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An intriguing pH-triggered FRET-based biosensor emission of a pyrazoline–doxorubicin couple and its application in living cells†

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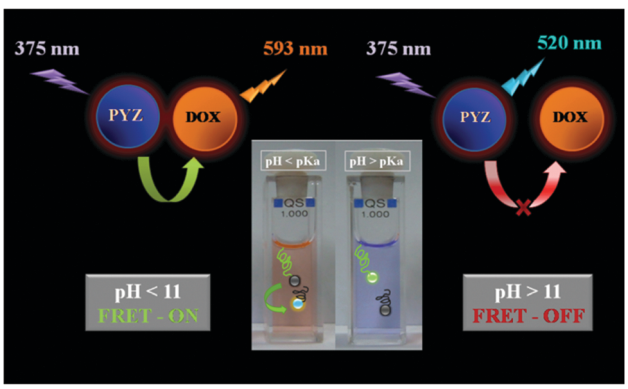
A unique pH-driven Förster resonance energy transfer (FRET) based biosensor emission by a pyrazoline–doxorubicin pair has been deciphered with a bioimaging application in a live HepG2 cell whereas conformational switching of both molecules at elevated pH reveals a fascinating twist (FRET-OFF) *via* strong fluorescent exciplex formation.

Owing to intense and inherent contemplation to the biomedical research fields and medical diagnostics, researchers have pursued optical biosensors employing stimuli-sensitive fluorogenic drugs that exploit various modalities due to their quick, non-invasive, disposable, easily miniaturized, simple functionality and visual signalling.[1] In living organisms, pH and temperature have crucial impacts on the cell and tissue activities, and abnormal pH and temperature deviations in bio-microenvironments are typically associated with pathophysiological processes.[2] Most of the existing pH-assisted optical chemosensors,[3] which have been developed for *in vivo* cell imaging purposes, are based on pH-responsive intensity changes in single-emission windows. However, single-emission detection is problematic for precise analyses under biological conditions as fluorescence suffers strong perturbation[4] owing to various environmental factors. To alleviate this difficulty, ratiometric measurements with greater precision compared to that of using a single wavelength were developed through simultaneously recording fluorescence intensities at two wavelengths and calculating their ratios.[5] The Förster resonance energy transfer (FRET) principle, which refers to the non-radiative energy transfer between fluorescent donors and acceptors, has been typically employed for the construction of ratiometric pH sensors which efficiently eradicate the interference from background signals and the fluctuation of detection conditions.[6]

Recently, a very interesting pH-triggered DNA nanomachine labeled with a FRET donor and acceptor at the two terminals was reported.[7] The designed DNA duplex adopts an extended conformation at pH 7.3 and a "closed state" at pH 5. It was also demonstrated that in an acidic environment, the cleavage of the pH labile bond between the fluorophores stops the energy transfer and results in an emission enhancement from the donor fluorophore, opening possibilities for interesting biosensing applications with judiciously designed FRET pairs (Scheme 1).

A few FRET-based pH sensing methods, including entrapping a pH-sensitive fluorophore on quantum dots or in nanogels, have been reported in the literature.[8] However, their pH sensitive capacity is restricted to 4.0–8.0, thus restricting their biological applications above pH 8.0.[9] Notwithstanding that this modest degree of research concerning those systems are well developed, crucial motifs are yet to be explored for efficient pH tunable FRET-couple particularly in the arena of non-invasive photo-probing for tumor cells in aqueous media along with profuse response for bioimaging purposes. To address this concern, we introduce a synthesized pyrazoline derivative[10] (PYZ, Scheme 2a)



Scheme 1 Schematic illustration of the pH-controlled FRET processes based on a PYZ–DOX conjugate and the mechanism of FRET ON–OFF switchover, and a photograph of the visually detectable solution of DOX above and below pK_a.

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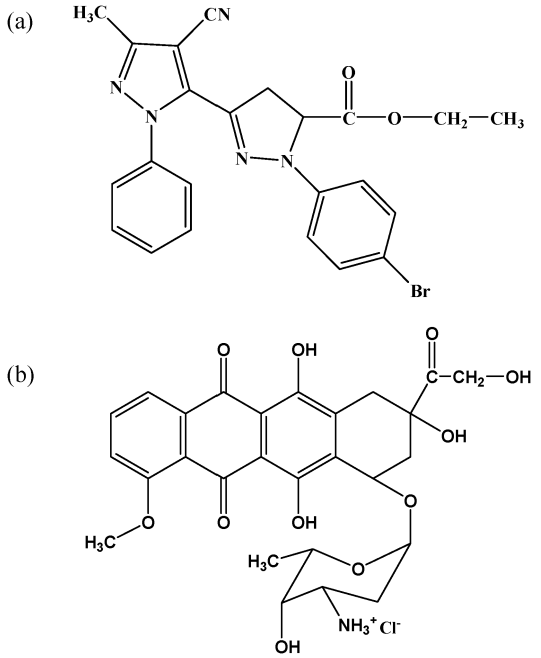

Scheme 2 Chemical structure of (a) PYZ (b) DOX.

as the donor fluorophore being coupled with widely used anticancer drug doxorubicin (DOX, Scheme 2b) as the acceptor. Being an important class of heterocycles, pyrazoline derivatives have attracted profuse interest not only in the arena of medicinal chemistry because of their antifungal and antimicrobial activity,[11] but also in materials chemistry for their role in nanocrystal investigation or as brightening agents[12] and so forth. Doxorubicin, affiliated to the anthracycline group of intrinsically fluorescent antibiotics, is recognized to show chemotherapeutic activity.[13] In this communication, we have successfully designed a new pH induced FRET biosensor using a PYZ–DOX couple that can control the FRET efficiency by simply monitoring the medium pH. Our results are not limited to FRET ON–OFF sensing upon varying the basic pH window, but is a breakthrough in the quest for pH-assisted superior emission enhancement of pyrazoline derivatives with stringent bathochromic shifts in the emission maximum which is rationally employed in the twisting behaviour (FRET-OFF) in response to their underlying conformational transition in mapping the environmental pH changes.

At a neutral pH, a very feeble green emission band of PYZ with a peaking maximum at 485 nm is noticed upon selective excitation at 375 nm. Interestingly, with the increase in pH, the emission profile shows dramatic changes leading to the appearance of a greatly red-shifted emission band centered at ~521 nm accompanied by a large enhancement in fluorescence emission intensity (Fig. 1). The observed change in the emission maximum of PYZ can be attributed to the structural change of the emitting species at high pH. At pH above 11, the ester group in the PYZ moiety is starting to dissociate to form carboxylate anion which would make the PYZ charge negative. This leads to the red-shift of the emission maximum because of intersystem π–π interactions. The pH-dependent structural orientation of PYZ is also supported

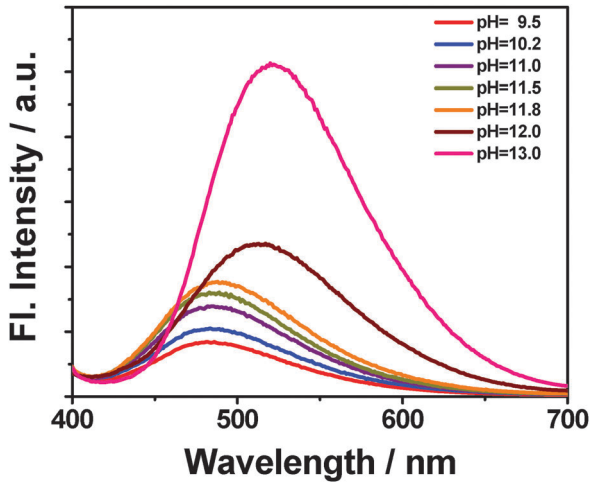

Fig. 1 Emission spectra of PYZ at different values of pH. λ_{exc} = 375 nm.

by NMR study. The NMR spectra of PYZ in $CDCl_3$ and $CDCl_3$ enriched with NaOD are shown in Fig. S1, ESI.† CH_2Me in the ethyl ester group under ordinary conditions absorb in the region $\delta = 4.21$ ppm.[1] In PYZ, we have found that the CH_2Me peak appears at δ = 4.197 ppm in $CDCl_3$. But the peak position of CH_2Me in $CDCl_3$ enriched with NaOD appears in the region δ = 3.811 ppm. Therefore, it is reasonable to suppose that the ethyl ester moiety in PYZ undergoes hydrolysis at high pH.

The basic absorption features of DOX in buffer solutions having different values of pH (ranging from 2 to 13) have been used[13] to explain the conformational change. A ratiometric plot (A_{550}/A_{480}) shows that the application range of DOX can be found to be from pH 7.0 to 11.0 (Fig. S2, ESI†).

Fig. 2 shows the pH tunable FRET profile of PYZ upon addition of DOX. The modulated FRET efficiency as a function of medium pH through environment dependent emission and

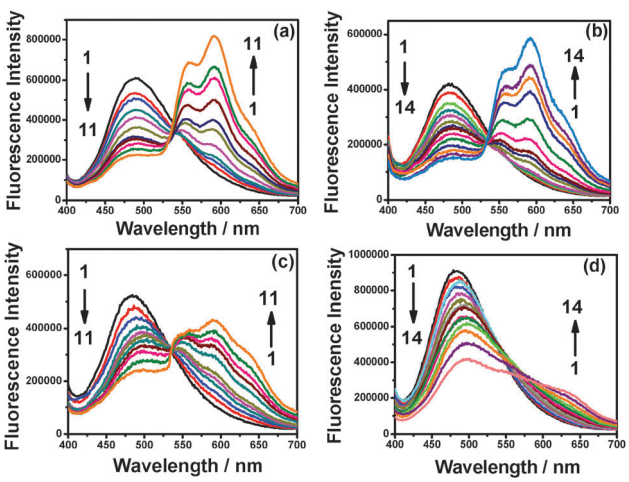

Fig. 2 Emission profile of PYZ at different concentrations of DOX at (a) pH = 7.0, curves 1 to 11 correspond to 0.0 to 29.0 mM DOX; (b) pH = 9.5; curves 1 to 14 correspond to 0.0 to 29.8 mM DOX; (c) pH = 10.2; curves 1 to 11 correspond to 0.0 to 31.9 mM DOX; (d) pH = 10.7; curves 1 to 14 correspond to 0.0 to 34.1 mM DOX, respectively. λ_{exc} = 375 nm.

Table 1 Various energy transfer parameters of PYZ–DOX couple

pH	$J \times 10^{12}$ (dm^3 cm^3 mol^{-1})	R_0 (nm)	r_0 (nm)	E
7.0	3.699	4.80	5.52	0.37
9.5	6.563	4.94	4.49	0.64
10.2	5.799	5.02	4.89	0.53
10.7	6.723	5.48	5.34	0.54

absorption of the donor and accepter, respectively, is determined from the energy transfer parameters summarized in Table 1. Using FRET, the actual distance r_0 between PYZ and DOX at different values of pH could be calculated by the following equation

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r_0^6}$$

where E denotes the efficiency of energy transfer between the donor and the acceptor, and R_0 (Förster radius) is the critical distance when the efficiency of transfer is 50%;[14] F and F_0 denote the steady state fluorescence intensities of PYZ in the presence and absence of the quencher, respectively.

Owing to the pH dependence of the drug absorption spectrum, the spectral overlap between the drug absorption and the PYZ emission is modulated as the pH is changed. This spectral overlap integral J is directly related to the critical FRET distance, R_0, which in turn affects the energy transfer efficiency, as shown in equation

$$R_0^6 = 8.79 \times 10^{-25}\, \chi^2 n^{-4} \Phi J$$

where χ^2 is the orientation factor related to the geometry of the donor and acceptor of dipoles and $\chi^2 = 2/3$ for a random orientation as in fluid solution; n is the average refractive index of medium in the wavelength range where the spectral overlap is significant; Φ is the fluorescence quantum yield of the donor; and J is the spectral overlap integral between the emission spectrum of the donor and the absorption spectrum of the acceptor. This can be calculated by the equation

$$J(\mathrm{pH}) = \frac{\int_0^\infty F(\lambda)\varepsilon_{\mathrm{pH}}(\lambda)\lambda^4 \mathrm{d}\lambda}{\int_0^\infty F(\lambda)\mathrm{d}\lambda}$$

where λ is wavelength, $\varepsilon_{\mathrm{pH}}(\lambda)$ is the pH-dependent molar absorptivity and $F(\lambda)$ is the normalized PYZ donor emission. With increasing pH of the medium, the distance (r_0) between the donor and acceptor passes through a minimum (Fig. S3, ESI†). As pH is increased, spectral overlap as well as R_0 grows larger and the FRET efficiency increases enormously up to a critical pH, after which the efficiency decreases again with further increase of pH. This allows one to tune the sensing ability of the sensor and exploit it to its maximum efficiency level by simply monitoring the medium pH (Fig. 3).

At pH above 10.9, the FRET becomes switch-OFF and an enhancement of donor emission takes place along with a dramatic red shift. The structureless features and the shift of the bathochromic emission as a function of DOX concentration are attributed to a strongly fluorescent exciplex. In an alkaline medium, such twisting behaviour is rare. Thus, we should be able to evaluate FRET ON–OFF sensing activity by controlling the pH between the PYZ–DOX couple molecules exposed to a

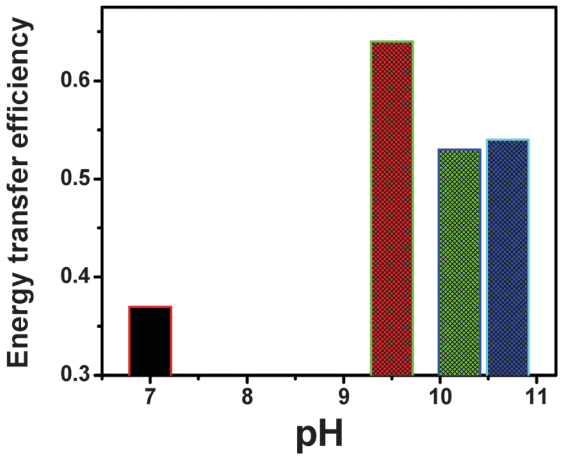

Fig. 3 Energy transfer efficiency diagram at different pH.

physiologically normal intracellular pH and those released in an enhanced extracellular pH environment.

The sensing action of the DOX–PYZ couple is imparted by modulation of the FRET efficiency arising from the pH-induced conformational change of DOX. Owing to the pH dependence of the drug absorption spectrum, the spectral overlap between the pH-sensitive drug absorption and the PYZ emission is modulated as the pH is changed. Since the phenolic –OH moiety of DOX is deprotonated and becomes an open chain configuration at pH > pK_a, it is expected that DOX is highly soluble in water and has an expanded chain conformation. As observed, for the free drug (DOX) in homogeneous solution[13] the DOX absorption band is greatly red-shifted and suppressed under basic pH. Consequently, the conformational change of DOX from the closed structure to the open chain structure results in an increase in the distance between the FRET donor and acceptor, which enables the FRET-OFF to be induced.

To rationalize the bioimaging application of this PYZ–DOX couple, we have taken fluorescence confocal images in living cells. Incubation of HepG2 cells with PYZ (5 μM) for 30 min at 37 °C was followed by the addition of DOX (30 μM) and the mixture was then incubated for another 30 min. The results are shown in Fig. 4. One can clearly observe significant confocal imaging changes of the medium upon addition of DOX for 30 min at 37 °C. HepG2 cells incubated with PYZ initially display a fluorescent blue image, but the fluorescence image immediately becomes a strong fluorescent red image in the presence of DOX. The results suggest that this sensor can efficiently penetrate the cell membrane and can be used for imaging in living cells and potentially *in vivo*.

Conclusions

In closing, we have successfully designed a new pH responsive FRET biosensor by introducing a PYZ–DOX couple that can image and deliver anticancer drugs to cancer cells and control the delivery of drugs to the targeted tumor cells based on the mechanism of Förster resonance energy transfer by simply monitoring the medium pH. At elevated pH, the FRET becomes switch-OFF and the two chromophores form a stable fluorescent exciplex at the expense of FRET efficiency. Proficient cell

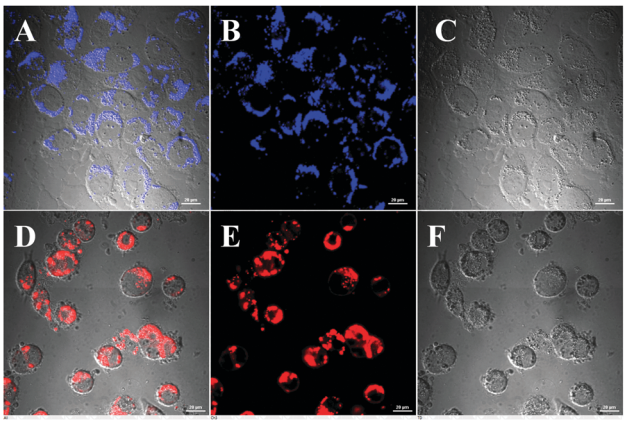

Fig. 4 Confocal fluorescence images of live HepG2 cells. (B) Cells incubated with 5 μM PYZ for 30 min at 37 °C. (E) Further incubated with addition of 30 μM DOX for 30 min at 37 °C. (C) and (F) are the brightfield images of live HepG2 cells shown in corresponding panel B and E, confirming their viability. The left image of each panel represents the merged images of the HepG2 cells and the corresponding fluorescent drug (A and D). The colors rendered in the fluorescence micrographs were λ-encoded (real color). λ_{exc} = 405 nm. Scale bar = 20 μm.

permeability of this sensor towards live HepG2 cells will help in understanding biological processes at the molecular level. Immediate goals to expand the utility of the PYZ–DOX couple for studies of medicinal biology include the optimization of the sensitivity and selectivity of the drug delivery to tumor cell lines, as well as an improvement of optical brightness and dynamic ranges for imaging applications.

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Notes and references

1 (*a*) X. Zhou, F. Su, H. Lu, P. Senechal-Willis, Y. Tian, R. H. Johnson and D. R. Meldrum, *Biomaterials*, 2012, **33**, 171; (*b*) T. R. Martz, J. J. Carr, C. R. French and M. D. DeGrandpre, *Anal. Chem.*, 2003, **75**, 1844.
2 X. Wan and S. Liu, *J. Mater. Chem.*, 2011, **21**, 10321.
3 (*a*) J. Kneipp, H. Kneipp, B. Wittig and K. Kneipp, *Nano Lett.*, 2007, **7**, 2819; (*b*) B. R. Lee, K. T. Oh, Y. T. Oh, H. J. Baik, S. Y. Park, Y. S. Youn and E. S. Lee, *Chem. Commun.*, 2011, **47**, 3852.
4 Y. Kurishita, T. Kohira, A. Ojida and I. Hamachi, *J. Am. Chem. Soc.*, 2010, **132**, 13290.
5 K. Kikuchi, H. Takakusa and T. Nagano, *TrAC, Trends Anal. Chem.*, 2004, **23**, 407.
6 (*a*) H. S. Peng, J. A. Stolwijk, L. N. Sun, J. Wegener and O. S. Wolfbeis, *Angew. Chem., Int. Ed.*, 2010, **49**, 4246; (*b*) C. Pietsch, R. Hoogenboom and U. S. Schubert, *Angew. Chem., Int. Ed.*, 2009, **48**, 5653; (*c*) L. Albertazzi, B. Storti, L. Marchetti and F. Beltram, *J. Am. Chem. Soc.*, 2010, **132**, 18158; (*d*) J. Yin, H. B. Hu, Y. H. Wu and S. Y. Liu, *Polym. Chem.*, 2011, **2**, 363.
7 S. Modi, M. G. Swetha, D. Goswami, G. D. Gupta, S. Mayor and Y. Krishnan, *Nat. Nanotechnol.*, 2009, **4**, 325.
8 (*a*) Y. L. Chiu, S. A. Chen, J. H. Chen, K. J. Chen, H. L. Chen and H. W. Sung, *ACS Nano*, 2010, **4**, 7467; (*b*) P. T. Snee, R. C. Somers, G. Nair, J. P. Zimmer, M. G. Bawendi and D. G. Nocera, *J. Am. Chem. Soc.*, 2006, **128**, 13320; (*c*) M. Suzuki, Y. Husimi, H. Komatsu, K. Suzuki and K. T. Douglas, *J. Am. Chem. Soc.*, 2008, **130**, 5720.
9 (*a*) T. Bagar, K. Altenbach, N. D. Read and M. Bencina, *Eukaryotic Cell*, 2009, **8**, 703; (*b*) R. Gupta, Q. K. Beg and P. Lorenz, *Appl. Microbiol. Biotechnol.*, 2002, **59**, 15; (*c*) X. Chen, Y. Zhou, X. Peng and J. Yoon, *Chem. Soc. Rev.*, 2010, **39**, 2120; (*d*) J. Lowther, M. W. Robinson, S. M. Donnelly, W. Xu, C. M. Stack, J. M. Matthews and J. P. Dalton, *PLoS One*, 2009, **3**, e369.
10 A. Mukherjee and K. K. Mahalanabis, *Heterocycles*, 2009, **78**, 911.
11 (*a*) R. Sivakumar, R. V. Pradeepchandran, K. N. Jayaveera, R. Vijaianand and P. Kumarnallasivan, *Int. J. Health Nutr.*, 2010, **1**, 1; (*b*) M. Shaharyar, A. A. Siddiqui, M. A. Ali, D. Sriram and P. Yogeeswari, *Bioorg. Med. Chem. Lett.*, 2006, **16**, 3947.
12 (*a*) S. W. Oh, D. R. Zhang and Y. S. Kang, *Mater. Sci. Eng.*, 2004, **C24**, 131; (*b*) M. Wang, J. Zhang, J. Liu, C. Xu and H. Ju, *J. Lumin.*, 2002, **99**, 79.
13 D. K. Rana, S. Dhar, A. Sarkar and S. C. Bhattacharya, *J. Phys. Chem. A*, 2011, **115**, 9169.
14 J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, Springer, New York, 3rd edn, 2006.

