Analyst

This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the **Information for Authors**.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard **Terms & Conditions** and the **Ethical guidelines** still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

Journal Name

ARTICLE

Small Quinolinium-Based Enzymatic Probes via Blue-to-Red Ratiometric Fluorescence

Pan Wang,[a] Jiajun Du,[a] Huijing Liu,[b] Guoqiang Bi[b]* and Guoqing Zhang[a]*

A small fluorescence ratiometric probe consisting of a *single* dye species, *N*-methyl-6-hydroxyquinolinium (MHQ), and coupled enzymatic substrates, exhibits a dramatic colour change (deep blue to red) and possesses a huge response ratio (over 2000 fold) upon specific regconition of target enzymes. Such dramatic responses are attributed to the excited-state proton transfer processes in MHQ molecules in water. Here the detections of β-Galactosidase and porcine pancreatic lipase are successfully demonstrated and this class of molecules has the potential to be developed as "naked-eyes" probes in vitro.

Introduction

Fluorogenic sensing techniques are widely used in modern enzymology due to the intrinsic benefits of fluorescence microscopy such as high sensitivity and low invasiveness. However, only limited varieties of fluorogenic molecules have been developed (mostly xanthene-based), including derivatives of resorufin,[1,2] coumarin,[3-8] fluorescein[9-16] and rhodamine.[17-21] Despite the high brightness that xanthene dyes exhibit, their predominantly π-π* transitions generally produce small Stokes shifts, making these dyes susceptible to concentration- and aggregation-caused quenching (ACQ). The effect of ACQ may lead to the false reading of intensity-based experimental data.

Ratiometric fluorescent sensing (RFS) takes advantage of the relative intensity change of optically-resolvable bands in the presence of a target analyte and correlates such ratiometric responses quantitatively with the concentration of the target. This sensing module is independent of probe concentrations and is unlikely to be affected by ACQ. There are many different operating mechanisms that can be used to design RFS schemes,[22] however, multi-band emission from a single dye species is particularly valuable since it is generally more synthetically accessible, versus its dyad counterparts.[23] A series of single-component dual-emissive oxygen sensors have been developed by Fraser and Zhang et al.[24,25] and have been successfully used to image tumour oxygen *in vivo*.[26] Tang and Liu et al. reported an RFS method for alkaline phosphatase,[27] which also employs a single dye species. In
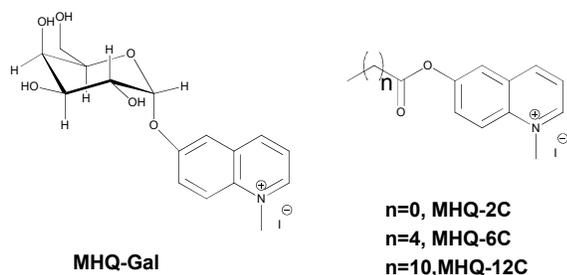
combination with aggregation-induced emission (AIE),[28] the two fluorescence bands stem from the water-soluble dye-substrate conjugate and dye residue aggregates, respectively. We recently also developed a macrocyclic RFS probe that is able to recognize Hg^{2+} based on the size and oxidation potential of the metal ion.[29] In addition to the single component aspect, another key criterion to RSF design is that the wavelength change must be significant enough to avoid cross-talks among different emission bands. Finally, the size of the RFS probes should not impose conspicuous influences on the detecting environments, thus the smaller the RFS the better.

Inspired by the work of Tang and Liu et al.,[27] here we demonstrate the utility of a single-component, synthetically facile and small-sized RSF probe with more dramatic emission colour change (deep blue to red) for the detection enzymes. The core fluorophore, *N*-methyl-6-hydroxyquinolinium (MHQ, Fig. 1), has been known to exhibit two emission bands, due to a locally excited state and another one stemming from excited-state proton transfer in protic solvent.[30] Similar to Tang's AIE work which results in a huge Stokes shift, the proton transfer can also generate essentially non-overlapping bands in aqueous environments and can thus be used for RFS. β-Galactosidase (Gal) and lipase are two classes of enzymes that are vitally important in biology. Bacterially derived Gal, often coupled with other reporter genes, is one of the most widely used tools in molecular biology.[31] Lipases are biotechnologically relevant enzymes and have found extensive applications in food, dairy, personal care and pharmaceutical industries.[32] In food sciences, the level of lipases is strictly regulated since the presence of such enzymes can cause rancidification during storage or transportation. In clinical sciences, the level of Gal excreted from the kidney is an important indicator in the diagnosis for renal diseases[33] whereas the concentration of lipase in serum is also an vital sign for pancreatitis.[34] As a result, qualitative as well as quantitative detection in vitro of these enzymes can be both scientifically and practically useful.

a. *Heifei National Laboratory for Physical Sciences at the Microscale and Department of Polymer Science and Engieering,* b*CAS Key Laboratory of Brain Function and Disease and Department of Neurobiology and Biophysics, School of Life Sciences, University of Science and Technology of China, 230026 Hefei, P. R. China.*

Results and discussion

Fig. 1 Chemical structures of fluorescent ratiometric probes for β-Galactosidase and porcine pancreatic lipase.

Shown in Fig.1, the β-Galactosidase (Gal) probe **MHQ-Gal** and porcine pancreatic lipase (PPL) probes **MHQ-2C**, **MHQ-6C**, and **MHQ-12C** were synthesized via facile coupling reactions (Electronic Supplementary Information, ESI). The counter ion for all MHQ derivatives is iodide (for the ease of synthesis), which does not impose a significant quenching effect thanks to both the strong charge-screening capability of water molecules and the low detection concentrations of the probes (i.e., [iodide] <1 m*M*). All the probes exhibit good aqueous solubility except for **MHQ-12C**, which forms nanoscopic particles, confirmed by the dynamic light scattering experiment (d ~ 300 nm). Under a broad-band 365-nm UV lamp, all MHQ probes exhibit fairly strong emissions (Φ_F = ~0.1-0.2) in the deep blue region in a pH = 7.3 phosphate buffer (Table 1, Fig. 1 and Fig. S1). For **MHQ-Gal** (100 µg/mL) under the same UV excitation, when Gal was added, the emission of the solution started to shift to the red region visible to the naked eye (Fig. S2). The process could be completed within 10 min at room temperature (25°C±2°C). For the three PPL probes, however, upon addition of PPL (ranging from 0.1 to 0.4 mg/mL), only the **MHQ-12C** nanodispersion exhibited visible colour change within a few minutes. It took ~6 h for **MHQ-6C** solution to change fluorescence emission colour (visually comparable to that of **MHQ-12C** after 10 min); while the fluorescence of **MHQ-2C** did not visibly change even after 6 h. The results are consistent with previous observations that lipases tend to work much more efficiently towards hydrophobic substrates.[35,36] The strategy of altering the length of alkyl chains can possibly provide a convenient means for the study of microenvironments in enzymology.

The spectroscopic data are presented in Fig. 2 for both **MHQ-Gal** and **MHQ-12C**, after corresponding enzyme additions, new absorption shoulders in the red-shifted regions were observed (Fig. 2), indicating the presence of free MHQ molecules as a result of the enzymatic cleavage of the glycosideic and ester bonds, respectively. Based on previous reports, the red-shifted absorption is originated from the zwitterionic **MHQ** species. Correspondingly, a huge stokes shift in the steady-state emission spectrum could be noted for both solutions due to excited-state proton transfer (ESPT) of **MHQ** in protic solvents. [30] This dramatic spectroscopic change is very advantageous for FRS since it avoids cross communications of
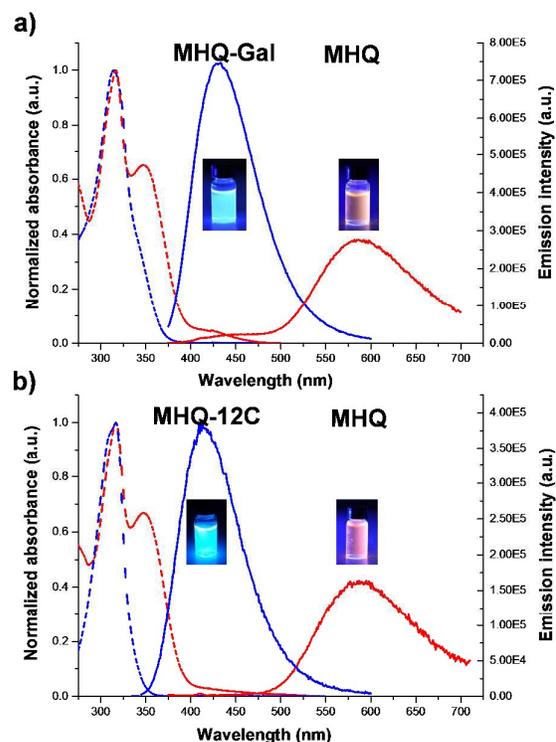
Fig. 2 (a) Absorption (dotted line) and steady-state emission (solid line) spectra of β-Galactosidase probe **MHQ-Gal** (blue line) and hydrolyzed product MHQ (red line). (b) Absorption (dotted line) and steady-state emission (solid line) spectra of porcine pancreatic lipase probe **MHQ-12C** (blue line) and hydrolyzed product MHQ (red line).

the two channels to the maximal extent. For **MHQ-Gal**, the Stokes shift is 6250 cm^{-1} (or 0.77 eV) and for **MHQ-12C** as much as 7090 cm^{-1} (or 0.88 eV). To the best of our knowledge, these are the largest Stokes shift values for any RFS probes ever reported. The significant excited-state energy loss is typical for ESPT; specifically in this case it involves the work of moving a proton in between a water molecule and an **MHQ** molecule.[30]

The Gal and PPL activities were then examined with these probes over a time period of 30 min via the monitoring of fluorescence changes in solution, as shown in Fig. 3. The change was already conspicuous 2 min after the addition of Gal and PPL, respectively. In both cases, a well-behaved isoemissive point could be obtained, confirming that only two emissive species, i.e., blue fluorescent probe substrates and **MHQ**, were present in the solutions after the enzymes were added. At the end of the experiment (*t* = 30 min), it was calculated that the ratio change for Gal was 334 fold (I_{588nm}/I_{430nm}) and 2296 fold for PPL (I_{588nm}/I_{415nm}). These huge numeric values suggest that the contrast of these MHQ-based enzymatic probes is very high.

       ARTICLE
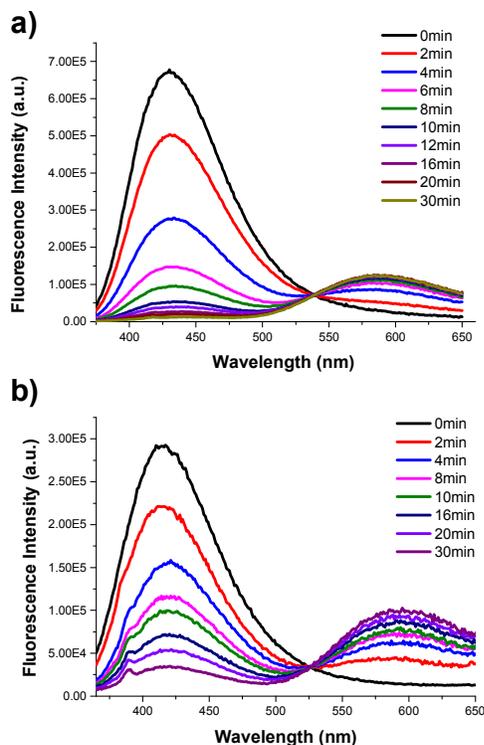


Fig. 3 (a) Steady-state emission spectra of **MHQ-Gal** (100μg/ml) in the presence of Gal (0.1mg/mL) over a period of 30 min (λ_{ex} = 360 nm). (b) Steady-state emission spectra of **MHQ-12C** (50μg/ml) in the presence of PPL (0.2mg/mL) over a period of 30 min (λ_{ex} = 340 nm).

The reactivity of the two enzyme substrates *in vitro* was evaluated via the Michaelis-Menten kinetics. The fluorescent substrates of varying concentrations were incubated with the two corresponding enzymes and then monitored via spectroscopy. The fluorescence intensities at the red maxima showed a gradual increase as the enzymatic hydrolysis of substrates commenced. The single-hit kinetics along with the kinetic constants are shown in Fig.4., where **MHQ-Gal** is demonstrated as a reasonably good substrate with k_{cat}/K_m = 2.57×10^5 $M^{-1}s^{-1}$ and K_m = 5.07μM. Comparing to the fluorescence ratiometric β-Galactosidase substrate previously reported (k_{cat}/K_m=4.07×10^4 $M^{-1}s^{-1}$,K_m=0.15 mM),[37] the significantly higher k_{cat}/K_m indicates that **MHQ-Gal** is a better substrate for β-Galactosidase. We ascribe the higher number to the small-sized quinolinium dye, which exerts less steric impact during the catalytic cycles. For **MHQ-12C**, the corresponding constants are k_{cat}/K_m = 9.56×10^3 $M^{-1}s^{-1}$ and K_m= 7.56 μM, respectively. Compared with a reported fluorescence substrate for pig liver esterase (PLE) based on Rhodamine 110 (k_{cat}/K_m = 1.9×10^3 $M^{-1}s^{-1}$, K_m = 0.47 μM), **MHQ-12C** exhibits higher catalytic efficiency but lower enzyme affinity,[38] presumably because lipases bind more tightly to hydrophobic substrates and our probe is more hydrophilic due to the presence of the positive charge.
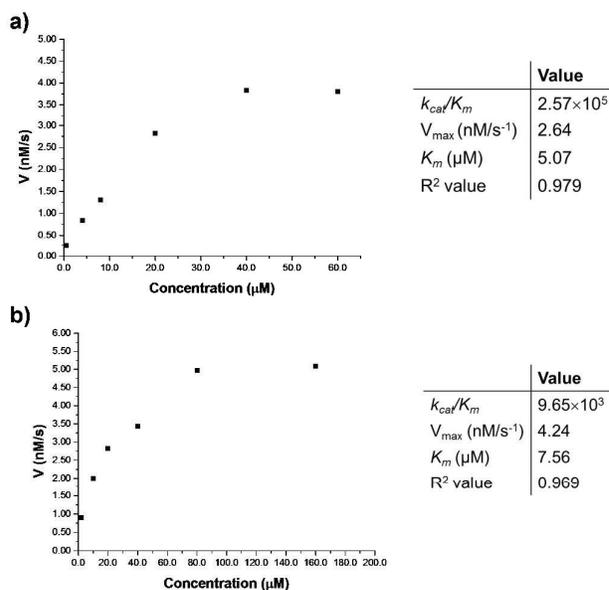
Fig. 4 (a) Michaelis–Menten saturation curve for Gal-catalyzed hydrolysis reaction of **MHQ-Gal** showing the relation between the **MHQ-Gal** concentration and hydrolysis rate. Key parameters (b) Michaelis–Menten saturation curve for PPL-catalyzed hydrolysis reaction of **MHQ-12C** showing the relation between the **MHQ-12C** concentration and hydrolysis rate.
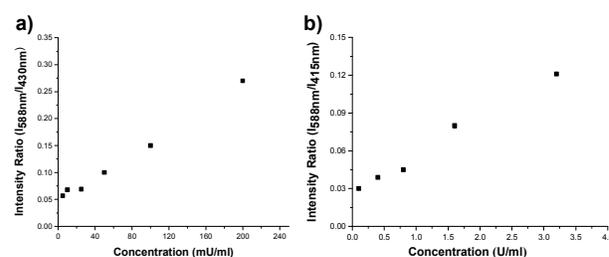
Fig. 5 Quantitative detection. a) Comparison of the fluorescence ratio 588nm/430nm of **MHQ-Gal** after a 60-min incubation at 37°C in pH7.3 buffer with increasing amounts of β-galactosidase (λ_{ex} = 360 nm). b) Comparison of the fluorescence ratio of 588 nm/415 nm of **MHQ-12C** after a 60-min incubation period at 37°C in a pH 7.3 buffer solution with increasing amounts of porcine pancreatic lipase(λ_{ex} = 340 nm).

Finally, we attempt to establish a quantitative relationship between the enzyme concentration and fluorescence intensity ratio at a specific incubation time (e.g., 60 min). β-Galactosidase of different concentrations up to 200 mU/mL was incubated with **MHQ-Gal**. Shown in Fig. 5, the plot is rather linear in terms of fluorescence intensity ratio vs. enzyme concentration from 5 to 200 mU/mL over an incubation period of 60 min with the presence of 25 μg/mL **MHQ-Gal**. The limit of detection concentration for Gal is 5 mU/mL (ESI). Similarly, a reasonably linear plot could also be observed for the fluorescence intensity ratio vs concentration over a range of 0.1 to 3.2 U/mL PPL over an incubation period

of 60 min in the presence of 50 µg/mL **MHQ-12C**. The limit of detection concentration for PPL is 0.1 U/mL (ESI). Therefore, the two MHQ substrates not only serve as "naked-eyes" probes but can also be in used the quantitative examination of enzymes.

Conclusions

In summary, we have demonstrated the use of small molecular N-methyl-6-hydroxyquinolinium (**MHQ**) as a core fluorophore to detect enzymes via ratiometric fluorescence sensing (RFS). Different substrate moieties were covalently attached to the **MHQ** fluorophore and were exposed to β-Galactosidase and porcine pancreatic lipase (PPL), respectively. It was found that these synthetically facile substrates exhibited dramatic spectroscopic shifts from the deep blue region to the red, which is presumably due to the presence of excited-state proton transfer process. The time-dependent spectroscopic evolution and enzymatic kinetics were investigated as well. Compared to previously reported enzymatic probes, the advantages of using **MHQ** are obvious in terms of the ease of synthesis and the dramatic fluorescence colour change and could be potentially scaled up for industrial production.

Acknowledgements

Notes and references

[1] J. Hofmann and M. Sernetz, *Anal. Chim. Acta.*,1984, 163, 67-72.
[2] Y. Zhang, W. Chen, D. Feng, W. Shi, X. Li and H. Ma, *Analyst.*, 2012, 137, 716–721.
[3] Chilvers. K. F, Perry. J. D, James. A. L and Reed. R. H, *J. Appl. Microbiol.*,2001, 91, 1118-1130.
[4] K. R. Gee, W. S. Sun, M. K. Bhalgat, R. H. Upson, D. H. Klaubert, K. A. Latham, and R. P. Haugland, *Anal. Biochem.*,1999, 273, 41-48.
[5] P. Babiak and J. L. Reymond, *Anal. Chem*., 2005, 77, 373-377.
[6] E. Leroy, N. Bensel and J. L. Reymond, *Med. Chem. Lett*., 2003, 13, 2105–2108.
[7] L. D. Lavis, T. Y. Chao and R. T. Raines, *ChemBioChem*., 2006, 7, 1151-1154.
[8] J. P. Goddard and J. L. Reymond, *Curr. Opin. Chem. Biol*., 2004, 15, 314–322.
[9] V. A. Rakhmanova and R. C. MacDonald, *Anal. Biochem.,* 1998,257, 234–237.
[10] N. C. Yanga and M. L. Hu. *Anal. Biochem.*, 2004, 325,337–343.
[11] T. Egawa,Y. Koide,K. Hanaoka,T. Komatsu,T. Teraiab and T. Nagano, *Chem. Commun.*, 2011, 47, 4162–4164.
[12] Y. Urano, M. Kamiya, K. Kanda, T. Ueno, K. Hirose, and T. Nagano, *J. Am. Chem. Soc.*, 2005, 127, 4888-4894.
[13] M. Kamiya, H. Kobayashi, Y. Hama,Y. Koyama, M. Bernardo, T. Nagano, P. L. Choyke, and Y. Urano, *J. Am. Chem. Soc.*, 2007, 129, 3918-3929.
[14] M. Kamiya, D. Asanuma, E. Kuranaga, A. Takeishi, M. Sakabe, M. Miura,T. Nagano, and Y. Urano, *J. Am. Chem. Soc.*, 2011, 133, 12960–12963.
[15] L. D. Lavis, T. Y. Chao and R. T. Raines, *Chem. Sci.*, 2011, 2, 521–530.
[16] L. Tian, Y. Yang, L. M. Wysocki, A. C. Arnold, A. Hu, B. Ravichandran, S. M. Sternson,L. L. Looger, and L. D. Lavis, *Proc. Natl. Acad. Sci.USA.*, 2012, 109, 4756-4762.
[17] M. Kamiya, D. Asanuma, E. Kuranaga,A. Takeishi, M. Sakabe,M. Miura, T. Nagano,and Y. Urano, *J. Am. Chem. So*c., 2011, 133, 12960–12963.
[18] D. Asanuma, M. Sakabe, M. Kamiya, K. Yamamoto, J. Hiratake, M. Ogawa, N. Kosaka. P. L. Choyke, T. Nagano, H. Kobayashi and Y. Urano, *Nat.Commun.*, 2015, 6, 6463.
[19] L.D. Lavis, T. Y. Chao, and R. T. Raines, *ACS Chem. Biol*., 2006, 1, 252-260.
[20] H.S.Hendrickson, *Anal. Biochem.*, 1994, 219, 1-8.
[21] S. S. Chandran, K. A. Dickson and R. T. Raines, *J. Am. Chem. So*c., 2005, 127, 1652-1653.
[22] M. H. Lee, J. S. Kim and J. L. Sessler, *Chem. Soc. Rev.,* 2015, 44, 4185-4191.
[23] T. Komatsu, K. Kikuchi, H. Takakusa, K. Hanaoka, T. Ueno, M. Kamiya, Y. Urano and T. Nagano, *J. Am. Chem. Soc*., 2006, 128, 15946-15947.
[24] G. Zhang, J. Chen, S. J. Payne, S. E. Kooi, J. N. Demas and C. L. Fraser, *J. Am. Chem. Soc.*, 2007, 129, 8942-8943.
[25] X. Zhang, T. Xie, M. Cui, L. Yang, X. Sun, J. Jiang and G. Zhang, *ACS Appl. Mat. Interfaces.*, 2014, 6, 2279-2284.
[26] G. Zhang, G. M. Palmer, M. W. Dewhirst and C. L. Fraser, *Nat. Mater.*, 2009, 8, 747-751.
[27] Z. Song, R. T. K. Kwok, E. Zhao, Z. He, Y. Hong, J. W. Y. Lam, B. Liu and B. Z. Tang, *ACS Appl. Mat. Interfaces.,* 2014, 6, 17245-17254.
[28] Y. Hong, J. W. Y. Lam and B. Z. Tang, *Chem. Soc. Rev.,* 2011, 40, 5361-5388.
[29] X. Xu, C. O. Trindle, G. Zhang and L. Pu, *Chem. Commun.*, 2015, 51, 8469-8472.
[30] E. A. Gould, A. V. Popov, L. M. Tolbert, I. Presiado, Y. Erez, D. Huppert and K. M. Solntsev, *Phys. Chem. Chem. Phys.*, 2012, 14, 8964-8973.
[31] J. Alam , and J. L.Cook, *Anal. Chem.,1990,* 188, 245-254.
[32] R. Gupta, N. Gupta, P. Rathi, *Appl. Microbiol. Biotechnol.*, 2004, 64, 763-81.
[33] N. Dance, R. G. Price, W. R. Cattell, J. Lansdell, B. Richards,*Clinica Chimica Acta.* 1970, 27, 87–92.
[34] V. Keim, N. Teich, F. Fiedler, W. Hartig, G. Thiele, J.Mössner, *Pancreas.*, 1998, 16, 45-49.
[35] F. Hasan, A. A. Shah , A. Hameed, *Biotechnol. Adv.*, 2009, 27, 782–798.
[36] Y. B. Ali, R. Verger, A. Abousalham, *Methods Mol. Biol*., 2012, 861, 31-51.
[37] T.Komatsu, K. Kikuchi, H. Takakusa,K. Hanaoka, T. Ueno,M. Kamiya, Y. Urano, and T. Nagano. *J. Am. Chem. Soc*., 2006, 126, 15946-15947.
[38] S.S. Chandran, K.A. Dickson and R. T. Raines, *J. Am. Chem. Soc.*, 2005, 127, 1652-1653.