ARTICLE

OBC
www.rsc.org/obc

Optical glucose detection across the visible spectrum using anionic fluorescent dyes and a viologen quencher in a two-component saccharide sensing system†‡

David B. Cordes, Aaron Miller, Soya Gamsey, Zach Sharrett, Praveen Thoniyot, Ritchie Wessling and Bakthan Singaram*

Department of Chemistry and Biochemistry, University of California, Santa Cruz, CA 95060, USA. E-mail: singaram@chemistry.ucsc.edu

Received 21st December 2004, Accepted 9th March 2005
First published as an Advance Article on the web 29th March 2005

A very general system is described in which anionic fluorescent dyes possessing a wide range of absorbance and emission wavelengths are used in combination with a boronic acid-modified viologen quencher to sense glucose at pH 7.4 in buffered aqueous solution. The present study demonstrates this capability with the use of eleven anionic fluorescent dyes of various structural types. Signal modulation occurs as the monosaccharide binds to the viologen quencher and alters its efficiency in quenching the fluorescence of the anionic dyes. The degree of quenching and the magnitude of the glucose signal were found to correlate roughly with the number of anionic groups on the dye. Optimal quencher : dye ratios were determined for each dye to provide a fairly linear signal in response to changes in glucose concentration across the physiological range.

Introduction

The development of fluorescent synthetic chemosensors for the detection of carbohydrates has attracted much attraction for over the past decade.[1–5] The majority of these sensing systems consist of a single molecule possessing both a fluorophore and a boronic acid receptor. The ability of boronic acids to reversibly bind saccharides has been well known for over forty years[6] and has since been extensively reviewed. Typically, upon glucose binding, the resulting changes in the electronic and/or steric states of the sensor molecule cause modulations in the fluorescence emission of the fluorophore that can be correlated with monosaccharide concentration. In these single molecule systems, the difficulty associated with synthetically combining a boronic acid receptor with the desired fluorophore in a single moiety can be a significant drawback since synthetic transformation can cause unpredictable changes in the photophysical properties of the dye. In a two-component saccharide sensing system, however, the fluorophore units can be interchanged without any synthetic modification of the receptor. Such a system can therefore make use of numerous commercially available fluorescent dyes.

In our two-component saccharide sensing system, boronic acid-functionalized viologen quenchers are used as the saccharide receptor unit.[7–10] Viologens have been found to quench the luminescence of both simple dyes[11–13] and macromolecular systems[14–17] through an ionic attraction between quencher and fluorophore to form a non-fluorescent complex. Upon saccharide binding to the boronic acid of the viologen quencher, a negatively charged boronate ester is formed. We hypothesize that this causes a diminished electrostatic attraction between quencher and dye resulting in a fluorescence increase. The putative general sensing mechanism is represented in Scheme 1 with the viologen quencher *m*-BBVBP^{4+}.[18]

Various anionic dyes were explored to demonstrate the flexibility of the two-component system. The ability to use any desired fluorophore in an optical sensing system is a considerable advantage since certain applications may require the use of particular excitation or emission wavelengths. For example, biological and particularly *in vivo* applications normally preclude the use of damaging UV wavelengths, whereas these may be appropriate for food processing or *in vitro* biochemical analysis. Previously, we have used the well-studied anionic dye pyranine (HPTS) and several of its derivatives, including a polymerizable sulfonamide immobilized in a thin film hydrogel, to sense monosaccharides.[9,10] The present study describes the use of various anionic dyes to sense glucose across the visible spectrum.

Results and discussion

Structurally diverse ionic dyes were selected to provide a wide range of emission wavelengths while providing structural diversity. All the dyes possess at least two sulfonic or carboxylic acid groups, except fluorescein-SA which has one of each, and all are expected to exist in their anionic forms at pH 7.4. The eleven dyes used in this study are shown in Fig. 1.

Quenching studies

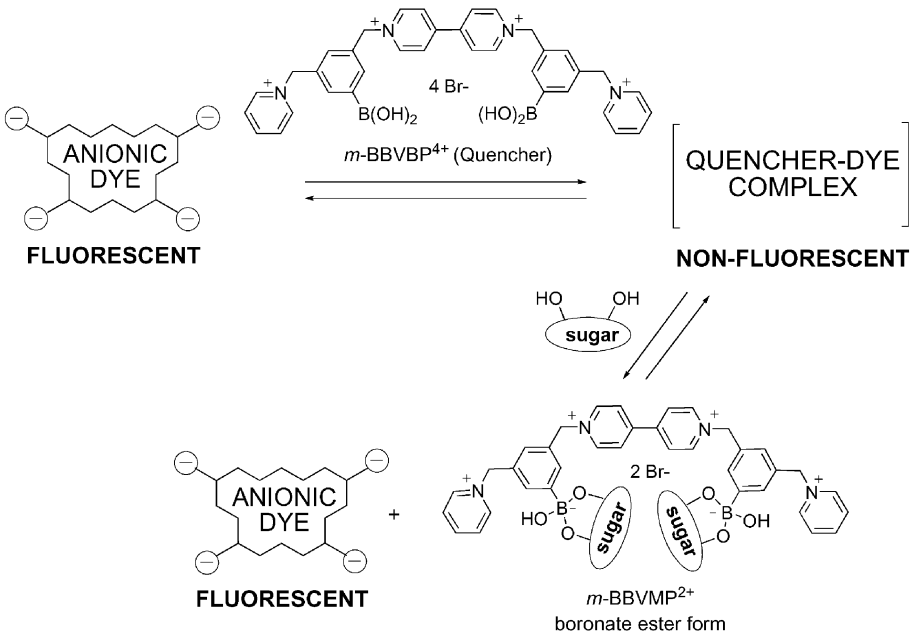
Studies of several boronic acid-modified viologen quenchers possessing both bipyridinium and phenanthrolinium cores have already been reported.[8,10] The ability of these viologens to quench the fluorescence of anionic HPTS was demonstrated and found to be most significant when there was a large number of cationic groups on the quencher. Among the quenchers studied, the 4+ charged bipyridium *m*-BBVBP^{4+} exhibited superior fluorescence quenching while providing a moderately strong glucose signal. In the present study, *m*-BBVBP^{4+} was used to quench the fluorescence of the dyes shown in Fig. 1. Based on our previous results, we expected stronger quenching to occur for dyes possessing a greater number of anionic groups. Generally we found this to be the case, with highly negatively charged HPTS, HPTS(Lys)$_3$, PTCA, MPTS and the porphyrin dyes, TSPP and TCPP, providing the most significant quenching. Dyes with fewer anionic groups quenched less effectively. A Stern–Volmer plot of quenching of selected dyes by *m*-BBVBP^{4+} at pH 7.4 is given in Fig. 2.

Graphical methods and UV-visible absorbance spectroscopy were used to determine the mechanism responsible for the fluorescence quenching of selected dyes. For most dyes, quenching involves mainly a static mechanism in which association between

† Dedication: dedicated to the memory of Herbert C. Brown.

‡ Electronic supplementary information (ESI) available: additional experimental details. See http://www.rsc.org/suppdata/ob/b4/b418953a/.

DOI: 10.1039/b418953a



Scheme 1 General sensing mechanism.

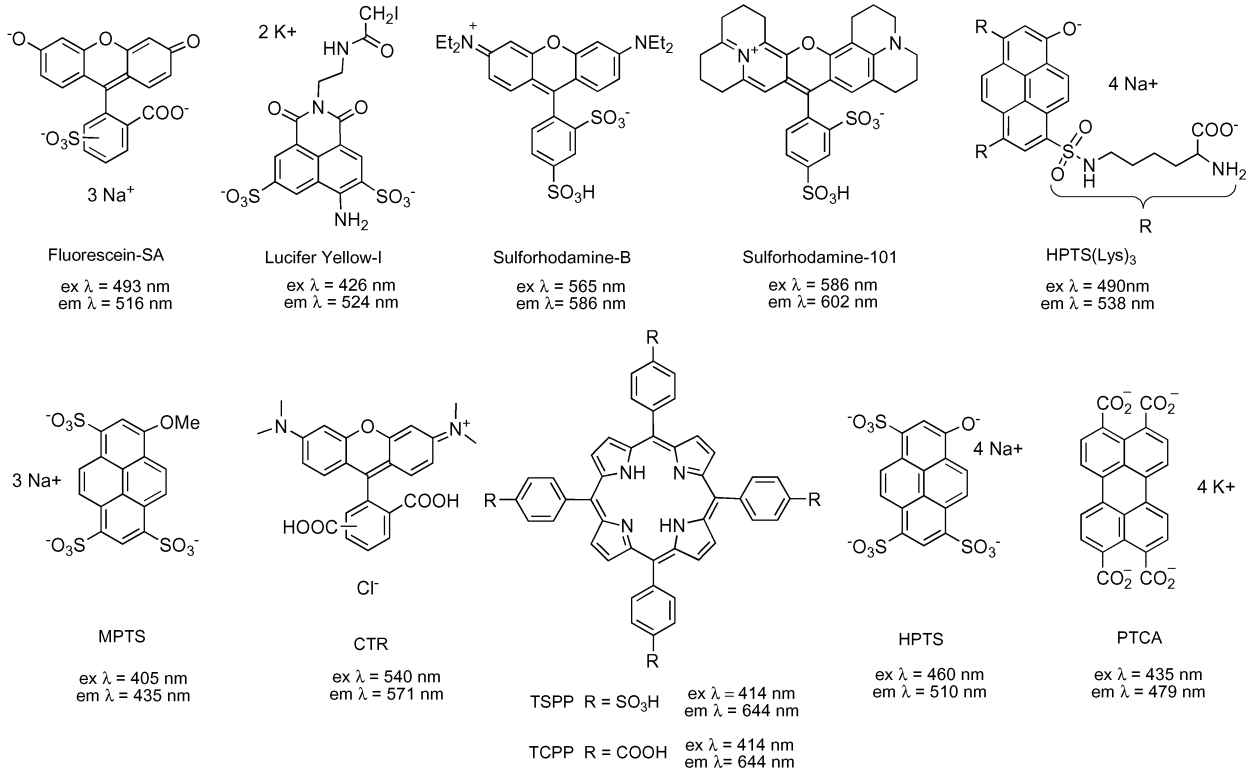


Fig. 1 Selected ionic dyes for use in saccharide sensing. All compounds are shown as purchased or prepared. Fluorescein-SA = fluorescein-5-(and-6-)sulfonic acid; lucifer yellow-I = lucifer yellow iodoacetamide; SR-B = sulforhodamine-B; SR-101 = sulforhodamine-101; MPTS = methoxypyrenetrisulfonic acid trisulfonate; CTR = carboxytetramethylrhodamine; TSPP = tetrakis(4-sulfophenyl)porphine; TCPP = tetrakis(4-carboxyphenyl)porphine; HPTS = hydroxypyrenetrisulfonic acid trisulfonate; PTCA = perylenetetracarboxylic acid tetracarboxylate.

dye and quencher provides an essentially non-fluorescent complex. An alternate quenching process, dynamic quenching, can occur *via* deactivating collisions between the dye and quencher in solution.[19] To determine the contribution of each of these processes to the overall quenching we relied on a "sphere of action" model[20] modified from earlier work by Frank and Vavilov[21] that provides quenching constants for both static (K_{sv}) and dynamic (V) processes (eqn. (1)).

$$(F_o/F) = (1 + K_{sv}[Q])e^{V[Q]} \quad (1)$$

F_o is the initial fluorescence and F is the fluorescence after addition of quencher. For most of the dyes studied, we determined that the dynamic, or collisional, quenching component contributed only a small portion of the overall quenching (Table 1). An exception was the HPTS(Lys)$_3$ dye, which was observed to undergo a significant degree of dynamic quenching. We speculate that this may occur due to the lengthy lysine units attached to the pyrene core. This would be expected to significantly increase the radius of gyration in solution and, thus, the likelihood of deactivating collisions with the quencher.[20]

Table 1 Quenching data

	Static quenching		Dynamic quenching
Dye	K_{sv} [a]	K_{uv} [b]	V [c]
HPTS	50 000 ± 4000	(45 000 ± 5000)	3800 ± 700
MPTS	22 000 ± 1000	(18 000 ± 1000)	300 ± 90
PTCA	16 000 ± 4000	(9000 ± 1000)	4500 ± 200
TCPP	14 400 ± 100	(22 000 ± 4000)	0
$HPTS(Lys)_3$	10 900 ± 200	(15 000 ± 4000)	14 000 ± 1000
TSPP	4600 ± 100	(3200 ± 400)	100 ± 40
Fluorescein-SA	1960 ± 70	(1500 ± 600)	30 ± 10

[a] K_{sv} (static Stern–Volmer quenching constant determined from fluorescence data). [b] K_{uv} (apparent binding constant for quencher–dye complexation from UV-vis data). [c] V (dynamic or collisional quenching constant derived from fluorescence data); errors are reported as standard deviations based on three trials. All values are reported in M^{-1} units.

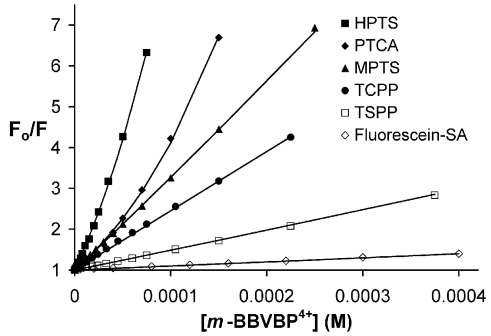


Fig. 2 Stern–Volmer plot of fluorescence quenching of selected ionic dyes by *m*-$BBVBP^{4+}$ at pH 7.4. All dyes were studied at [4×10^{-6} M] except SR-B and SR-101 [1×10^{-7} M].

The dyes CTR, SR-B, SR-101 and LY-I were only weakly quenched by *m*-$BBVBP^{4+}$ and we were unable to determine if the small degree of quenching was due to a static or dynamic mechanism. Each of these dyes bears only two anionic groups and we speculate that the quenching efficiency of these dyes is poor due to their relatively weak electrostatic attraction for the cationic quencher. In all of the cases of weak quenching we observed nearly linear Stern–Volmer plots indicating only a single mechanism at work. Relatively, small quenching constants ranging from 80 to 260 M^{-1} were obtained from Stern–Volmer analysis of the fluorescence quenching of these dyes.[22] All of the dyes showing strong quenching interactions with *m*-$BBVBP^{4+}$ displayed changes in their UV-visible spectra as they were titrated with quencher solution. Representative spectra are shown in Fig. 3.

These spectra strongly indicate complex formation between dye and quencher. The isosbestic points present in each spectra suggest a single complex being formed from each quencher–dye pair with what we speculate is 1 : 1 stoichiometry. Stability constants (K_{uv}) for complexes between the strongly quenched dyes and *m*-$BBVBP^{4+}$ were calculated from Benesi–Hildebrand plots of the UV-vis absorbance data and were found to roughly follow the same order as the Stern–Volmer quenching constants obtained from fluorescence studies (See Table 1). When quenching does occur by a static mechanism, as in the present case, the K_{sv} obtained from fluorescence should match closely the apparent quencher–dye stability constant (K_{uv}) derived from UV-vis absorbance data ($K_{sv} \approx K_{uv}$). The dyes showing weak quenching showed no perturbation in their UV-vis absorbance spectra as they were titrated with quencher; as discussed, we believe this is due to the weak electrostatic attraction of these dyes for the quencher. Despite the differences in quenching, all of the dyes studied were successfully used to sense glucose.

Glucose sensing studies

The binding isotherm derived from the fluorescence signal was determined by adding glucose to a quenched solution of dye. As glucose is added to the quenched solution, the fluorescence increases as the quenching becomes less efficient due to glucose binding the boronic acid of the quencher. Experiments show that the shape of the binding isotherm can be conveniently manipulated by adjusting the ratio between quencher and dye, an added advantage of the two-component system. Increasing the quencher : dye ratio raises the concentration at which the signal response saturates by lowering the apparent binding affinity between dye and quencher. In this way, we can avoid the common problem of rapid saturation that occurs in many one-component saccharide sensors. This allows us to obtain a fairly linear glucose response over the physiological range. Similar behavior was observed for the monosaccharides fructose and galactose, with the monosaccharide selectivity observed in our study roughly corresponding with the 33 : 3 : 1 ratio of binding affinities for fructose, galactose and glucose determined for simple phenylboronic acid.[23] In cases where only weak

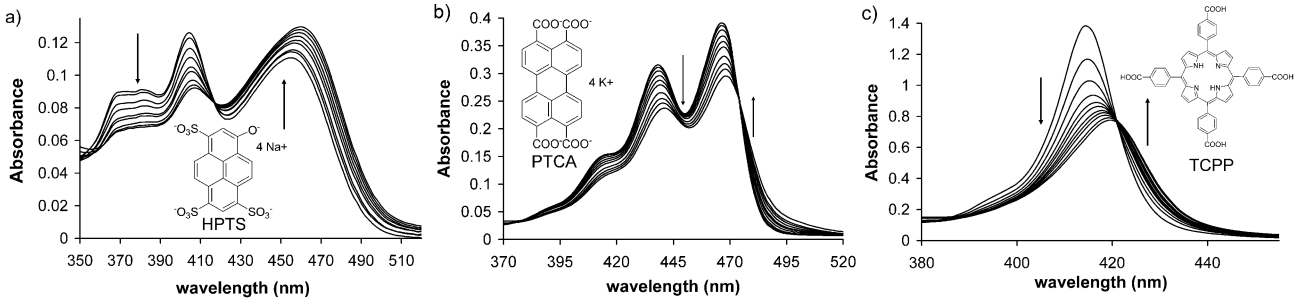


Fig. 3 Changes in UV-visible absorbance spectra upon titration with *m*-$BBVBP^{4+}$. a) HPTS (1×10^{-5} M), b) PTCA (1×10^{-5} M) and c) TCPP (4×10^{-6} M). Final concentration of *m*-$BBVBP^{4+}$ for a) 6.25×10^{-5} M, b) 1.13×10^{-4} M and c) 1.13×10^{-3} M.

fluorescence quenching was observed, we generally had to use very high quencher : dye ratios to obtain linearity in the glucose response signal. The effects of adjusting the quencher : dye ratio for TSPP with *m*-BBVBP^{4+} are shown in Fig. 4.

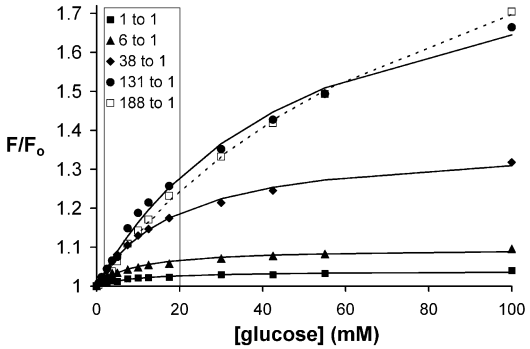


Fig. 4 Glucose response curves for addition of glucose to quenched solutions of TSPP at pH 7.4 with varying *m*-BBVBP^{4+} : TSPP ratios. The dashed line indicates the optimal quencher : dye ratio at 188 : 1. The physiological glucose range is boxed. F_o = initial quenched fluorescence; F = fluorescence after addition of glucose. Note: 10 mM [glucose] = 180 mg dL^{-1}.

Quencher : dye ratio optimization experiments were carried out for all of the dyes in the study to obtain a linear signal response in the physiological range. The optimized quencher : dye ratios and apparent binding constants for quencher binding glucose are given in Table 2, along with percentage increases in signals observed for increasing the glucose concentration from 0 to 30 mM.

As in the quenching experiments, we expected to find a strong charge dependence with regard to glucose sensing, with the dyes carrying more anionic groups providing greater signals. Generally, we observed that these dyes did provide stronger glucose signals and that the magnitude of signal response could be roughly correlated with the number of anionic groups on the dye (Fig. 5).

Conclusions

In conclusion, we have demonstrated a general glucose sensing system, operable at pH 7.4, using a broad range of anionic fluorescent dyes in combination with a boronic acid-modified viologen quencher. The mechanism relies on the ability of the viologen to quench the fluorescence of the anionic dye. The signal is produced when quenching is diminished upon addition of glucose, which binds to the boronic acids of the viologen quencher. The system operates at pH 7.4 and its sensitivity for sensing glucose in the physiological range can be adjusted by altering the ratio between quencher and dye. We found that both the degree of quenching and the magnitude of the glucose signal were generally greater for dyes possessing a greater number of anionic groups. The generality of this two-component sensing system and the ease with which desired fluorophores can be introduced into the sensing scheme allows for considerable versatility in the design of saccharide sensing systems. We are actively investigating the use of polymerizable analogs of these dyes for use in glucose sensing hydrogels and are exploring the application of this two-component sensing system towards other analytes.

Experimental

General

The following fluorescent dyes were purchased from Molecular Probes or Aldrich and used as received: fluorescein-SA = fluorescein-5-(and-6-)sulfonic acid (MP#F1130); lucifer

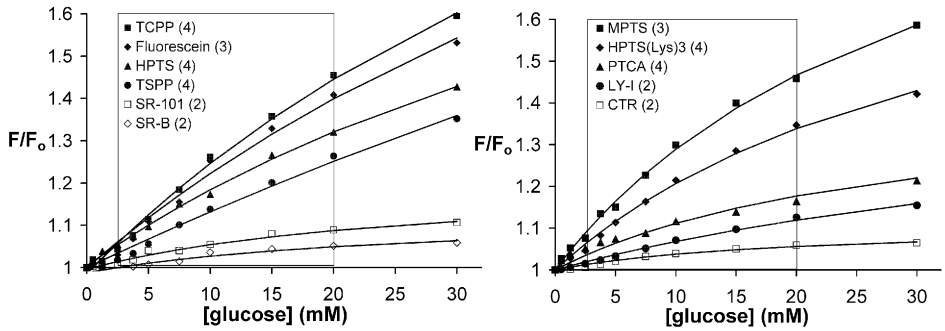


Fig. 5 Glucose response of various ionic dyes combined with *m*-BBVBP^{4+} at pH 7.4. F_o = initial fluorescence; F = new fluorescence. All dyes were [4 × 10^{-6} M] except SR-101 and SR-B which were [1 × 10^{-7} M]. Quencher : dye ratios and apparent binding constants are given in Table 2. The number of anionic groups (acids and phenols) is given in parentheses next to the dye name. The physiological glucose range is boxed. The data were distributed for improved clarity. Note: 10 mM [glucose] = 180 mg dL^{-1}.

Table 2 Glucose sensing data

Dye	Optimal quencher : dye (Q : D) ratio	Apparent glucose binding constants at optimal Q : D ratio (K_{app})/M^{-1}	Signal increase from 0 to 30 mM (%)
TCPP	188 : 1	0.030 ± 0.005	59
MPTS	250 : 1	38 ± 5	59
Fluorescein-SA	1300 : 1	22 ± 3	53
HPTS	31 : 1	27 ± 6	43
HPTS(Lys)$_3$	625 : 1	28 ± 5	42
TSPP	188 : 1	14 ± 4	33
PTCA	38 : 1	50 ± 10	22
Lucifer yellow-I	1250 : 1	13 ± 3	16
Sulforhodamine-101	125 000 : 1	32 ± 3	9
CTR	12 500 : 1	60 ± 10	7
Sulforhodamine-B	125 000 : 1	23 ± 1	6

Optimal quencher : dye ratios for linear signal response to glucose in 0 to 30 mM concentration range and apparent glucose binding constants at that ratio.

yellow-I = lucifer yellow iodoacetamide (MP#L1338); SR-B = sulforhodamine-B (Aldrich#23,016-2); SR-101 = sulforhodamine-101 (Aldrich#28,491-2); MPTS = methoxypyrenetrisulfonate (MP#335); CTR = carboxytetramethylrhodamine (MP#C300); TSPP = tetrakis(4-sulfophenyl)porphine (MP#T6932); TCPP = tetrakis(4-carboxyphenyl)porphine (MP#T6931). The preparation of HPTS(Lys)$_3$ has already been described.[9] Perylenetetracarboxylic acid (PTCA) was prepared by simple alkaline hydrolysis of perylene dianhydride with potassium hydroxide and used as the tetrapotassium carboxylate salt. Details for the preparation and characterization of *m*-BBVBP^{4+} are given in the supporting information.‡ All other reagents including HPTS dye were purchased from Aldrich Co. and used as received. All solvents were HPLC grade. DMF was stirred over CaH_2 for 2 days and filtered immediately before use. All reactions were conducted under an inert atmosphere of argon using air sensitive techniques. Measurements of pH were carried out on a Mettler Toledo MP 220 pH meter. ^{11}B NMR spectra were measured on a Bruker 250 using BF_3:Et_2O as an external standard. ^{13}C and ^{1}H NMR spectra were measured on a Varian 500 referenced to TMS.

Fluorescence emission and UV-vis absorption studies

All studies were carried out in pH 7.4 buffer solution prepared with water purified *via* a Nanopure Ultrafiltration system. Buffer solution (pH 7.4, 0.1 ionic strength) was freshly prepared using KH_2PO_4 and Na_2HPO_4. Fluorescence spectra were taken on a Perkin-Elmer LS50-B luminescence spectrometer. The absorption spectra were taken on a Hewlett Packard 8452A Diode Array Spectrophotometer. All studies were carried out at 20 °C without exclusion of air. Excitation and emission wavelengths for each dye are given in Fig. 1. For fluorescence titration experiments, the added volume did not exceed 3% of the total volume and the sample absorbance for fluorescent measurements was below 0.1.[24] All experiments except the quencher : dye optimization studies were carried out in triplicate and the error is reported as the standard deviation. All data was analyzed using the Solver (non-linear least-squares curve fitting) in Microsoft Excel.[25]

Absorbance studies to show complex formation

Measurements were done *in situ* by taking the absorbance spectra of the dyes HPTS, HPTS(Lys)$_3$, PTCA, MPTS and fluorescein-SA at a series of quencher concentrations. The emission of each dye (2 mL of 1×10^{-5} M in buffer, TCPP and TSPP at 4×10^{-6} M) was first obtained, then aliquots of quencher (0.005, 0.05, 0.01 or 0.1 M) were added, the solution shaken for 60 s and the new absorbance was measured. In order to minimize the volume of quencher solution being added, more highly concentrated quencher stock solutions were used for the titrations of dyes requiring high quencher concentrations to achieve significant perturbation in their absorbance spectra. Association constants (K_{UV}) were calculated by means of Benesi–Hildebrand plots and eqn. (2):

$$b/(\Delta A) = 1/(S_t K_{UV}\, \Delta\epsilon[L]) + 1/(S_t \Delta\epsilon) \quad (2)$$

with association constants determined using eqn. (3):

$$K_{UV} = (y\text{-intercept})/(\text{slope}) = -(x\text{-intercept}) \quad (3)$$

where b is the y-intercept, ΔA is the change in absorbance at the monitored wavelength, S_t is the substrate (dye) concentration, $\Delta\epsilon$ is the change in the molar absorptivity and [L] is the ligand (quencher) concentration.[26] Absorbance spectra and plots are provided as supporting information.‡

Fluorescence emission studies

Fluorescence intensity was taken as the area under the emission curve for all studies. Stern–Volmer constants were calculated by fitting the data with eqn. (1). Apparent glucose binding constants were calculated by fitting the data with eqn. (4):

$$F_{calc} = (F_{min} + F_{max} K[\text{glucose}])/(1 + K[\text{glucose}]) \quad (4)$$

where F_{calc} is the calculated fluorescence intensity, F_{min} is the initial fluorescence intensity of the quenched dye, F_{max} is the calculated intensity at which the fluorescence increase reaches its maximum and K is the apparent binding constant.[27]

Quencher : dye ratio optimization for glucose sensing

The fluorescence measurements were done *in situ* by taking the emission spectra of a 1 : 1 quencher : dye solution then adding an aliquot of buffered 1 M glucose solution and measuring the new fluorescence emission after shaking for 60 s. Additional aliquots were added and measurements taken until a glucose concentration of 30 mM was obtained. The overall process was then repeated at successively higher ratios until an optimal quencher : dye ratio could be determined.

Glucose sensing

The fluorescence measurements for each dye were done *in situ* by taking the fluorescence emission spectra of the quencher : dye solution at its optimized ratio. An aliquot of buffered 1 M glucose solution was then added and the new fluorescence emission was measured after shaking for 60 s. Additional aliquots were added and measurements taken until a glucose concentration of 30 mM was obtained. Representative spectra are provided in supporting information.‡

Acknowledgements

We thank Glumetrics, Inc., operating through the BioStar Industry–University Cooperative Research program, for continuing financial support.

References

1 H. Fang, G. Kaur and B. H. Wang, *J. Fluoresc.*, 2004, **14**, 481.
2 R. J. McNichols and G. L. Cote, *J. Biomed. Opt.*, 2000, **5**, 5.
3 J. R. Chen, Y. Q. Miao, N. Y. He, X. H. Wu and S. J. Li, *Biotechnol. Adv.*, 2004, **22**, 505.
4 H. S. Cao and M. D. Heagy, *J. Fluoresc.*, 2004, **14**, 569.
5 T. D. James and S. Shinkai, *Topics in Current Chemistry*, ed. S. Penadés, Springer-Verlag, Berlin, Heidelberg, 2002, vol. 218.
6 J. P. Lorand and J. O. Edwards, *J. Org. Chem.*, 1959, **24**, 769.
7 J. N. Camara, J. T. Suri, F. E. Cappuccio, R. A. Wessling and B. Singaram, *Tetrahedron Lett.*, 2002, **43**, 1139.
8 J. T. Suri, D. B. Cordes, F. E. Cappuccio, R. A. Wessling and B. Singaram, *Langmuir*, 2003, **19**, 5145.
9 J. T. Suri, D. B. Cordes, F. E. Cappuccio, R. A. Wessling and B. Singaram, *Angew. Chem., Int. Ed.*, 2003, **42**, 5857.
10 F. E. Cappuccio, J. T. Suri, D. B. Cordes, R. A. Wessling and B. Singaram, *J. Fluoresc.*, 2004, **14**, 521.
11 E. B. de Borba, C. L. C. Amaral, M. J. Politi, R. Villalobos and M. S. Baptista, *Langmuir*, 2000, **16**, 5900.
12 K. Nakashima and N. Kido, *Photochem. Photobiol.*, 1996, **64**, 296.
13 Z. G. Zhao, T. Shen and H. J. Xu, *J. Photochem. Photobiol., A*, 1990, **52**, 47.
14 B. S. Gaylord, S. J. Wang, A. J. Heeger and G. C. Bazan, *J. Am. Chem. Soc.*, 2001, **123**, 6417.
15 D. L. Wang, X. Gong, P. S. Heeger, F. Rininsland, G. C. Bazan and A. J. Heeger, *Proc. Natl. Acad. Sci. USA*, 2002, **99**, 49.
16 L. H. Chen, D. W. McBranch, H. L. Wang, R. Helgeson, F. Wudl and D. G. Whitten, *Proc. Natl. Acad. Sci. USA*, 1999, **96**, 12287.
17 N. DiCesare, M. R. Pinto, K. S. Schanze and J. R. Lakowicz, *Langmuir*, 2002, **18**, 7785.
18 4,4′-*N*,*N*′-Bis-[benzyl-(3-methylene-pyridinium bromide)-5-(boronic acid)]-dipyridinium dibromide.
19 J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, Kluwer Academic/Plenum Publishers, New York, 1999, 2nd edn.

20 D. L. Wang, J. Wang, D. Moses, G. C. Bazan and A. J. Heeger, *Langmuir*, 2001, **17**, 1262.
21 I. M. Frank and S. I. Vavilov, *Z. Phys. Chem. (Munich)*, 1931, **69**, 100.
22 See supporting information for details.
23 G. Springsteen and B. H. Wang, *Tetrahedron*, 2002, **58**, 5291.
24 A. Credi and L. Prodi, *Spectrochim. Acta, Part A*, 1998, **54**, 159.
25 E. J. Billo, *Excel for Chemists: A Comprehensive Guide*, John Wiley & Sons, New York, 2001, 2nd edn.
26 K. A. Connors, *Binding Constants: The Measurement of Complex Stability*, John Wiley & Sons, New York, 1987.
27 C. Cooper and T. D. James, *J. Chem. Soc., Perkin Trans. 1*, 2000, 963.