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Interactions of therapeutically active plant flavonols with biological targets : Insights from fluorescence spectroscopic studies†

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Abstract : Plant flavonols have attracted much recent attention in view of their novel therapeutic properties (effective against various free radical mediated and other diseases) which make them promising alternatives to conventional therapeutic drugs. However, till date, not much is known, regarding their mode of interactions and binding affinities with relevant biological targets. This article presents perspectives highlighting the usefulness of the exquisitely sensitive 'two color' fluorescence behavior of flavonols (which arise due to highly efficient photoinduced excited state intramolecular proton transfer (ESIPT) reactions) for exploring their interactions, at the molecular level, with biomembranes and proteins, which are the principal biological targets of such drug molecules. In this context, we made exploratory studies on the interactions of some representative therapeutically important flavonols with model and natural membranes (composed of phosphatidylcholine liposomes and red blood cell ghost membranes respectively) and serum albumin proteins. Since the ESIPT process is highly sensitive to external hydrogen bonding perturbation effects, the relative contribution between the two colors is strongly modulated by the local environment of the fluorophore, with dramatic changes in the emission yield, energy, anisotropy (r), lifetime (τ) and related parameters of both ESIPT tautomer and normal fluorescence bands. This provides multiparametric fluorescence probing opportunities, revealing salient details about the nature and location of binding sites as well as quantitative estimates of partition coefficients/binding constants. This promising new approach may be expected to open up new avenues for the 'screening' of the most appropriate flavonoid derivatives, from among numerous structural variants found in nature, as well as the design of relevant synthetic derivatives with improved features.

Keywords : 3-Hydroxyflavone, fisetin, robinetin, quercetin, fluorescence spectroscopy, excited-state intramolecular proton transfer, phosphatidylcholine liposome, bovine serum albumin.

Introduction

Flavonols and related phenolic compounds of the flavonoid group (with a common structure of diphenylpropanes, C6-C3-C6, consisting of two aromatic rings linked through three carbons)[1] are present in flowers, fruits, leaves and other parts of plants and are ubiquitous in plants of higher genera. These phytochemicals carry out many important functions which are essential for the survival of plants in the natural environment. These include their role (as pigments/copigments) in the color of flowers and fruits (responsible for attracting insects and other pollinating vectors), in the photoprotection of sensitive molecules of the photosynthetic system (by screening harmful solar UV B radiation), as scavengers of free radicals/singlet oxygen, as signaling agents in plant - microbe symbiosis of agronomical significance (via triggering of nod genes in nitrogen fixing bacteria e.g. Rhizobium, causing root nodule formation in host plants e.g. pea, clover, and alfalfa), as leaf surface fungitoxins etc.[2].

Recent interest in flavonoids stems, in particular, from two different contexts of enormous importance. First, flavonols (which are most widespread among naturally occurring flavonoids) have emerged as one of the best known molecular systems exhibiting intramolecular excited state proton transfer (ESIPT) and dual fluorescence behavior[3-5]. Thus they can serve as useful models for mechanistic

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studies on ESIPT and related photophysical aspects. Secondly such compounds possess novel therapeutic properties (with high potency and low systemic toxicity) which make them promising alternatives to conventional therapeutic drugs. Interest on this latter aspect dates back to 1936, when Szent-Gyorgyi first drew attention to the therapeutically beneficial role of dietary flavonoids (some common sources being citrus fruits, berries, apple, onion, broccoli, soy products, tea, and red wine)[6]. Recent years have witnessed revitalized attention on this aspect with an explosive growth of research on various bioactive flavonoids effective against a wide range of diseases including cancers, tumors, AIDS, and various free radical mediated disorders (e.g. atherosclerosis, ischemia, neuronal degeneration etc.). In this context the question of possible target molecules and the mode of interactions with these targets constitute an important focus of current pharmacological research on flavonoids. There is mounting evidence that biomembranes, and various enzymes as well as other proteins are the principal targets of flavonoids in relation to their important protective functions.

In this article, we present perspectives illustrating the potential usefulness of the intrinsic fluorescence emission of flavonols for probing their interactions with such biological targets. This is based on research carried out in our laboratory during the past decade, for which we chose representative membrane systems and proteins as testing grounds. We used the simple model flavonol (of synthetic origin) 3-hydroxyflavone (3-HF), together with the polyhydroxy substituted naturally occurring flavonols namely quercetin (3,5,7,3′,4′-OH flavone, the most abundant dietary flavonoid), fisetin (3,7,3′,4′-OH flavone, a common source of which are strawberries), and robinetin (3,7,3′,4′,5′-OH flavone, found in e.g. hard wood).

Excited-state intramolecular proton transfer (ESIPT) and 'two color' fluorescence of flavonols :

Flavonols undergo ultrafast photoinduced ESIPT reaction (via the intramolecular hydrogen bond between the C=O and 3-OH groups) resulting in the transformation of the initially excited (N*) state to the tautomer (T*) form. This leads to 'two color' fluorescence, in the blue-violet and yellow-green regions, originating from the N* and T* states respectively (Fig. 1)[3–5]. The ESIPT process in flavonols is highly sensitive to the external hydrogen bonding interference of the environment on the internal hydrogen bond of the molecules and consequently, the relative contributions between the two colors is strongly modulated by the local environment of the fluorophore. In flavonol derivatives where the N* form shows strong charge transfer (CT) character, solvent dipolar relaxation effects are prominent. In such situations, while the yellow-green fluorescence serves as a 'proton transfer' probe (sensing H-bonding effects), the blue-violet fluorescence serves as a 'polarity probe' (sensing pola-

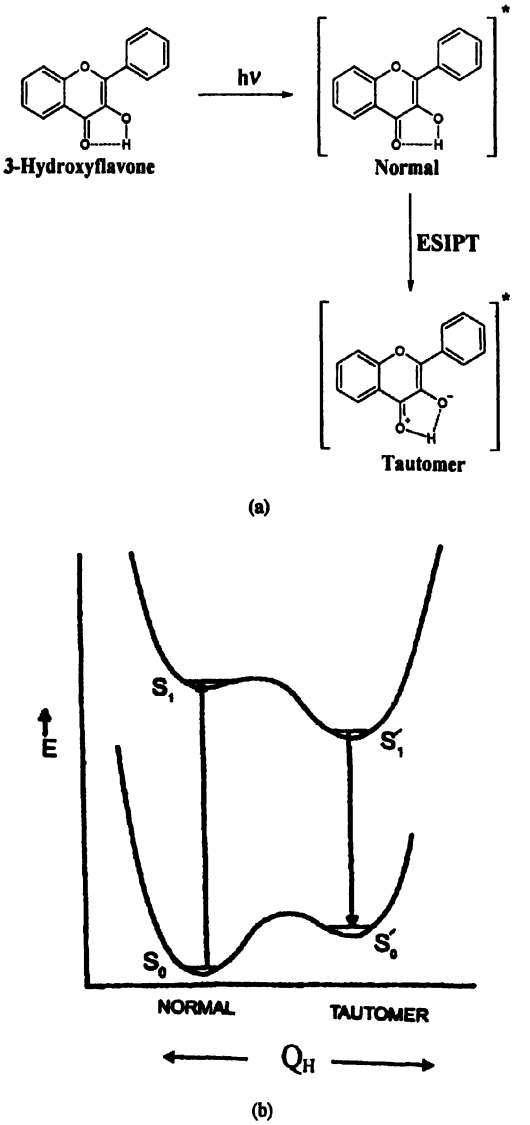

Fig. 1. (a) Photoinduced ESIPT in 3-hydroxyflavone. (b) Schematic diagrams showing : Proton transfer double-well potential with Q_H as the proton transfer coordinate (Refs. 3, 4).

rity of the fluorophore environment), thus permitting multiparametric use of the same fluorophore. A remarkable situation occurs in flavonols where 5-OH and 3-OH groups are simultaneously present (e.g. quercetin), where the C(4)=O·····HO—C(5) hydrogen bond interferes with the C(4)=O·····HO—C(3) hydrogen bond, thus preventing efficient ESIPT[7,8] and consequently the fluorescence quantum yield is intrinsically low, but strong fluorescence signals can be elicited in special situations e.g. upon binding to target proteins with specific structural motifs which disrupt the internal hydrogen bond involving the 5-OH group. These features form the underlying basis of the high sensitivity of flavonol emission to the surrounding environment and their prospective applications as exquisitely sensitive fluorescent molecular probes for exploring their interactions with various biological targets[2].

We explored the fluorescence emission behaviors of selected flavonols (with representative spectral characteristics) in model as well as natural membranes (phosphatadylcholine liposomes and goat red blood cell (RBC) ghost membranes) and serum albumin proteins (bovine serum albumin, BSA, and human serum albumin, HSA).

Membrane-flavonol interactions

Flavonols in model membranes (liposomes) :

Here we describe studies on three representative flavonols, namely 3-HF, robinetin, and fisetin. All these compounds are antioxidants with powerful inhibitory activities against lipid peroxidation[9,10,13] and therefore it is of considerable importance to obtain relevant details about their binding sites in membranes, partition coefficients and related aspects. Figs. 2a and 2b shows the influence of liposomal membrane environments on the ESIPT and dual fluorescence properties of 3-HF and robinetin.

Upon gradual addition of lipid (EYPC), the emission profile shows significant changes with substantial enhancement in the emission intensity of the tautomer fluorescence band. The remarkably enhanced tautomer fluorescence (which is known to be exquisitely sensitive to external H-bonding perturbation effects) tends to indicate that the flavonol molecules are incorporated in relatively hydrophobic fatty acyl chain regions of the liposomes, where the chromone moiety (which is the part of the molecule mainly relevant to the ESIPT process) is shielded from the water molecules. The ratio of the intensities of the tautomer (I_T) to normal (I_N) emission bands, I_T/I_N, is an useful parameter for monitoring the enhancement in relative yield of the ESIPT tautomer emission and provides a convenient indicator of the hydrophobicity of the microenvironment of flavonols[9,10]. Here, the saturating value of the I_T/I_N ratio for 3-HF is ~12 whereas that of the polyhydroxy substituted derivative, robinetin, is ~8. The relatively lower value of this parameter for robinetin suggests that its average environment in liposomes is comparatively less hydrophobic than 3-HF, which is consistent with the presence of multiple OH groups in robinetin,

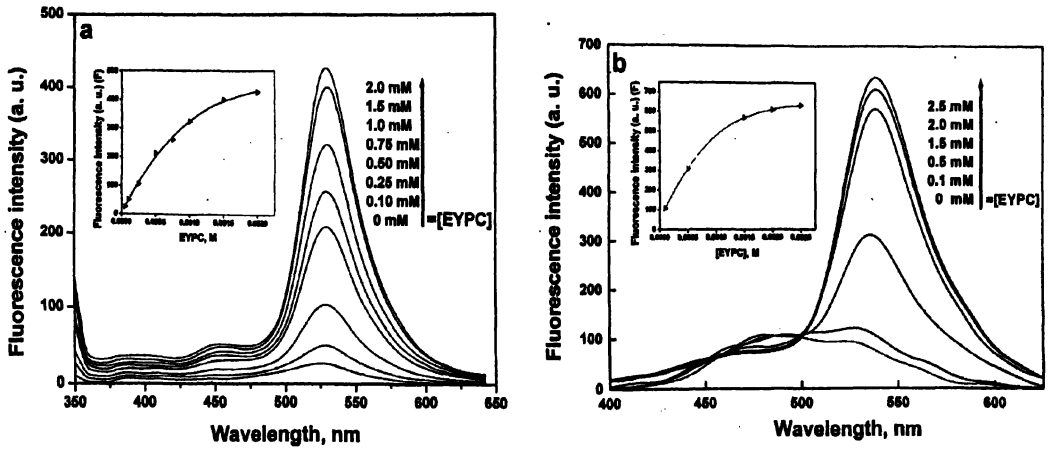

Fig. 2. Fluorescence emission spectra of (a) 3-HF and (b) robinetin, in EYPC liposomes for varying concentrations of EYPC, λ_{ex} = 340 nm for 3-HF and 370 nm for robinetin. Insets show the variation of ESIPT tautomer fluorescence intensities of the respective flavonoids with increasing EYPC concentrations. [3-HF] = 2.5 × 10^{-5} *M*, [robinetin] = 1.5 × 10^{-5} *M* (Refs. 9, 10).

which might be expected to promote, at least in part, exposure to the polar regions of the membranes.

The partition coefficient (K_p) is calculated from the slope and $1/F$ intercept of the double-reciprocal linear plot of $1/F$ against 1/[EYPC], according to the equation,

$$\frac{1}{F} = \frac{55.6}{K_p . F_{max}} \times \frac{1}{[\text{Lipid}]} + \frac{1}{F_{max}}$$

where F is the intensity of the tautomer fluorescence for a particular lipid concentration and F_{max} is the maximum fluorescence resulting from total probe incorporation into the membrane[11]. K_p was found to be 4.2×10^4 for 3-HF and 8.65×10^4 for robinetin (at 25 °C).

We also performed fluorescence polarization studies and estimated the anisotropy (r) parameter, which is a good indicator of the rigidity of the microenvironment of fluorophores, with zero or very low values of r in fluid solution where the fluorophore can freely rotate, and high r values signifying restricted motional freedom[12]. Progressive increase in anisotropy of the tautomer emission is observed with increasing lipid concentration (Figs. 3a and 3b) which is consistent with the picture that more and more fluorophore molecules are occurring in liposome bound state. Temperature dependence of r is illustrated in case of fisetin for two different phospholipids, EYPC and DPPC (Fig. 4). Significantly, the r vs temperature plots show characteristic sigmoidal shape, revealing the thermotropic phase transition of the phospholipids from gel to liquid crystalline states. Phase transition temperatures (T_m, 42 °C for DPPC and -12 °C for egg PC) estimated from the midpoints of the sigmoidal shaped curves, are in reasonably good agreement with existing literature data based on different physical methods[13-16]. The high values for the anisotropy (r) observed (e.g. 'r' = 0.31 at -14 °C in EYPC liposomes, Fig. 4), together with the fact that this parameter is proving to be a sensitive monitor of the thermotropic phase transitions of the phospholipids, are consistent with the picture that the fisetin molecules are localized in motionally constrained sites in the hydrophobic bilayer.

Fluorescence lifetime serves as a sensitive monitor of the local environment of fluorophores. We observed biexponential fluorescence decay kinetics for both mono and poly-hydroxy flavonols which suggest heterogeneity in microenvironments of the fluorophores in the membrane matrix. Moreover, in liposomes the individual components of the fluorescence decay are found to be significantly higher from the corresponding values in water. For example, when robinetin, is incorporated in EYPC liposomes, the average fluorescence lifetime increases by ~50% for the normal and ~200% for the tautomer emission, relative to the lifetimes in water[10]. These observations can be explained in terms of decreased water exposure in the membrane lipid bilayer with associated decrease in non-radiative deactivation rates.

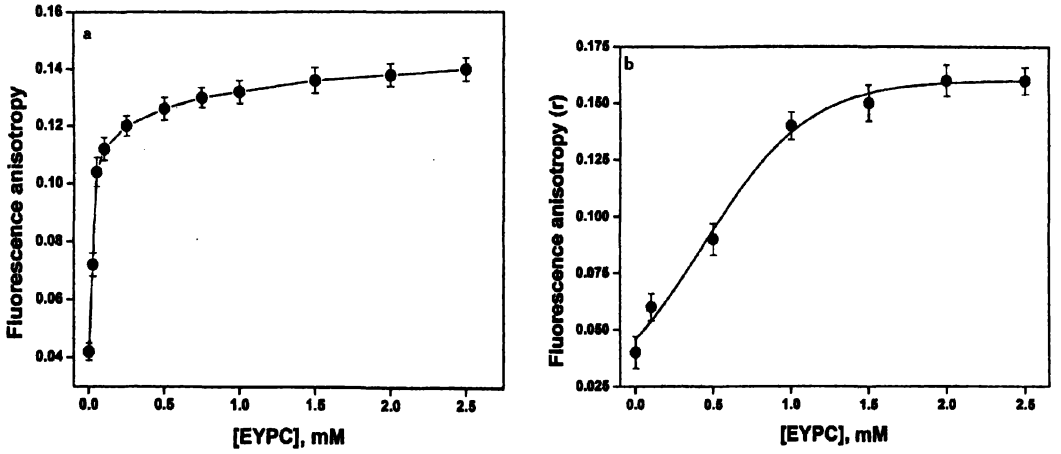

Fig. 3. Variation in fluorescence anisotropy of (a) 3-HF (λ_{ex} = 340 nm, λ_{em} = 525 nm) and (b) robinetin (λ_{ex} = 370 nm, λ_{em} = 540 nm) with increasing concentrations of EYPC.

Flavonols in RBC ghost membranes :

The fluorescence emission spectra of 3-HF and fisetin incorporated in RBC ghost membranes (along with refer-

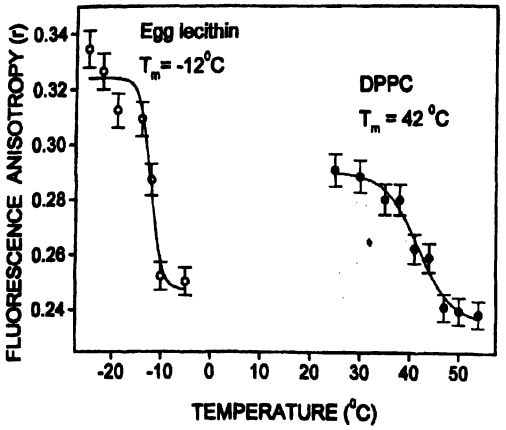

Fig. 4. Variation of fluorescence anisotropy (*r*) of ESPT fluorescence of fisetin in EYPC (open circles) and DPPC (solid circles) liposomes as a function of temperature. λ_{ex} = 370 nm, λ_{em} = 535 nm (Ref. 13).

ence spectra in aqueous buffer) are presented in Figs. 5a and 5b respectively. In buffer (in the absence of membranes) the emission spectrum of 3-HF shows well resolved dual fluorescence bands (consisting of a blue-violet normal fluorescence with λ_{em}^{max} = 408 nm and a green fluorescence with λ_{em}^{max} = 508 nm), the weak band at shorter wavelengths being due to Raman scattering from the aqueous solvent medium. Upon binding to RBC membranes the emission profile shows dramatic changes with significant enhancement in the emission intensity of the green fluorescence band, which shows λ_{em}^{max} at 524 nm, which corresponds to the value in typical hydrocarbon solvents[3]. Thus it is evident that in RBC membranes 3-HF is predominantly present in environments of low polarity, presumably in the lipid dominated region of the membrane, where external H-bonding perturbation is minimal, which facilitates an efficient ESIPT process with high quantum yield of the tautomer (green) fluorescence band. While dual fluorescence behavior is observed for both 3-HF and fisetin, the spectra are much less resolved in the latter case, where strong overlap occurs between the ESIPT tautomer and normal (non-proton transferred) emissions, the latter possessing notable charge transfer character[13,17,18]. Interestingly, in contrast to 3-HF, where the tautomer emission is strongly enhanced upon incorporation in RBC ghost membranes, only a slight increase in intensity occurs in case of fisetin. Hence, we infer that unlike 3-HF, fisetin molecules are localized in relatively polar regions of the RBC membranes, presumably at the lipid-water interface. Thus the differences observed in the protective effects of the various flavonoids towards lipid peroxidation and hypotonic hemolysis (data not shown, see Ref. 18 for details) can be at least partly attributed to the difference in the degree of the penetrations of the flavonoid molecules in intact erythrocytes and ghost membranes.

Protein-flavonol interaction :

Several reports indicate that proteins (including different enzymes) are frequently the targets for therapeuti-

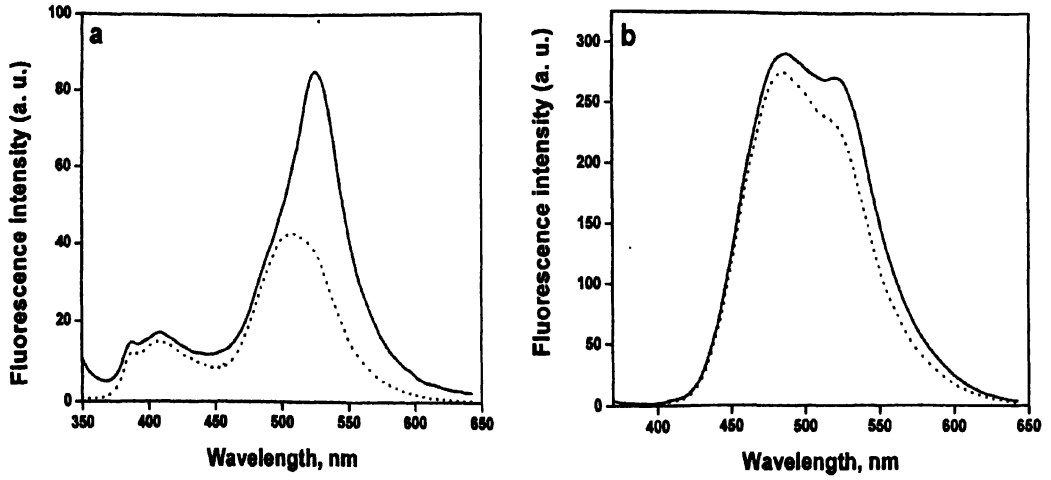

Fig. 5. (a) Fluorescence emission spectra of (a) 3-HF and (b) fisetin in RBC ghost membranes (containing 25 μg protein/ml) (–) and in PBS (·····), λ_{ex} = 340 nm for 3-HF and 360 nm for fisetin. [Flavonol] = 2×10^{-5} *M* (Ref. 18).

cally active flavonoids of both natural and synthetic origin[8]. For example, quercetin (3,5,7,3′,4′-OH flavone), the most abundant dietary flavonoid, is known to bind to human plasma proteins and inhibit the activities of enzymes e.g. different kinases[19-21] and DNA topoisomerases[22]. Another naturally occurring flavonol, fisetin (3, 7,3′,4′-OH flavone) has been found to inhibit protein kinase C, a signal transducing enzyme[23], and HIV-1 proteinase[24], a virally encoded protein which is indispensable for the maturation and processing of AIDS virus, and, more recently, was shown to be effective in preventing glycosylation of hemoglobin[25]. With this scenario in mind, we explored interactions of various intrinsically fluorescent flavonoids with serum albumins (bovine serum albumin, BSA, and human serum albumin, HSA), which we chose as model proteins for assessing the usefulness of the intrinsic fluorescence of flavonols in exploring protein-flavonol interactions.

Figs. 6a and 6b display typical fluorescence emission and excitation spectra (monitored at 535 nm) of 3-HF in presence of BSA, together with relevant reference spectra which are included for comparison. With increasing BSA concentration the tautomer (green) emission intensity is dramatically enhanced and the λ_{em}^{max} corresponds to that for the polar aprotic solvents. Moreover, in the presence of BSA, the excitation spectrum consists of a vibrationally resolved band with λ_{ex}^{max} = 345 nm, together with a conspicuous vibrational shoulder at 363 nm. Such features correspond to the spectroscopic signature in a predominantly aprotic environment, and sharply contrasts with the smooth (i.e. devoid of vibrational structure) excitation band typically observed in polar protic environments[26,27]. Furthermore, a band with λ_{ex}^{max} ~280 nm (corresponding to tryptophan absorption) appears in the excitation profile, indicating Förster-type fluorescence resonance energy transfer (FRET) between the tryptophan (s) (donor) present in BSA and the 3-HF molecule (acceptor), which suggests proximity of the 3-HF binding site to the tryptophan residue(s).

The interaction of quercetin with HSA exemplifies a rather interesting situation where protein binding 'activates' strong fluorescence in a fluorophore which is weakly fluorescent intrinsically[8]. With increase in protein concentrations, we observe gradual induction of pronounced dual fluorescence behavior of quercetin (Fig. 7a). The intensity ratio of tautomer : normal fluorescence, I_T/I_N, decreases rapidly with increasing protein concentration (shown in Fig. 7a inset) until [HSA] ~18 μM, beyond which it tends to level off. These observations can be rationalized in terms of interference with the internal H-bonds of quercetin, i.e. with C(4)=O·····HO–C(5) (which facilitates non-radiative deactivation[8]) and C(4)=O····· HO–C(3) (which permits the ESIPT process) at the binding site in HSA. Furthermore, similar to the case of BSA–3-HF interaction discussed earlier, occurrence of FRET (tryptophan-quercetin) is evident from the excitation pro-

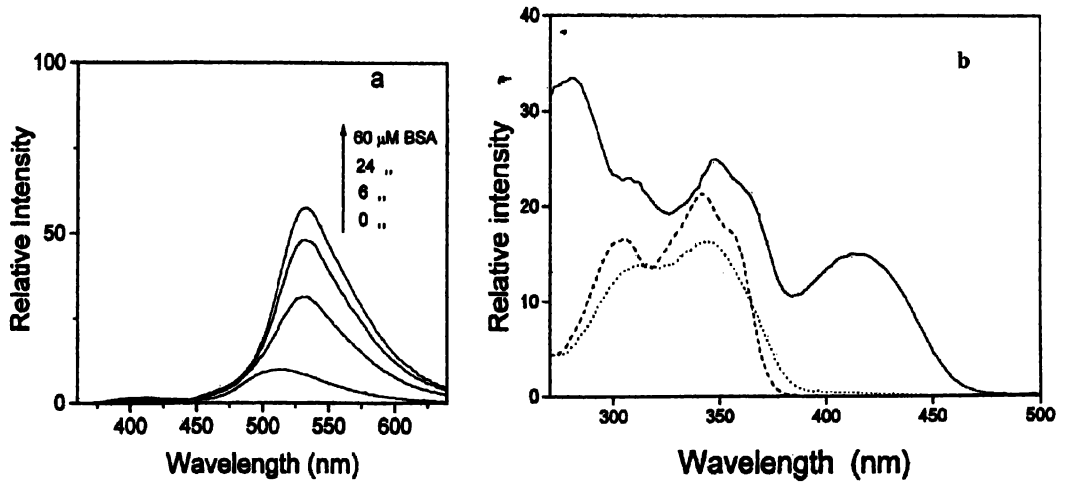

Fig. 6. (a) Emission spectra of 3-hydroxyflavone (3-HF) containing varying concentration of bovine serum albumin (BSA). (b) Excitation spectra of 3-HF in bovine serum albumin (BSA) (unbroken curve), ethyl acetate (dashed curve), and buffer (dotted curve) for the neutral species. λ_{em} = 535 nm, [3-HF] = 5 μM (Ref. 26).

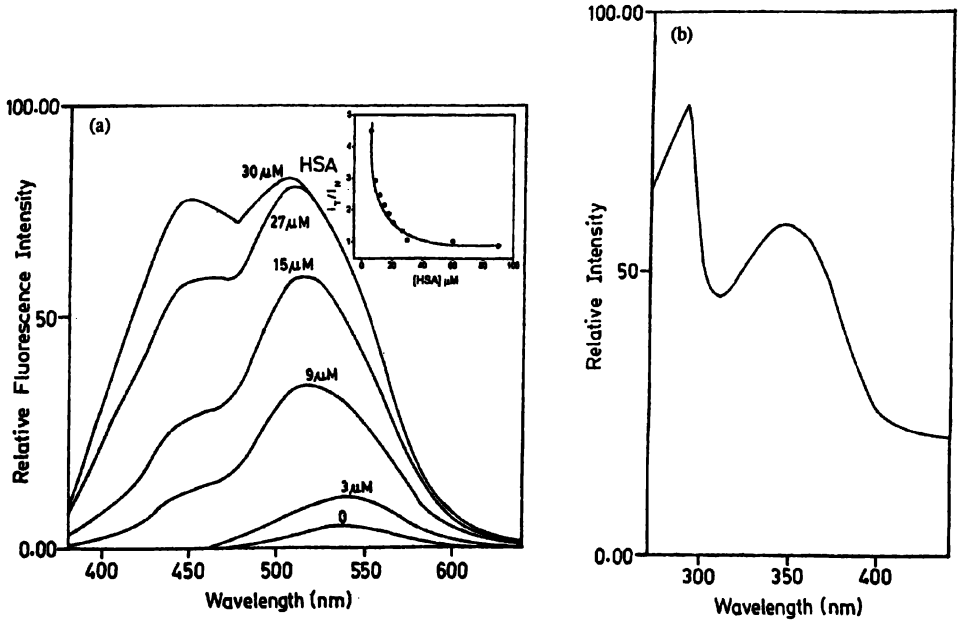

Fig. 7. (a) Fluorescence emission spectra of quercetin in the presence of various concentrations of HSA (0 → 30 μM) (λ_{ex} = 370 nm). Inset : I_T/I_N vs [HSA] plot. (b) Fluorescence excitation spectra of quercetin in the presence of HSA (≈18 μM), λ_{em} = 460 nm, [quercetin] = 15 μM (Ref. 8).

file (Fig. 7b). Since HSA contains a single tryptophan residue (at position 214) located in the interdomain cleft region of the protein (known from X-ray crystallographic studies[28]), we can infer that the binding site of the flavonoid must be located in this region, proximal to trp-214. Further, fluorescence polarization studies reveal high anisotropy values for protein bound flavonoids in case of both BSA and HSA (e.g. see Fig. 8 for HSA-quercetin binding, where $r \approx 0.18$ at [HSA] = 30 μM) characteristic of motionally constrained sites. Gutzeit and co-workers have shown the potential utility of such fluorescence (in quercetin and other medicinally important flavonoids) activated by protein binding to detect specific target proteins in drosophila follicles[29] and the nuclei of human leukemia cells[30] and have emphasized the general utility of this approach for quercetin and other related fluorogenic flavonoids of medical relevance.

Apart from such qualitative understanding on protein-flavonoid interactions we obtained quantitative estimates via titration studies, monitoring variations in fluorescence intensity and anisotropy parameters with increasing protein concentrations. Based on such measurements, and using Scatchard's procedure[32], binding constants (K) were estimated to be typically of the order of 10^5 M^{-1} which agrees well with existing literature data based on other techniques.

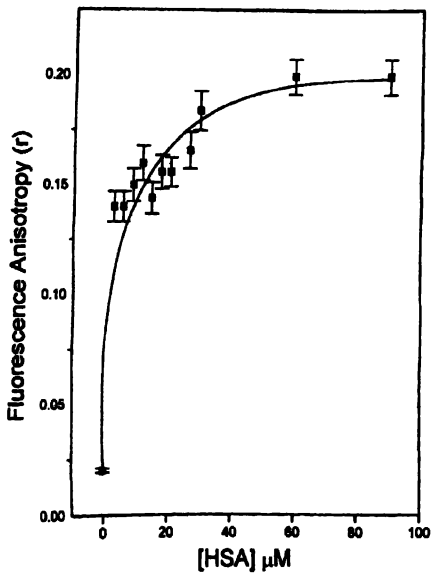

Fig. 8. Variation in the fluorescence anisotropy (r) of quercetin with increasing protein (HSA) concentration (λ_{ex} = 370 nm; λ_{em} = 530 nm). [Quercetin] = 15 μM (Ref. 8).

Concluding remarks

The spectroscopic research described in this article exemplify novel uses of flavonols as their own 'reporters' (by exploiting their exquisitely sensitive intrinsic fluorescence properties) for probing and characterizing their interactions with lipid membranes and protein targets. We can envision that expanding applications of this promising new approach would open the door to new avenues for the 'screening' and 'design' of the most suitable derivatives from among numerous available structural variants of this new generation of therapeutic drugs.

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Abbreviations

3-HF, 3-hydroxyflavone; ESIPT, excited-state intramolecular proton transfer; EYPC, egg yolk phosphatidylcholine; DPPC, dipalmitoyl phosphatadylcholine; RBC, red blood cell; HSA, human serum albumin; BSA, bovine serum albumin.

References

1. J. B. Harborne, in "Plant Flavonoids in Biology and Medicine II : Biochemical, Cellular and Medicinal Properties", eds. V. Cody, E. Middleton, J. B. Harborne and A. Beretz, Alan R. Liss, New York, 1988.
2. P. K. Sengupta, A. Banerjee and B. Sengupta, in "Photo/Electrochemistry and Photobiology in the Environment", Energy and Fuel, Research Signpost, India, 2006 and references cited therein.
3. P. K. Sengupta and M. Kasha, *Chem. Phys. Lett.*, 1979, **68**, 382.
4. M. Kasha, *J. Chem. Soc., Faraday Trans. 2*, 1986, 82; 1986, 2379.
5. A. P. Demchenko, A. S. Klymchenko, V. G. Pivovarenko and S. Ercelen, in : eds. R. Krayenhof, A. J. W. G. Visser and H. C. Gerritsen, "Fluorescence Spectroscopy, Imaging and Probes – New Tools in Chemical, Physical and Life Sciences", Springer Series on 'Fluorescence Methods and Applications', Vol. 2, Springer Heidelberg, Germany, 2002, pp. 101-110.
6. St. Rusznyák and A. Szent-Györgyi, *Nature*, 1936, **138**, 27.
7. B. Sengupta and P. K. Sengupta, *Biopolymers (Biospectroscopy)*, 2003, **72**, 427 and references cited therein.
8. B. Sengupta and P. K. Sengupta, *Biochem. Biophys. Res. Commun.*, 2002, **299**, 400.
9. S. Chaudhuri, K. Basu, B. Sengupta, A. Banerjee and P. K. Sengupta, *Luminescence*, 2008, **23**, 397.
10. S. Chaudhuri, B. Pahari, B. Sengupta and P. K. Sengupta, *J. Photochem. Photobiol. B : Biology*, 2009 (In Press).
11. Z. Huang and R. P. Haugland, *Biochem. Biophys. Res. Commun.*, 1991, **181**, 166.
12. J. R. Lakowicz, "Principles of Fluorescence Spectroscopy", 3rd ed., Springer-Verlag, New York, Inc., 2006.
13. B. Sengupta, A. Banerjee and P. K. Sengupta, *FEBS Lett.*, 2004, **570**, 77.
14. J. Guharay, S. M. Dennison and P. K. Sengupta, *Spectrochim. Acta (A)*, 1999, **55**, 1091.
15. D. Melchior, E. Bruggemann and Steim, *J. Biochim. Biophys. Acta*, 1982, **690**, 81.
16. J. Guharay, R. Chaudhuri, A. Chakrabarti and P. K. Sengupta, *Spectrochim. Acta (A)*, 1997, **53**, 457 and references cited therein.
17. B. Sengupta, A. Banerjee and P. K. Sengupta, *J. Photochem. Photobiol. B : Biology*, 2005, **80**, 79.
18. S. Chaudhuri, A. Banerjee, K. Basu, B. Sengupta and P. K. Sengupta, *Int. J. Biol. Macromol.*, 2007, **41**, 42.
19. M. Gschwendt, F. Horn, W. Kittstein, G. Furstenberger, E. Besemfekder and F. Mark, *Biochem. Biophys. Res. Commun.*, 1984, **124**, 63.
20. A. K. Srivastava, *Biochem. Biophys. Res. Commun.*, 1985, **131**, 1.
21. W. F. Matter, R. F. Brown and C. Vlahos, *Biochem. Biophys. Res. Commun.*, 1992, **186**, 624.
22. F. Boege, T. Straub, A. Kehr, C. Boesenberg, K. Christiansen, A. Andersen, F. Jakob and J. Kohrle, *J. Biol. Chem.*, 1996, **271**, 2262.
23. P. C. Ferricola, V. Cody and E. Middleton, *Biochem. Pharmacol.*, 1989, **381**, 1617.
24. R. S. Brinkworth, M. J. Stoermer and D. P. Fairlie, *Biochem. Biophys. Res. Commun.*, 1992, **188**, 631.
25. B. Sengupta and J. Swenson, *Biochem. Biophys. Res. Commun.*, 2005, **334**, 954.
26. J. Guharay, B. Sengupta and P. K. Sengupta, *PROTEINS : Structure, Function and Genetics*, 2001, **43**, 75.
27. M. Sarkar and P. K. Sengupta, *Chem. Phys. Lett.*, 1991, **179**, 68.
28. D. C. Carter and X. M. He, *Nature*, 1992, **358**, 209.
29. H. O. Gutzeit, Y. Henker, B. Kind and A. Franz, *Biochem. Biophys. Res. Commun.*, 2004, **318**, 490.
30. M. Böhl, C. Zupalla, S. V. Takalov, B. Hoflack and H. O. Gutzeit, *Anal. Biochem.*, 2005, **34**, 295.
31. D. Freifelder, "Physical Biochemistry", 2nd ed., W. H. Freeman and Company, New York, 1982.