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Exploring flavonoid-DNA interactions *via* photoinduced proton transfer and two color fluorescence studies: Perspectives and emerging frontiers

Pradeep K Sengupta

Department of Biophysics, Molecular Biology and Bioinformatics, University of Calcutta, 92 Acharya Prafulla Chandra Road, Kolkata 700 009, India
E-mail: pradeepk.sengupta@saha.ac.in; pradeepsinp@yahoo.co.in

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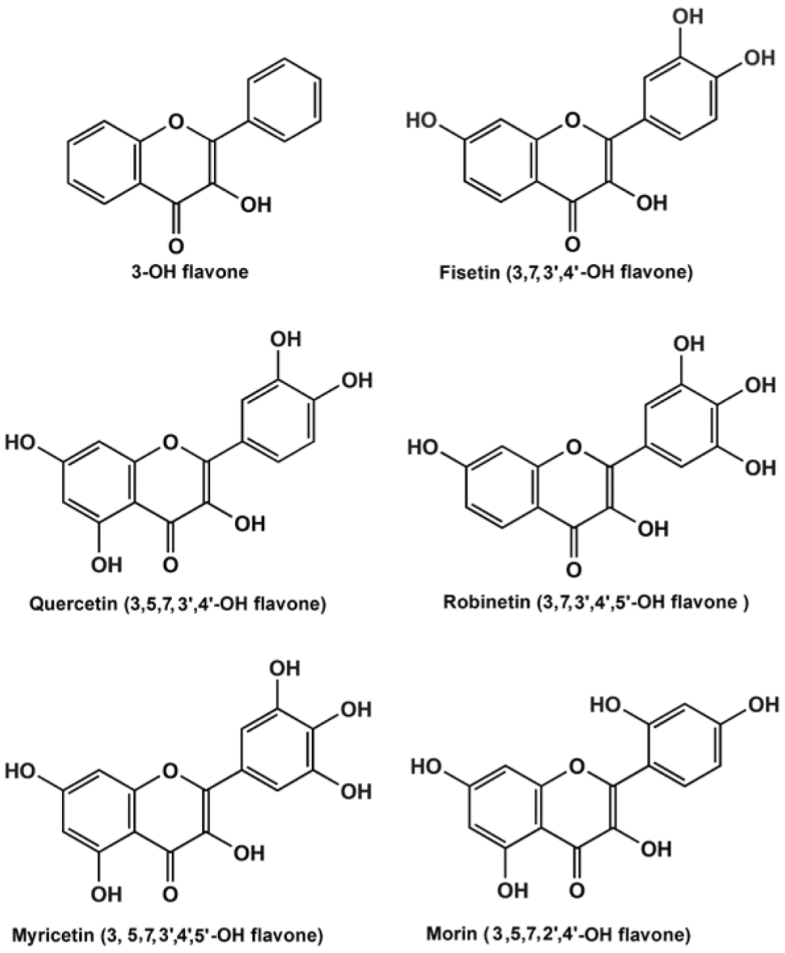
Plant flavonols are the best known class of naturally occurring organic molecules exhibiting ultrafast excited state intramolecular proton transfer (ESIPT) leading to 'two color' fluorescence emissions, which are exquisitely sensitive to the environment. On a different scenario, flavonols and related polyphenolic compounds of the flavonoid group, have attracted enormous attention as novel prophylactic and therapeutic drugs for free radical mediated and other human diseases. Their high potency and low cyto-toxicity make them viable alternatives to conventional therapeutics. In this context, identification of their physiological target molecules, mode of interactions with such targets, structure-affinity relationships and related aspects, have been key areas of investigations. This article presents perspectives, illustrating the potential usefulness of bioactive plant flavonols as their own fluorescence 'reporters' for exploring their interactions with duplex and quadruplex DNA molecules of potential interest as therapeutic targets. Representative and recent findings from our laboratory, and other relevant studies, exemplifying novel uses of flavonols as multiparametric fluorescence probes, are appropriately highlighted.

Keywords: Bioactive plant flavonols, photoinduced excited state intramolecular proton transfer (ESIPT), two color fluorescence, multiparametric probes, duplex DNA, quadruplex DNA, fluorescence lifetime, anisotropy decay

Photoinduced excited state proton transfer (ESPT) reactions in aromatic molecules represent one of the most fundamental and widely studied class of reactions in photochemistry[1,2]. Pioneering researches by Förster and Weller in the 1950's first established the occurrence of ESPT in organic molecules, and two distinct classes of reactions, namely intermolecular and intramolecular ESPT were recognized[3-5]. Initially, Förster reported studies on intermolecular ESPT in 2-naphthol. He showed that the pK_a* of the hydroxyl group of 2-naphthol decreased substantially in the singlet excited (S_1) state relative to that in the ground (S_0) state[3]. Subsequently, Weller discovered that methyl salicylate exhibited an unusually large Stokes shifted fluorescence due to an intramolecular ESPT[5]. In the years that have followed these seminal discoveries, occurrence of both inter- and intramolecular ESPT have been recognized in a large number of molecular systems, with diverse applications in chemistry and biology[1,2].

Excited state intramolecular proton transfer (ESIPT) is the reaction occurring between hydrogen bond donor and acceptor groups located in close proximity within the same molecule. In this process a proton rapidly migrates from one group to the other, leading to the formation of a tautomer species where a large reorganization takes place in the electronic structure, leading to dramatic shift of the fluorescence spectrum to longer wavelengths. Such large Stokes shifted fluorescence is a key feature of ESIPT. ESIPT is of one of the most important electronic excited state relaxation processes which occur following absorption of UV/visible light by molecules, and is therefore of enormous intrinsic interest from fundamental photophysical and photochemical perspectives. Moreover, molecules which exhibit the simultaneous occurrence of the normal (non proton transferred) fluorescence (with normal Stokes shift) and the ESIPT tautomer fluorescence (with large Stokes shift), offer attractive opportunities for use as fluorescence sensors of intermolecular interactions with target molecules, since the switch between the two forms of emission can be modulated by target binding[1].

Among the various organic molecular systems exhibiting ESIPT, flavonols (3-hydroxyflavone (3HF) and its derivatives (see Scheme I for typical chemical structures), of both natural and synthetic origin, stand



Scheme I — Chemical structures indicating –OH substitution patterns for typical flavonols

out for their unique features, and till date, are perhaps the best known and most widely investigated prototype molecules for investigating mechanistic aspects of ESIPT, as well as for their many, wide and varied applications in chemistry, biology and physics[1]. The discovery of ESIPT in flavonols was originally reported from Kasha's laboratory in 1979 (Ref 6). In such molecules a proton is transferred from the 3-OH group to the adjacent 4-C=O group across the internal hydrogen bond linking these two groups. The circumstances leading to discovery of ESIPT in flavonols is rather interesting in retrospect, and worth recollecting. This discovery was made in course of a spectroscopic study on 3HF (the structural backbone and chromophore of naturally occurring flavonols), which was performed in order to resolve a prevailing spectroscopic anomaly, namely why flavonols, which had their lowest (S_0-S_1) electronic absorption bands in the near-UV region, showed unusually large Stokes shifted green-yellow fluorescence upon excitation with a UV (365 nm) lamp. Such fluorescence has been serving as a standard diagnostic test in phytochemical analysis, for the detection of plant flavonols (*e.g.* kaempferol, quercetin, fisetin, *etc.*) following their chromatographic separation from raw plant extracts[6,7,9]. The recognition that ESIPT occurs in 3HF and its naturally occurring derivatives immediately resolved the long standing spectroscopic anomaly relating to their large Stokes shifted fluorescence, and furthermore, stimulated a remarkable outburst of further research on such molecules *via* steady state as well as laser kinetic spectroscopic techniques, and this has continued and rapidly proliferated during nearly the past 4 decades since the initial discovery[1,7-10]. The range of applications reported in relation to the ESIPT and fluorescence properties of flavonols is rather impressive, which include their use as prototype molecules for mechanistic studies of ESIPT, as

fluorescence probes for exploring their interactions with different biomolecular targets encompassing *in vitro, ex vivo* and *in vivo* situations, as prospective laser dyes, as wavelength shifters for near UV optical sensors, as an aid to improve reaction yields in synthetic organic chemistry, and for estimating physical and chemical parameters such as temperature and *p*H[7,8,11,27].

Chemistry, natural distribution, and biomedical importance of flavonols

Flavonols and other phenolic compounds of the flavonoid group have a common structure of diphenylpropanes, which are based on a 15-carbon skeleton (abbreviated as C6-C3-C6) consisting of two phenyl rings (A and B) linked through an oxygen containing heterocyclic ring (C). They are ubiquitous in higher plants, where they are produced as secondary metabolites, and occur in flowers, fruits, leaves and other parts of the plant body. Based on the degree of unsaturation and oxidation of the central three carbon segment, flavonoids are divided into different chemical classes, namely flavones, flavonols, anthocyanidins, isoflavones, *etc*. Over 6000 structurally distinct flavonoids have been identified in plants. These phytochemicals serve as pigments/ copigments in flowers and fruits (playing the useful role of attracting pollinating vectors), and as photo-screens in leaves (providing a protective role toward photosynthetic organelles against harmful solar UV B radiation), and fulfill additional useful functions which are vital for the survival of plants in the natural environment. Flavonoids are abundant in common plant based food and beverages (such as onion, citrus fruits, berries, apple, soy products, broccoli, tea and red wine), and are assuming burgeoning attention as nutraceuticals[12,13]. The potential health benefits of dietary flavonoids were first recognized in 1936 by István Rusznyák and Albert Szent-Györgyi (Nobel laureate in Physiology or Medicine, 1937)[14]. In recent years, there has been a remarkable renaissance of interest on flavonoids as prophylactic and therapeutic drugs which are effective against a broad range of free radical mediated and other human diseases including cancers, cardiovascular diseases, atherosclerosis, diabetes, neurodegenerative diseases, ischemia, allergies, and AIDS. The high potency and low cytotoxicity of such phytochemicals make them potentially viable alternatives to conventional therapeutics. In this context, the question of their physiological targets (relevant to their therapeutic activities) and the mode of interaction with such targets loom large and are major aspects of inquiry from the biomedical context[7,12,13]. Flavonols (3-hydroxyflavones) comprise the most common class of dietary flavonoids and are therefore of especial interest. Their structural diversity stems from the presence and distribution of –OH (and other) substituents in the A and B rings of the molecules (as shown in Scheme I)[7,12].

Excited–state intramolecular proton transfer (ESIPT) and 'two color' fluorescence as exquisitely sensitive multi-parametric fluorescence probes: an overview

A convenient way of representing ESIPT in 3HF and its derivatives is in terms of proton transfer across an asymmetric double well potential involving four energy states[6,7,15,29], as depicted in Figure 1. Figure 2 shows the steps involved in photoinduced ESIPT in a typical polyhydroxy substituted natural flavonol, fisetin (3,7,3',4'-OH flavone) which is responsible for its 'two color' fluorescence behavior[7]. The photoinduced ESIPT reaction occurs from the 3-OH group to the proximally located C=O group, across the intramolecular hydrogen bond linking the two groups. As a result, the initially excited normal (**N***) form is transformed to the ESIPT phototautomer (**T***) form. Emission originating from the N* and T* forms

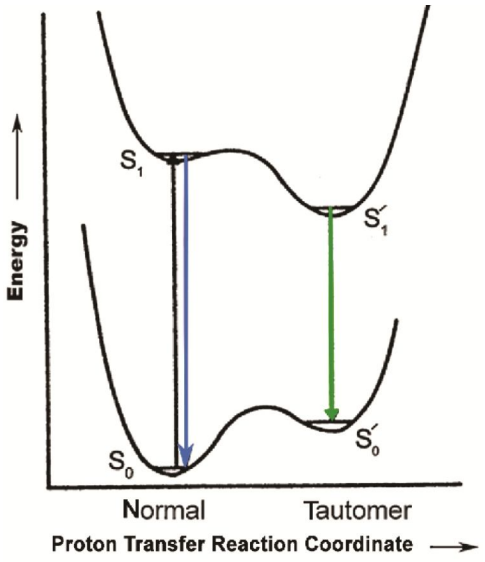


Figure 1 — Schematic diagram showing photo-induced proton transfer across an asymmetric double well potential involving four energy states. The arrows indicate initial photo-excitation (black), normal (non proton transferred) fluorescence (blue), and tautomer (generated by ESIPT reaction) fluorescence (green) (Ref 7, 15, 29).

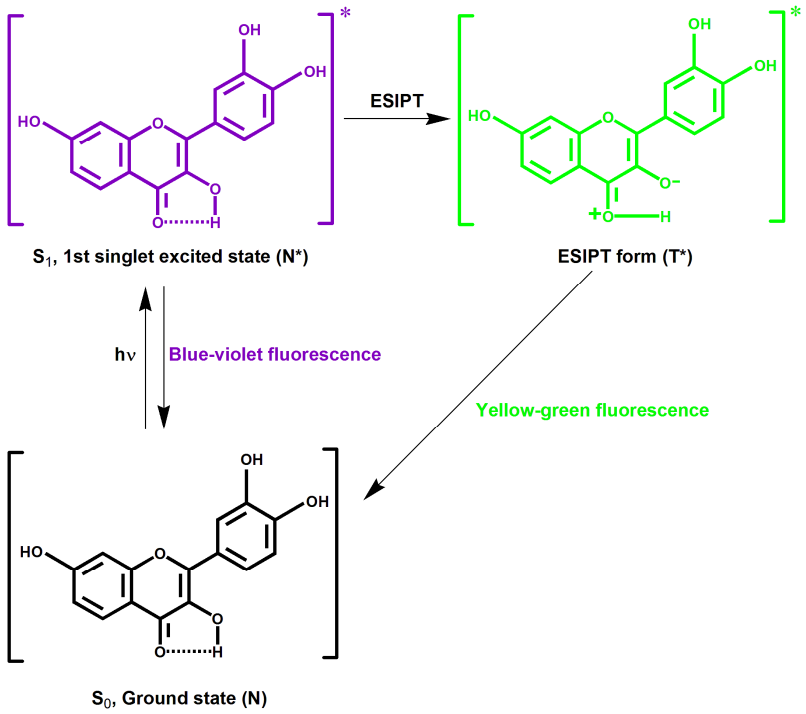


Figure 2 — Photoinduced ESIPT in a typical dietary plant flavonol, fisetin, leading to 'two color' fluorescence behavior (Ref 7)

results in 'two color' fluorescence, consisting of a blue-violet normal fluorescence with a normal Stokes shift and a yellow-green fluorescence with a dramatically large Stokes shift. Stokes shifts in the range 5000 cm^{-1}-6000 cm^{-1} are typically observed for the ESIPT tautomer fluorescence of different flavonols[10]. This ESIPT reaction is ultrafast and occurs in sub-picosecond timescales. For unsubstituted 3HF, the time constant for ESIPT was found to be 35 fs in fluid solution in a typical aprotic solvent at room temperature[16], and *ca.* 40 fs in Shpol'skii matrix at cryogenic temperatures[17]. Interestingly, the ESIPT reactions in 3 HFs are extraordinarily sensitive to external hydrogen bonding perturbation of the environment on the internal (C(4)=O……HO—C(3)) hydrogen bond of the molecule[7,15]. Consequently, the relative contributions between the two colors is strongly modulated by the local environments of the fluorophores. Moreover, in some flavonol derivatives (such as fisetin and robinetin), the N* form shows strong charge transfer (CT) character, resulting in pronounced solvent dipolar relaxation effects, manifested by polarity dependent spectral shifts of the normal fluorescence[18,19]. In such derivatives, the yellow-green ESIPT tautomer fluorescence serves as a 'proton transfer' probe (sensing H-bonding effects), and the blue-violet normal fluorescence serves as a 'polarity probe' (sensing the polarity of the fluorophore environment). This enables multiparametric use of the same fluorophore[7]. An especially interesting situation occurs in flavonols where 5-OH and 3-OH groups are simultaneously present (*e.g.* in quercetin (3,5,7, 3', 4'-OH flavone), the most commonly occurring dietary flavonol), where the C(4)=O……HO—C(5) hydrogen bond interferes with the C(4)=O……HO—C(3) hydrogen bond, which prevents efficient ESIPT, and thus the fluorescence quantum yield is intrinsically low. However, moderately strong fluorescence can be elicited in specific situations *e.g.* on binding to bio-relevant targets, such as proteins[20,21] and DNA[22,23] with appropriate structural motifs, or in low temperature solvent glass matrix[24], where the internal hydrogen bond involving the 5-OH group is disrupted, facilitating the ESIPT process across C(4)=O……HO—C(3). These features constitute the basis for the high sensitivity of flavonol emission to the surrounding environment, enabling their wide applications as exquisitely sensitive multi-parametric fluorescent molecular probes[7]. Motivated by this scenario, we, as well as other workers have published extensive spectroscopic studies illustrating the usefulness of flavonols as their own fluorescent 'reporters' for exploring their binding with

representative bio- relevant targets encompassing proteins, duplex and quadruplex DNA, model and natural biomembranes, and the encapsulations of these polyphenols encapsulation in nano-vehicles for drug delivery[7,18-23,25-38]. The aim of this article is to present perspectives on the interactions of representative bioactive plant flavonols with various duplex and quadruplex DNA molecules explored *via* the exquisitely sensitive 'two color' fluorescence of the flavonols, keeping in view the biomedical importance of this research.

Spectroscopic Methods

Fluorescence emission and excitation profiles presented in this review were obtained using standard procedures[1]. Besides the emission and excitation profile shape, structure, and intensity maxima, a parameter that has been frequently used for characterizing 'two color' fluorescence of flavonols is the tautomer/normal emission intensity ratio ($I_{tautomer}$ /I_{normal}), which provides a convenient measure of the hydrophobicity of the local environment of the fluorophore. The steady state fluorescence anisotropy (*r*) values were obtained using the expression,

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}}$$

where I_{VV} and I_{VH} are the vertically and horizontally polarized components of probe emission with excitation by vertically polarized light at the respective wavelength and G defines the instrumental correction factor (polarization characteristics of the photometric system) calculated as

$G=I_{HV}/I_{HH}$

Each intensity value used in this expression represents the computer-averaged values of several successive measurements.

Time resolved fluorescence decay measurements were carried out with a time correlated single photon counting (TCSPC) spectrometer, using excitation with sub-nanosecond pulses from laser diode excitation sources. An emission monochromator was used to block the scattered light and isolate the emissions. Fluorescence intensity decay curves were deconvoluted with the instrument response function and fitted to a multiexponential decay function,

$F(t) = \sum_i \alpha_i exp\ (-t/\tau_i)$

where $F(t)$ represents the fluorescence intensity at time *t*, and α_i and τ_i are the amplitudes and decay times of the individual components in the multiexponential decay profile such that $\sum_i \alpha_i = 1$. The goodness of fit was estimated by using χ^2 values and Durbin-Watson parameter. Average lifetimes $\bar{\tau}$ were calculated from the decay times and preexponential factors using the expression:

$$\bar{\tau} = \frac{\sum_i \alpha_i \tau_i^2}{\sum_i \alpha_i \tau_i}$$

For measurement of fluorescence depolarization kinetics the parallel (I_{VV}) and perpendicular (I_{VH}) components were collected as a function of time in an alternating manner until the difference of fluorescence counts collected reaches ~5000. The time dependent fluorescence anisotropy values *r(t)* were calculated using the expression (1):

$$r(t) = \frac{I(t)_{VV} - GI(t)_{VH}}{I(t)_{VV} + 2GI(t)_{VH}}$$

where I_{VV}, I_{VH} and G are defined as for the steady state anisotropy measurements. When freely rotating spherically symmetrical molecules are excited with polarized light, anisotropy decays as a function of time according the following equation

$$r(t) = r_0 e^{-t/\tau_{rot}}$$

where r_0 is the limiting anisotropy, which is the anisotropy value just after photo excitation (*i.e.* at $t = 0$) and τ_{rot} is rotational correlation time, which is a parameter of molecular rotation (*i.e.* how fast or slow the molecule rotates in the environment).Rotational diffusion depolarizes the fluorescence anisotropy from the initial r_0 value to a final randomized value r_{∞}. High values of τ_{rot} indicate strong motional constraint in the fluorophore microenvironment, and is therefore useful in diagnosing the binding of small molecule ligands to macromolecular targets[1,7]. To consolidate the findings from steady state and time resolved fluorescence spectroscopy, and to obtain structural insights, we relied on electronic circular dichroism (CD) and vibrational Raman spectroscopy. Computational methods, involving molecular docking studies, were used for obtaining information about the DNA-flavonol interations in atomistic detail, and to evaluate binding energetics and related parameters.

DNA-flavonol interactions

Background

Since DNA is the carrier of genetic information[40], it has been a typical choice for studies on interactions with various therapeutic drugs, from a variety of

considerations. DNA-flavonoid interactions and related studies are of enormous interest primarily because of mounting evidence indicating that dietary flavonoids, which are powerful antioxidants, can repair a range of free radical induced oxidative damage in DNA, and thus offer protection against single strand breaks and base damage in double stranded DNA[41]. The molecular and electronic structures of the flavonoids appear to modulate the free radical scavenging and oxidative damage protection activities[42]. Besides double stranded DNA with canonical Watson-Crick structure[40], much recent interest has also focused on non-canonical (non B) DNA structures, such as quadruplex (QD) DNAs. QD DNAs occur in guanine rich regions of the genome, and are especially prevalent at telomere ends and oncogene promoter regions[43]. The G quartet structure, characterized by Hoogsteen type base pairing[25,43], is depicted in Figure 3. QD DNAs occur with different base sequences, and also with different topologies, such as parallel, anti-parallel and combinations thereof. Small molecule ligands which can target QD DNAs and stabilize such structures, are potentially useful as novel cancer therapeutics[25,43-45]. The prospects of flavonols as QD DNA ligands are quite attractive, in view of their low systemic toxicity, as well as noninvasive sensing opportunities by exploiting their exquisitely sensitive fluorescence properties[7,25]. Keeping these in view, a series of studies have been recently performed by the present author and his collaborators on the interactions of flavonols and related molecules possessing intrinsic fluorescence, with duplex and quadruplex DNA and other non-canonical DNA structures[22,23,25,32,37,46]. Our contributions in this field[22,23,25,32,46], along with other recent studies of related interest[35,36,47], will be briefly highlighted in this perspective article.

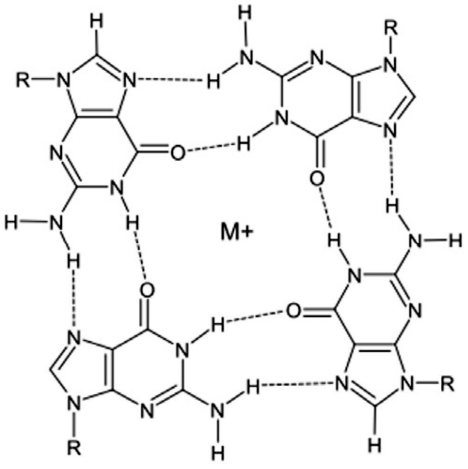


Figure 3 — Structure of the G-quartet, where four guanines are hydrogen bonded in a square arrangement (Ref 25, 43)

Double stranded (Calf thymus) DNA-flavonol interactions

For our initial spectroscopic studies we chose the flavonol fisetin (3,7,3',4'-OH flavones), which is present in common dietary sources (such as strawberry and cucumber)[32]. Fisetin has been reported to possess multi-functional medicinal activities, and is a prototype molecule exhibiting ESIPT and 'two color' fluorescence. Addition of double stranded (ds) calf thymus (CT) DNA induces dramatic changes in the emission profile of fisetin. Dual fluorescence bands with emission maxima at 480 nm and 524 nm appear with the $I_{tautomer}/I_{normal}$ ratio reaching a value of 1.545 at the highest DNA concentration used (Figure 4). Thus a predominantly hydrophobic binding site (where external H-bonding perturbation effects are minimal, facilitating the ESIPT process) of the flavonol in the DNA matrix is clearly evident. We also performed a competitive binding study using

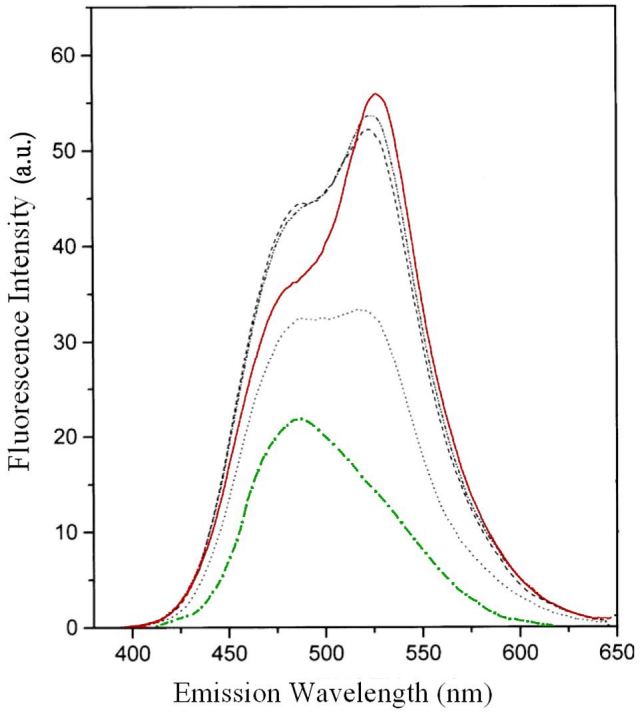


Figure 4 — Fluorescence emission spectra (λ_{ex} = 370 nm) of fisetin (12 μM) at different concentrations of double stranded DNA (calf thymus, CT) DNA (_._._) 0 μM, (……) 5 μM, (---) 15 μM, (_.._.._) 100 μM, (__) 200 μM (Adapted with modification from Ref 32)

Ethidium Bromide (EtBr, a classic DNA intercalator). Interestingly, the addition of EtBr caused a dramatic reversal of the emission profile which now resembled the situation before addition of DNA (Figure 5). When the same sample solution (containing fisetin +DNA+EtBr) was selectively excited at 480 nm (where EtBr absorbs maximally, but fisetin has no absorption) a strong orange fluorescence appeared with emission maximum at 580 nm, which is the characteristic fluorescence of EtBr bound to ds DNA. Thus it is evident that EtBr displaces fisetin from its binding site in DNA, implying that the fisetin molecules bind intercalatively between the DNA base pairs. Substantial increase in the fluorescence anisotropy was also found, indicating a constrained binding site for fisetin in the DNA matrix. In this connection it is noteworthy that Jana *et al*. have observed, that in contrast to fisetin and related polyhydroxy substituted natural flavonols, the mono-hydroxyflavone 3HF(of synthetic origin) shows a groove binding mechanism[35]. Recently Halder and Purakyastha synthesized a derivative of fisetin, which acts as a minor groove binder for ct DNA and as a loop binder for quadruplex DNA[36]. Such studies indicate the critical influence of substituents (in the flavonol molecules) in determining the DNA binding mode.

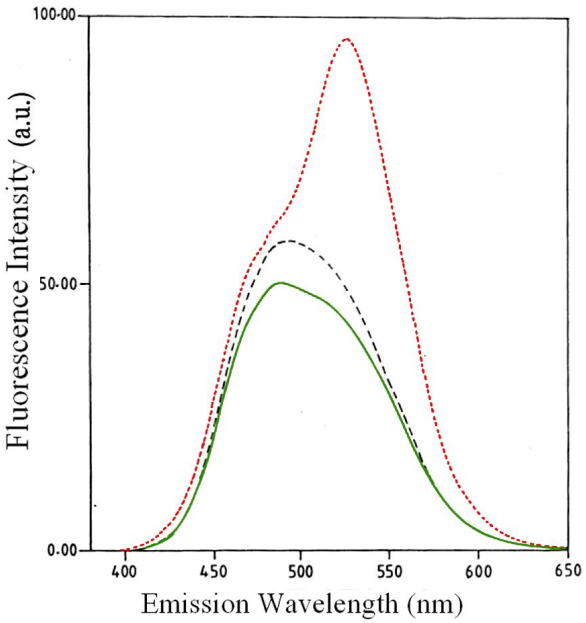


Figure 5 — Results of competitive binding studies using the classic intercalator Ethidium bromide (EtBr): Fluorescence emission spectra of fisetin (15 μM; λ_{ex} = 370 nm) (------) in absence of DNA, (…..) in the presence of DNA (200 μM), and (___) after addition of EtBr (15 μM) to the fisetin-DNA mixture (Adapted with modification from Ref 32)

Quadruplex DNA-flavonol interaction

Studies on quadruplex(QD) DNA-flavonol interactions have been the focus of much recent attention. The principal motivation for such work arises from a quest for small molecule ligands which can bind to QD DNA, with prospective applications as novel cancer therapeutic drugs. For our initial studies we chose to explore the interaction of fisetin with the quadruplex DNA $d(T_2AG_4)_4$ (which closely resembles the human telomeric DNA sequence). This research exemplifies applications of the intrinsic 'two color' fluorescence of the flavonol, along with other supporting spectroscopic and biophysical techniques. To obtain structural insights, structurally sensitive spectroscopic tools, namely electronic CD and vibrational Raman spectroscopic studies were performed, which revealed characteristic spectroscopic signatures for antiparallel quadruplex structure for this DNA[25]. Briefly, this consists of a positive peak at 295 nm, together with a negative peak at 262 nm in the CD spectral profile, and characteristic bands in the Raman spectrum, namely a 1582 cm^{-1} band (associated with quadruplex form) and shoulders at 684 cm^{-1}, and 1330 cm^{-1}, which are diagnostic of antiparallel topology (see Ref. 25 for details of CD and Raman spectral results and their interpretations). Highlights of findings from steady state fluorescence, time resolved intensity decay (providing fluorescence lifetime values), and time resolved anisotropy decay (providing rotational correlation times) studies are discussed here. From Figure 6 it can be seen that addition of QD DNA induces dramatic alterations in the emission characteristics of fisetin. In aqueous medium, the fluorescence spectrum of fisetin shows significant overlap between the normal and tautomer emissions, resulting in a broad emission band. With increasing concentration of $d(T_2AG_4)_4$, 'two color' fluorescence behavior becomes increasingly prominent, with the green-yellow ESIPT tautomer emission becoming dominant at high concentrations of DNA, signifying an aprotic binding site. This inference is further corroborated by the spectral characteristics of the excitation profile[25] (monitored for the ESIPT fluorescence) which reveals a weak but clearly perceptible vibrational shoulder typical of a predominantly aprotic environment (spectrum not shown). Furthermore, the normal emission

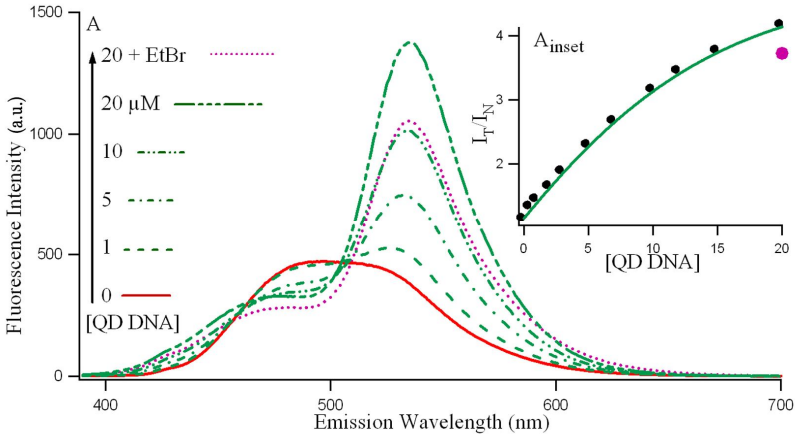


Figure 6 — Fluorescence Emission spectra of fisetin (15 μM) in the presence of different concentrations of quadruplex ($d(T_2AG_4)_4$) DNA. λ_{ex} = 370 nm (Adapted with some modification from Ref 25)

(which possesses a strong charge transfer (CT) character and therefore responsive to solvent dipolar relaxation induced spectral shifts[1,7]) undergoes a blue shift of 14 nm, with λ_{em}^{max} ~ 484 nm (in absence of DNA) changing to to λ_{em}^{max} ~ 470 nm in presence of 20 μM DNA. This provides another line of evidence for the hydrophobic nature of the binding site, and clearly exemplifies the usefulness of flavonols as multiparametric fluorescence probes. Time resolved fluorescence intensity decay studies show that in absence of DNA, fisetin ESPT tautomer species decay fits to a double exponential function with an average lifetime ($\bar{\tau}$) of 0.72 ns, which is in agreement with a previous literature data. Upon inclusion into DNA, there is a dramatic change in the decay profile with three discrete decay components now appearing. Compared to the free state of fisetin in aqueous system, $\bar{\tau}$ increases significantly with increasing DNA concentration, reaching a value of 5.21 ns, in the presence of 27 μM DNA. From these and supporting spectroscopic data (CD and Raman) and other biophysical studies[25], we picture that fisetin is bound at the diagonal loop region of the quadruplex (Figure 7), where it faces a relatively hydrophobic environment where non-radiative decay processes are reduced. This is also reflected in the higher emission yields noted in the steady state emission spectra[25].

Further insights were obtained from time resolved anisotropy decay studies on both the tautomer and normal fluorescence. Figure 8 shows a typical anisotropy decay profile for the ESPT tautomer (in presence of QD DNA). In methanol (chosen as a reference solvent) and DNA matrix (27 μM), the anisotropy decay of fisetin tautomer is found to be single exponential with rotational time constant (τ_{rot}) 0.21 ns and 16.79 ns respectively. The increase in the value of τ_{rot} from MeOH to DNA environment can be explained in terms of the increase in the restrictions in the motion of the fisetin molecules in the DNA matrix as well as the conformational swelling due to the overall motion of the fisetin-DNA complex. This result provides credible evidence that fisetin is firmly bound in a motionally constrained environment in the QD DNA matrix. Thus, the time resolved anisotropy decay measurements further reinforces the role of fisetin as a QD DNA ligand[25],and its promising prospect as a potential anti cancer drug with low systemic toxicity.

Our initial studies on QD DNA-fisetin interactions were subsequently extended by B. Sengupta *et al*. to other intrinsically fluorescent natural flavonols (in addition to fisetin) and their chromophores (of synthetic origin) as well as to different guanine rich (G_4) and cytosine rich (C_4) non canonical DNA structures, along with their duplex DNA counterparts[37]. This study provided critical insights on how structure and –OH substituent patterns on flavonoids affect their binding preferences toward non canonical *vis-à-vis* duplex DNA. Significantly, this research also exemplified application of the intrinsic fluorescence for a different class of flavonoid, namely a flavone (7- hydroxyflavone (7HF)) which, like flavonols, exhibit ESIPT and two color fluorescence. But in contrast to 3HFs, where the ESIPT is intrinsic

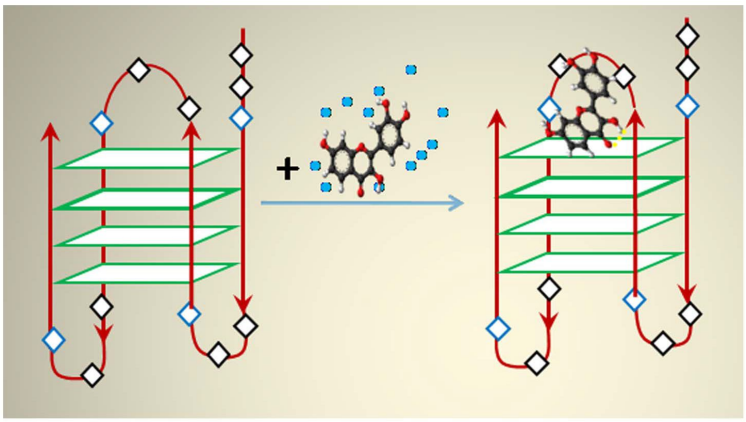


Figure 7 — Left: Intramolecular antiparallel quadruplex DNA, $(T_2AG_4)_4$ with adjacent parallel strands and diagonal loop. T,A,G bases are represented by black, blue and green blocks respectively. Right: Stacking interactions between the aromatic rings of the flavonol (fisetin) and G-quartet in the loop region in a 1:1 ratio. The blue dots denote water molecules surrounding fisetin in bulk aqueous buffer solution. The yellow dotted line denotes the intramolecular H bond in fisetin across which ESIPT tautomerization occurs in the DNA matrix. (Adapted from B Sengupta *et al*., PLoS ONE (2013), Ref 25, doi:10/1371/journal.pone.0065383.

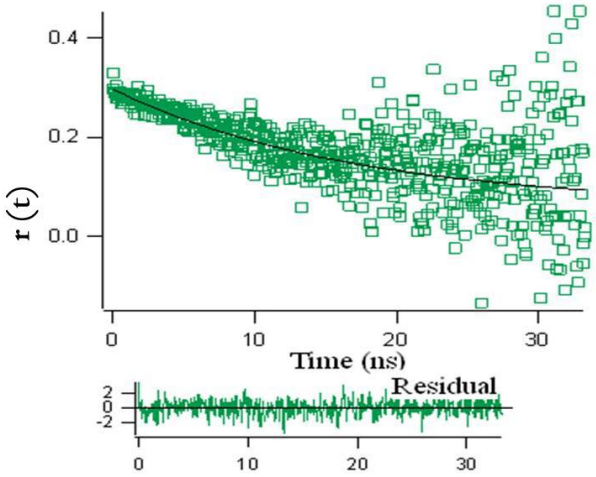


Figure 8 — Time resolved fluorescence anisotropy (r(t)) decay for fisetin ESPT tautomer fluorescence in quadruplex DNA(d$(T_2AG_4)_4$) matrix. The solid line shows the single exponential fit curve (Adapted with modification from Ref 25)

(occuring *via* an internal hydrogen bond), in 7HF, the ESIPT (between distal sites in the molecule) occurs *via* a solvent assisted process, and is thus mechanistically different[39].

Recent research efforts from our laboratory (Bhattacharjee *et al.*[22,23,46]) have emphasized the use of promoter quadruplex sequences with different sequence patterns (c-MYC, VEGF, c-KIT1, c-KIT2), along with the telomeric sequence (h-TELO). The repertoire of plant flavonols we have used include fisetin, quercetin, kaempferol and morin, which differ in their –OH substitution patterns on the A and B rings of the flavonols. The role of the –OH substitution patterns and the planarity of the C ring of the flavonols, as well as differences in the base sequences of the DNAs, in modulating the relative preferences for binding to different quadruplex sequences, as also in determining selectivity of recognition of quadruplex vis a vis duplex DNA structures have been critically assessed. For such assessments, we have largely relied on the the parameters of the 'two color' fluorescence of the flavonols, namely $I_{tautomer}$ /I_{normal} ratio, steady state fluorescence anisotropy, and time resolved intensity decay and anisotropy decay measurements (yielding fluorescence lifetimes and rotational correlation times respectively). Thus, in a typical study on the interaction of fisetin with a parallel G4 DNA (c-MYC)[46], the $I_{tautomer}$ /I_{normal} ratio increased rapidly with increasing concentration of G4 DNA, while only a very small increase was noted for the duplex structure. The fluorescence anisotropy parameter (which is an indicator of motional constraint[1,7]), increased from 0.03 (in aqueous buffer) to) 0.11 (in G4) and (0.06) for the duplex. Consistent with the steady state fluorescence data, time resolved measurements indicated a large increase in the average lifetime (τ_{avg}) for the ESIPT tautomer fluorescence, which increased from 0.73 ns (in aqueous buffer) to 3.73 ns (in presence of 30 µM G4) and 2.08 ns (in presence of 30 µM duplex), indicating a relatively hydrophobic environment in DNA. Anisotropy decay measurements lent further credence to this trend, with the rotational correlation time changing from 0.18 ns (for aqueous buffer) to 5.08 ns (for 30 µM G4), with a relatively modest change for the duplex. Thus the steady state as well as time resolved fluorescence data taken together, clearly support a more hydrophobic and motionally

constrained environment for fisetin in the G4 environment, as compared to that for the duplex, thus establishing that fisetin preferentially acts as a ligand for c-MYC quadruplex DNA. In further studies[22,23] quercetin and kaempferol (which, as previously noted, are weakly fluorescent intrinsically) were examined and found to show dramatic enhancement of their fluorescent yield in presence of appropriate G4 DNA target, namely VEGF (Vascular Endothelial Growth Factor) G4. This is reminiscent of early studies by us and by Gutzeit's group, where such fluorescence activation was observed for quercetin (which is weakly fluorescent intrinsically due to the simultaneous presence of 3-OH and 5-OH groups, hydrogen bonded to C=O), when quercetin is bound to a protein matrix. Using similar spectroscopic protocols as already noted, it was found that VEGF DNA showed preferential interaction toward quercetin, compared to other promoter G quadruplexes (c-MYC, c-KIT1, c-KIT-2) as well as telomeric and duplex DNA. This finding is rather interesting, considering the fact that all the promoter DNA sequences have a similar (parallel) secondary structure. Thus the preferential interaction toward VEGF DNA can be conceivably related to the bases in the loop region (mainly cytosine) of the VEGF DNA, which differ appreciably from the other promoter sequences[22].

In a recent study, a comparison was made of the binding interactions (with VEGF G4) of kaempferol (3,5,7,,4'-OH flavone) *vis-à-vis* morin (3,5,7, 2',4'-OH flavone), with the goal of exploring the role of B ring –OH substitution patterns, in modulating flavonol binding to G4 DNA[23]. It was found that while kaempferol shows preferential interaction with VEGF G4 DNA, as compared to other G4 sequences as well as duplex DNA, morin shows a significantly weaker level of interactions with duplex and G4 structures, exhibiting no structural selectivity toward any particular DNA structure. This contrasting behavior is presumably related to the presence of the additional 2'-OH substituent in the B-ring of the flavonol moiety, which leads to steric hindrance and a non-planar conformation for morin. Hence, it could be inferred that kaempferol (rather than morin) is an effective ligand for VEGF-G4 DNA.

Molecular docking calculations are of immense value in predicticting binding preferences by providing binding energy values. This is exemplified by representative data shown in Table I, which clearly reveals the relative binding preferences of fisetin (for c-MYC G4) and kaempferol and morin (for VEGF G4) *vis-à-vis* the corresponding duplex structures. It is noteworthy, that the docking predictions nicely corroborate the inferences from spectroscopic findings, based on 'two color' fluorescence studies.

Table I — Comparison of binding energies of some representative flavonols with G-quadruplex and duplex DNA predicted from molecular docking studies [a]

	Binding energy (Kcal/mol)	
Flavonol	G-quadruplex DNA	Duplex DNA
Fisetin	−7.20 [b]	−7.13
Kaempferol	−7.11 [c]	−6.64
Morin	−6.30 [c]	−6.37

[a] Data compiled from Bhattacharjee S. *et al*. (Ref. 23, 46)

[b] For c-myc quadruplex DNA

[c] For VEGF quadruplex DNA

Concluding Remarks

This article presents perspectives on photoinduced excited state intramolecular proton transfer (ESIPT) and 'two color' fluorescence properties of pharmacologically active plant flavonols, emphasizing applications to exploring DNA-flavonol interactions. Representative examples of studies on duplex (double stranded) DNA, as well as non canonical (quadruplex) DNA structures are presented. Based on steady state fluorescence, along with time resolved fluorescence intensity decay (providing fluorescence lifetimes) and anisotropy decay (providing rotational correlation time)studies, we demonstrate that the exquisitely sensitive 'two color' fluorescence of flavonols provide a powerful tool for noninvasive sensing of DNA-flavonol interactions. This leads to critical insights on the binding preferences, as well as the binding modes and affinities (typical binding constants of the order of 10^4-10^5 M^{-1}) of different flavonol derivatives toward duplex and quadruplex DNA structures. The studies on quadruplex DNAs highlight the importance of –OH substitution patterns, and planarity of the C ring of flavonol molecules, as also the base sequences of the DNAs, in modulating the preferential interactions of specific flavonols toward particular DNA sequences, as also in determining quadruplex *versus* duplex structure recognitions. The novel spectroscopic approach, based on the use of bioactive plant flavonols as their *own fluorescence reporters*, offers promising possibilities in relation to the quest for appropriate DNA binding flavonol derivatives with prospective applications as antioxidant based and anti-cancer drugs. Finally, one can envision the potential use of the sensitive 'two color' fluorescence of flavonols as a novel means for

detecting quadruplex DNA, for example in human cancer cells. For such ventures, two photon excitation microscopic approach (with its associated advantages of low scatter and high penetration)[1], which was recently exploited by Krasiva *et al*. for detecting fisetin fluorescence in neuronal cells and brain tissues of living animal models[11], might offer promising prospects.

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