staining patterns in cellulo, depending on the cell type and cell fixation method (Vummidi et al., 2013).

While this review has focused on small-molecule probes of G-quadruplex DNA, antibodies have also attracted significant attention for the visualization of G-quadruplex structures in cellulo. In recent years, a number of structure-specific antibodies for DNA and RNA G-quadruplexes have been developed for the imaging of G-quadruplexes in cells or tissues (Biffi et al., 2014a, 2014b; Henderson et al., 2013; Lam et al., 2013). Antibodies bind to G-quadruplexes with affinities in the nanomolar range (compared with typically low- to submicromolar affinities for small molecules), and the use of such antibodies has provided arguably the strongest evidence for the in vivo existence of G-quadruplexes in mammalian cells to date.

However, drawbacks of using antibodies as probes for G-quadruplex DNA include their relatively high cost, modest stability, and potential immunogenicity. Therefore, the search for effective small-molecule probes for G-quadruplex structures continues to attract widespread interest. This review also describes examples of where novel G-quadruplex probes were developed for use in the construction of oligonucleotide-based sensing assays. A number of these assays were demonstrated to function in diluted cell extracts or other biological media, indicating that the G-quadruplex probes were able distinguish G-quadruplex DNA from a variety of interfering substances.

In our view, it will be important to continue to strive for the development of G-quadruplex probes that are capable of recognizing G-quadruplex structures in living cells and tissues. Besides possessing high affinity and selectivity for G-quadruplex DNA, such probes should also possess ideal stability, biocompatibility, and membrane permeability. In addition, probes emitting in the red and near-infrared region (600–900 nm), also known as the ''optical window,'' are especially desirable, as the absorbance of photons by biological tissues falls to a minimum in this wavelength range. Moreover, the solubility, quantum yield, luminescence lifetime, and photostability of the molecules are also important factors that should be considered if the development of cellular G-quadruplex probes is to become a reality. Finally, we predict that the development of probes capable of distinguishing between different G-quadruplex topologies will continue to draw significant attention. Such structure-specific probes may be able to shed light on the mechanisms of interconversion of G-quadruplex structures in vivo, or in the interactions between endogenous proteins and G-quadruplex DNA in vivo, which are both highly dynamic but as yet poorly understood processes.

In conclusion, this review highlights examples of G-quadruplex probes that have been developed in the last three years. The continual development of more selective G-quadruplex probes, as well as those capable of monitoring G-quadruplex motifs in living cells, will be essential for further advancing our understanding of G-quadruplex structures in biology.

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