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Evaluation of Pyranine Derivatives in Boronic Acid Based Saccharide Sensing: Significance of Charge Interaction Between Dye and Quencher in Solution and Hydrogel

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In an ongoing program to synthesize a glucose sensing polymer that could be used for real time glucose monitoring *in vivo*, we have been exploring the use of boronic acid functional viologens as glucose responsive quenchers for fluorescent dyes. The present study focuses on the effect of ionic interactions between pyranine or its various sulfonamide derivatives and the viologen quenchers. Dyes bearing anionic groups were quenched more efficiently when compared to dyes with nonionic substituents. The anionic dyes in conjunction with the cationic quenchers exhibited a broader range of glucose response both in solution and when immobilized in a hydrogel. The interaction of glucose with the sensing components was similar whether they are soluble or immobilized.

KEY WORDS: Continuous glucose monitoring; HPTS; viologen; boronic acid.

INTRODUCTION

Glucose is a biological component of particular interest primarily due to the need for diabetic patients to tightly monitor and control their blood glucose levels. In recent studies, it has been shown that the current self monitored blood glucose (SMBG) tests used to monitor blood glucose levels do not give a completely accurate picture of blood glucose profiles often not detecting the highs and lows during the day [1]. The inherent shortcoming in present methods is that they only provide readings intermittently. Preferably, in order to get a complete picture of glucose profile, one needs a continuous monitoring technique. To address this shortcoming the glucose oxidase technique has been incorporated into devices for continuous glucose monitoring (CGM) in blood [1]. While this approach has proved somewhat effective for short term use in CGM, there are several problems associated with this approach. Most notably the devices still need to be calibrated with an SMBG test.

Consequently, there is a growing interest in synthetic chemosensors containing artificial glucose receptors and fluorescence signal transducers. It is known that aryl boronic acids react reversibly with saccharides to form boronate esters [2]. When attached to a fluorophore, boronic acids can function as receptors in saccharide sensing systems. Russell first described the use of this approach in a 1992 patent claiming a boronic acid functional azo dye as a glucose sensor [3]. Shortly thereafter, Yoon and Czarnik reported the first scientific study of a glucose responsive dye, boronic acid-appended anthracene, and showed that fluorescence intensity could be correlated to glucose concentration [4]. Since then there have been numerous reports of boronic acid containing molecules capable of detecting glucose by optical fluorescence methods [5–16], notably by Shinkai and coworkers [17–22]. Of particular note is the work done using aryl boronic acids appended to fluorescent dyes through tertiary amine [17] and pyridinium [8] coupling groups. These systems were

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designed for solution measurements and are not readily adaptable to CGM devices.

Our own work in this area involves a different approach in which the saccharide receptor is attached to a quencher and the fluorescent dye is an independent moiety [23–25]. Specifically, the quenchers which we are investigating are arylboronic acid functionalized viologens, such as 4,4′-*N*,*N*′-bis-(benzyl-2-boronic acid)-dipyridinium dibromide (*o*-BBV). Viologens, particularly 4,4′-bipyridinium salts such as methylviologen, have been shown to be excellent quenchers for many fluorescent compounds. For example, methylviologen has been shown to quench the fluorescence of pyranine, trisodium 8-hydroxypyrene-1,3,6-trisulfonate (HPTS, **5**, Scheme 2) [26] and other fluorescent dyes [27]. Methylviologen also is reported to quench water-soluble fluorescent polymers [28,29]. Benzylviologen and methylviologen are known to quench the fluorescence of various neutral aromatic fluorophores [30].

Our initial studies showed that quenching is largely due to ground state complex formation between positively charged viologens and negatively charged HPTS [23]. This is also true for viologens other then 4,4′-bipyridinium, specifically the 4,7-phenanthroliniums [24]. In the present study we report how the number of ionic groups on dye and quencher affect complex formation; and the impact this has on quenching and glucose sensing. This effect is studied with both soluble molecules in solution and as functional groups in a hydrogel polymer.

EXPERIMENTAL

General

Reagents were purchased from commercial sources and used without further purification unless noted. The water soluble Methoxy-PEG-NH_2 (MW = 5630) was obtained from Shearwater Polymers. Polyethylene glycol dimethacrylate (PEGDMA), 2-hydroxyethyl methacrylate (HEMA), and *N*-(3-aminopropyl)methacrylamide hydrochloride salt were obtained from Polysciences Inc. BOC protected lysine was purchased from Nova Biochem. Solvents were typically dried (methanol over sodium methoxide, THF over sodium/benzophenone, DMF over calcium hydride) and freshly distilled. All reactions were carried out under an inert atmosphere of argon using air sensitive techniques. Water was purified with a Nanopure Ultrafiltration System. Phosphate Buffer Solution (PBS) (0.1 ionic strength) was freshly prepared (pH 7.4 KH_2PO_4, Na_2HPO_4). Glucose solutions were also freshly prepared in 0.1 ionic strength pH 7.4 PBS. The dye is sensitive to pH changes and thus pH was kept with in ±0.02 of pH 7.40. HPTS has a pKa = 7.3 while the sulfonamide derivatives pKas are in the range of 6.6.

Instrumentation

^{1}H-NMR spectra were measured on a Varian 500 MHz or Bruker 250 MHz instrument. ^{11}B-NMR was measured on a Bruker 250 (80 MHz). NMR were referenced to internal solvent peak. Mass spec data was collected on a Mariner Biospectrometry workstation. pH measurements were carried out on a Bruker 989 pH meter; pH of all solutions was 7.40 ± 0.02 units. Absorbance measurements were carried out with a Hewlett Packard 8452A Diode Array Spectrophotometer. Fluorescence emission spectra were taken on a Perkin-Elmer LS50B luminescence spectrometer. Excitation for all fluorescence studies was 470 nm. Emission spectra were collected between 480–630 nm. All studies were carried out under ambient conditions (25°C, in air). For titration experiments the added volume did not exceed 2% of the total volume and absorbance for all fluorescent measurements was below 0.1. Solution and Hydrogel measurements were made after the signal had stabilized for at least 5 min.

Synthesis

*m-Bromomethylphenylboronic Acid (**2**)*

An oven-dried 1000-mL round bottom 3-necked flask, 250-mL addition funnel, and jacketed water-cooled condenser were assembled and cooled under argon (third neck fit with ground glass stopper), fit with a magnetic stir bar and charged with methylene chloride (800 mL), *m*-tolylboronic acid (**1**) (5.00 g, 36.8 mmol), and potassium carbonate (2.5 g). The addition funnel was charged with bromine (7 g, 44 mmol) and methylene chloride (100 mL). Tubing attached to the condenser was connected to a drying tube and then to an aqueous solution of sodium sulfite used to scrub for hydrogen bromide. A full spectrum sun lamp was placed 18 inches from the flask and the lamp output focused on the flask by using aluminum foil to make a cone. After several minutes this heat was sufficient to provide reflux. Upon reflux, bromine was added drop wise to the reaction mixture from the addition funnel over 1 hr. The reaction was allowed to continue under irradiation for an additional 1.5 hr. After cooling, the potassium carbonate was filtered from the reaction mixture. The supernatant was reduced under vacuum to a yellowish-brown solid and triturated with diethyl ether. The product (**2**) was filtered and washed. The white powder was dried under reduced

pressure (0.6 torr, 2 hr) and used without further purification. Yield: 5.62 g, 26.2 mmol (71%). ^{1}H-NMR (500 MHz, CD_3OD, ppm): 4.81 (s, 2.00 H), 7.362 (t, $^3J = 7.5$ Hz, $^3J = 6.5$ Hz, 1.12 H), 7.481 (dt, $^3J = 7.5$ Hz, $^4J = 1.4$ Hz, 1.08 H), 7.630 (d, $^3J = 6.5$ Hz, 1.00 H), 7.738 (s, 0.94 H). ^{11}B-NMR (60 MHz, CD_3OD): 29.8 (s).

*4,4′-N,N′-Bis-(benzyl-3-boronic acid)-dipyridinium Dibromide (m-BBV) (**4**)*

An oven-dried, 50-mL centrifuge tube was cooled under argon, fitted with a magnetic stir bar, and charged with 4,4′-dipyridyl (**3**) (0.469 g, 3 mmols), freshly prepared **2** (1.82 g, 7.5 mmols), and CH_3OH (7 mL). The tube was sealed with a rubber septum, and the homogenous solution was stirred at room temperature for 15 hr. The reaction vessel was centrifuged and the supernatant was cannulated to a separate flask. The remaining yellow solid was triturated with acetone:water (24:1, V/V, 25 mL), stirred vigorously on a vortex mixer, and centrifuged. The acetone solution was removed by cannula and the trituration process repeated two more times. The solid was then triturated with diethyl ether using the same process. The pale yellow solid remaining in the centrifuge tube, was dried under reduced pressure (0.6 torr, 2 hr) and used without further purification. Yield: 0.956 g, 1.63 mmols (54%). MP: decomposition > 230°C. ^{1}H-NMR (D_2O, ppm): 6.093 (s, 4H), 7.715, (dd, 2H, $J_1 = 7.5$ Hz, $J_2 = 7.5$ Hz), 7.788 (d, 1H, $J = 7.5$ Hz), 7.984 (s, 1H), 8.002 (d, 1H, $J = 7.5$ Hz), 8.662 (d, 4H, $J = 7$ Hz), 9.293 (d, 4H, $J = 7$ Hz). ^{11}B-NMR (MeOH, ppm): 29 (s).

*Trisodium-8-acetoxypyrene-1,3,6-trisulfonate (**6**)*

A 100 mL round-bottom flask was charged with 8-hydroxypyrene-1,3,6-trisulfonate trisodium salt (HPTS, **5**) (5.25 g, 10 mmol), sodium acetate (88 mg, 1 mmol), and acetic anhydride (∼55 mL). The mixture was refluxed for 48 hr, cooled, diluted with 10% acetic acid in THF, and filtered. The filter cake was washed with acetone (3×50 mL) and diethyl ether (2×30 mL), and warmed to 25°C under nitrogen. The pale brown solid was crushed, dried under reduced pressure (0.6 torr, 2 hr), and used without further purification. Yield: 5.069 g, 8.95 mmol (89%). ^{1}H-NMR (D_2O, ppm): 2.674 (s, 3H), 8.234 (d, 1H, $J = 9.6$ Hz), 8.464 (s, 1H), 9.091 (d, 1H, $J = 9.6$ Hz), 9.171 (d, 1H, $J = 9.8$ Hz), 9.235 (d, 1H, $J = 9.8$ Hz), 9.391 (s, 1H). FTIR (KBr pellet, cm^{-1}): 824, 1008, 1048, 1065, 1202 (br), 1522, 1498, 1638, 1752, 3452 (br). m.p.: >400°C.

*8-Acetoxypyrene-1,3,6-trisulfonyl Chloride (**7**)*

Pyranine derivative **6** (11.33 g, 20 mmol) was suspended in 30 mL of thionyl chloride to which 5 drops of dimethylformamide was added. The suspension was refluxed for 3 hr, during which time it became a brown solution. The solution was then cooled to 25°C under an argon atmosphere. Thionyl chloride was distilled off under vacuum leaving a yellow residue. The yellow residue was transferred to three separate centrifuge tubes along with 60 mL of dichloromethane. The suspensions were centrifuged and the supernatant solutions transferred to a dry round bottom flask. The residue remaining in the centrifuge tubes was washed an additional four times each with 10 mL portions of dichloromethane. The supernatent solutions were combined, added to 250 mL of pentane causing precipitation of a large amount of yellow solid. The supernatant was removed by a double ended needle. The yellow solid was dried under reduced pressure and used without further purification. Yield: 8.62 g, 15.5 mmol (78%). ^{1}H-NMR (500 MHz, $CDCl_3$, ppm): 2.682 (s, 3H), 8.833, (d, $J = 10$ Hz, 1H), 8.915 (s, 1H), 9.458 (d, $J = 10$ Hz, 1H), 9.509 (d, $J = 10$ Hz, 1H), 9.630 (s, 1H), 9.685 (d, $J = 10$ Hz, 1H).

*N,N′,N″-Tris-(1-amidoethyl-2-polyethylene Glycol-methoxy 8-acetoxypyrene-1,3,6-tris-sulfonamide (**8b**)*

A 250 mL round bottom flask equipped with a magnetic stir bar was charged with 170 mL of dry THF. Methoxy-PEG-amine (5.65 g, 5630 g/mol, 1 mmol) was added to the flask along with 0.5 g of granular CaH_2. The mixture was heated to 30°C for 24 hr with stirring. Diisopropylethylamine (0.6 mL, 3.4 mmol) was added to the flask and the mixture allowed to stir for an additional hour. The flask was cooled to room temperature and filtered through an air sensitive glass fritted filtration apparatus to remove excess CaH_2 and $Ca(OH)_2$. The THF solution was placed back into a 250 mL round bottom flask with magnetic stir bar and heated to 30°C with stirring. Pyranine derivative **7** (0.185 g, 0.3 mmol) was added to the warm THF solution. The solution immediately turned a deep blue color and faded to a red wine color over 15 min. The reaction was stirred at 30°C for 24 hr. The solvent was removed by rotary evaporation and the residue dissolved in 100 mL of 1 M HCl. The aqueous solution was extracted with methylene chloride (3×100 mL). The methylene chloride fractions were combined and the solvent removed by rotary evaporation to give a red solid, which was used without further purification. Yield: 5.5 g (97%). FTIR (KBr pellet, cm^{-1}): 842, 963, 1060, 1114, 1150, 1242, 1280, 1343, 1360, 1468, 1732, 2525, 2665, 2891.

*N,N′,N″-Tris-(1-amidoethyl-2-polyethylene Glycol-methoxy)-8-hydroxypyrene-1,3,6-tris-sulfonamide (**8a**)*

Compound **8b** (∼5.5 g) was dissolved in 100 mL of 1 M NaOH and stirred for 2 hr. The aqueous solution was neutralized to pH 7 and extracted with methylene chloride (3 × 100 mL). The methylene chloride fractions were combined and reduced to approximately 10 mL by rotary evaporation. The concentrated methylene chloride solution was added dropwise into 400 mL of vigorously stirred diethyl ether in an Erlenmeyer flask. The diethyl ether was filtered using a Buchner funnel. The product was isolated as an orange powder, which was used without further purification. Yield: 5.425 g, 0.31 mmol (94%). FTIR (KBr pellet, cm^{-1}): 842, 963, 1060, 1110, 1150, 1242, 1281, 1343, 1360, 1468, 2888. $\lambda_{max} = 535$ nm (pH 7.4 phosphate buffer).

*N,N′,N″-Tris-(1-amidopropyl-3-methacrylamide)-8-hydroxypyrene-1,3,6-tris-sulfonamide (**9a**)*

A 100 mL round bottom flask was charged with aminopropyl-3-methacrylamide HCl (2.68 g, 15 mmol) and 50 mL of acetonitrile to give a white suspension. Water was added dropwise while stirring untill all of the white suspension dissappeared giving two layers. Potassium carbonate was added and the suspension stirred for 15 min. The supernatent was transferred to a 500 mL round bottom flask and the potassium carbonate washed with 50 mL acetonitrile, which was then combined in the 500 mL round bottom flask.

A yellow solution of **7** (1.03 g, 1.8 mmol), 200 mL acetonitrile, and 20 mL dichloromethane was added under argon to the 500 mL round bottom flask containing the free amine in acetonitrile causing the solution to become dark red as a precipitate formed. The solution was stirred for 1 hr and the supernatant transferred and concentrated under vacuum to give a dark residue. The residue was extracted with water (1000 mL) and a 50:50 acetonitrile/ethylacetate solution (700 mL). The organic extract was washed with an additional 1000 mL water. The organic extract was dried over magnesium sulfate and concentrated on a rotary evaporator to give a red residue, which was dissolved in methanol. The methanol solution was concentrated and the resulting red residue was dried under reduced pressure to give a red solid, which was used without further purification. Yield: 420 mg, 0.5 mmol (28%). ^{1}H-NMR (500 MHz, CD_3OD, ppm): 1.617 (p, $J = 6.5$ Hz, 8H), 1.781 (s, 3H), 1.767 (s, 6H), 2.934 (p, $J = 6.5$ Hz, 9H), 3.158 (mult. 8H), 5.211 (t, $J = 1.5$ Hz), 5.229 (t, $J = 1.5$ Hz), 5.488 (s, 1H), 5.510 (s, 2H), 8.290 (s, 1H), 8.837 (d, $J = 9.5$ Hz, 1H), 8.913 (d, $J = 9.5$ Hz, 1H), 8.988 (d, $J = 1.5$ Hz, 1H), 9.201 (d, $J = 9.5$ Hz, 1H), 9.222 (s, 1H).

*N,N′,N″-Tris-(1-amidopropyl-3-methacrylamide)-8-acetoxypyrene-1,3,6-tris-sulfonamide (**9b**)*

Unprotected **9a** (100 mg, 0.1 mmol) was then suspended in 10 mL acetic anhydride and a catalytic amount of sodium acetate was added and the suspension refluxed for 2 hr. Acetic anhydride and acetic acid were removed under vacuum and the resulting brown residue was extracted with 20 mL acetonitrile. The extract was added dropwise to 150 mL diethyl ether causing the precipitation of a brown solid, which was used without further purification. Yield: 75 mg, 0.09 mmol, 86%. ^{1}H-NMR (500 MHz, CD_3OD, ppm): 1.617 (p, $J = 6.5$ Hz, 8H), 1.781 (s, 3H), 1.767 (s, 6H), 2.62 (s, 3H), 2.934 (p, $J = 6.5$ Hz, 9H), 3.158 (mult. 8H), 5.211 (t, $J = 1.5$ Hz), 5.229 (t, $J = 1.5$ Hz), 5.488 (s, 1H), 5.510 (s, 2H), 8.290 (s, 1H), 8.837 (d, $J = 9.5$ Hz, 1H), 8.913 (d, $J = 9.5$ Hz, 1H), 8.988 (d, $J = 1.5$ Hz, 1H), 9.201 (d, $J = 9.5$ Hz, 1H), 9.222 (s, 1H).

*Preparation of Hydrogel (**14**) Containing **12** and **9a***

A 10-mL volumetric flask was charged with 2-hydroxy ethyl methacrylate (3.525 g, 27.1 mmols), **12** (0.012 g, 0.0122 mmols), polyethylene glycol dimethacrylate (MW = 1000, 1.110 g, 1.11 mmols), 2,2′-azobis [2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044, 0.024 g, 0.0770 mmols) and **9b** (3 mL of 5×10^{-4} M solution in MeOH, 1.5×10^{-3} mmols). It was filled to the 10-mL mark with water. After the solution was vigorously stirred on a vortex mixer it was transferred, *via* pipette, to a 25-mL, cone-shaped round bottom flask and the flask was sealed with a rubber septum; it was deoxygenated with argon for 10 min. The monomeric solution was taken-up by syringe and the needle was capped with a rubber stopper. It was then transferred to an argon-filled glove box along with the polymerization chamber. The chamber was assembled by clamping two glass plates together with a Teflon spacer (0.002″) between them. Syringe ports were attached to one face of the mold to allow injection of the monomer mixture. A syringe was attached to the polymerization chamber and the solution was inserted into the cell, under argon, to fill the entire cavity. The chamber was sealed with two stainless steel plugs and the entire assembly was wrapped in a Ziploc® freezer bag. The bag was placed in an oven and heated at 40°C for 15 hr. The polymerization chamber was removed from the oven and the bag, and subsequently disassembled to afford a thin orange/green polymeric film. The polymeric film was

leached in a pH 8 NaOH solution for 12 hr and stored in pH 7.4 phosphate buffer.

*N,N′,N″-Tris-(1-amidobutryic acid)-8-hydroxypyrene-1,3,6-tris-sulfonamide (**10**)*

A round bottom flask was charged with 4-aminobutyric acid (5.156 g, 50 mmols). Methanol (50 mL) was added followed by sodium hydroxide (2 g, 50 mmols). The solution was stirred until it became homogeneous, at which point methanol was removed on a rotary evaporator. The tan solid was further dried of water by coevaporations with acetonitrile to give aminosodiumbutyrate. The crude material was used without further purification.

An oven dried round bottom flask was cooled under argon, fit with a magnetic stir bar, charged with **7** (460 mg, 0.83 mmols), and sealed with a septum. DMSO (20 mL) was added to give a homogenous yellow solution. A second oven dried round bottom flask was cooled under argon, fit with a magnetic stir bar, charged with the 4-aminosodiumbutyrate (415 mg, 3.32 mmols), and sealed with a septum. DMSO (20 mL) was added via double ended needle, and after a few minutes of stirring, the first solution containing **7** in DMSO was cannulated in drop wise to give a deep red homogeneous solution. After 6 hr approximately one third of the solution was removed, and DMSO was distilled off under vacuum. The resulting brown material was washed with a small amount of acetonitrile, which was filtered through cotton and dripped into Et_2O to precipitate a small amount of brown/red hygroscopic solid, which was used without further purification. ^{1}H-NMR (250 MHz, D_2O, ppm): 2 (p, 6H), 2.4 (t, 6H), 2.61 (s, 3H), 3 (t, 6H), 8.2 (d, 1H), 8.4 (s, 1H), 8.6 (d, 1H), 9.1 (d, 1H), 9.2 (d, 1H), 9.4 (s, 1H).

*Preparation of Hydrogel (**15**) Containing **12** and **13***

A 5-mL volumetric flask was charged with 2-hydroxy ethyl methacrylate (1.763 g, 13.55 mmols), **12** [31] (0.086 g, 0.081 mmols), polyethylene glycol dimethacrylate (MW = 1000, 0.555 g, 0.555 mmols), 2,2′-azobis [2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044, 0.012 g, 0.0385 mmols) and **13** (1 mL of 1.6×10^{-3} M solution in 1:1 MeOH:H_2O, 1.6×10^{-3} mmols). The volumetric flask was filled to the 5 mL mark with water, and the procedure for the preparation of hydrogel **14** was followed.

Solution Studies

Quenching Studies

Measurements were done *in situ* by taking the absorption and emission spectra of the dye (HPTS or a trisulfonamide derivative) at a series of quencher concentrations. The absorption and emission spectra for the dye (2 mL of 4×10^{-6} M in pH 7.4 PBS) was first obtained. Quencher (**4**, 5×10^{-3} M in pH 7.4 PBS; or **12**, 1×10^{-2} M in pH 7.4 PBS) was added (0.8–10 μL aliquots), the solution shaken for 60 s, and the absorption and emission measured. Emission was taken as the area under the curve from 480–630 nm for all studies. Stern-Volmer quenching constants were calculated using the sphere of action model, Eq. (1), where K_s is the static quenching, V is the dynamic quenching, and Q is the concentration of the quencher (**4** or **12**); or a modified Stern-Volmer equation, Eq. (2)

$$\frac{F_0}{F} = (1 + K_S[Q])e^{V[Q]} \tag{1}$$

where f is the percentage of fluorophore accessible to the quencher, K_s is the quenching constant, and Q is the concentration of quencher (**4** or **12**) [32]. Data was analyzed using Solver (non-linear least squares curve fitting) in Microsoft Excel, and regression statistics were determined using the SolvStat macro [33].

$$\frac{F_0}{\Delta F} = \frac{1}{fK_S[Q]} + \frac{1}{f} \tag{2}$$

Glucose Response Studies

Fluorescence measurements were done *in situ* by taking the absorption and emission spectra of dye/quencher solutions at a series of glucose concentrations. The absorption and emission of the dye (2 mL of 4×10^{-6} M in pH 7.4 PBS) were obtained. Quencher (**4** (35.2 μL 5×10^{-3} M) in pH 7.4 PBS; or **12** (0.8 μL 1×10^{-2} M) in pH 7.4 PBS) was added and the absorption and emission spectra were taken. Glucose was then added (2–10 μL aliquots of 1 M in pH 7.4 PBS), the solution was shaken for 60 s, and the absorption and emission measured. Emission was taken as the area under the curve from 480–630 nm for all studies. Glucose response was analyzed using Eq. (3) [24]. Data was analyzed using Solver (non-linear least squares curve fitting) in Microsoft Excel, and regression statistics were determined using the SolvStat macro [33].

$$\frac{F}{F_0} = \frac{F_{min} + F_{max}(K[Glu])}{1 + K[Glu]} \tag{3}$$

Hydrogel Studies

The bottom of a 10-mm path length, 3-mL quartz cuvet was cut and removed giving a cuvet with an open top and bottom. The openings were sealed with two disposable

polyethylene caps. Using a hot metal rod a 3.9 mm diameter hole was melted into the center of the caps. While the plastic was still malleable the threads of a 10–32 standard thread, 1/8″ I.D. hose end adapter were screwed into the plastic. The plastic was allowed to cool and the adapter was unscrewed. Teflon thread tape was applied to the threads. The adapter was then refit into the cuvet. A thin sheet of opaque plastic was then cut into a 35 × 9 mm rectangle and a window 6 × 15 mm was cut out of the center. Two pressure fittings were constructed from rubber septa. They were used to put pressure on the plastic mask to hold the polymer in place within the cuvet.

The flow-through-cell was assembled as follows. The caps were removed and the polymer film was placed in the center of the cuvet. The opaque plastic mask was placed directly over it, effectively framing the film with its window. The pressure fittings were then put in place using tweezers, one at the bottom of the cell and one at the top, oriented with the broad base against the plastic mask. The caps were lubricated with standard vacuum grease and inserted into the cuvet to seal the cell.

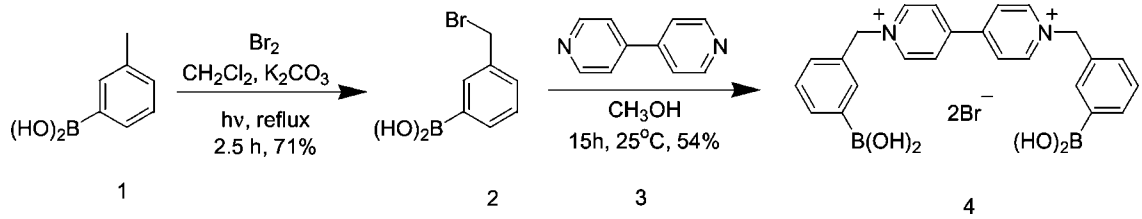
The cell was placed into a Perkin-Elmer LS50B spectrophotometer equipped with a front surface adapter. The cell was oriented so that its side, touching the polymer, was facing the excitation beam of the instrument (face first in the front surface adapter) and was angled at 30° relative to the incident beam. 1/8″ Tygon PTFE tubing was connected to the hose adapters of the flow-through-cell. The orientation of the front surface adapter was optimized so that the emission detector was sensing only the surface of the polymer. A peristaltic pump was used to circulate pH 7.4 phosphate buffer (ionic strength 0.1) through the cell at a rate of 30 mL per min. The time drive application of the Perkin-Elmer LS50B software was used for hydrogel **14** and the ratio application was used for hydrogel **15**. For the time drive application, the pulse function was set to pulse every 2 s and the integration was over 10 s for a total of 99,999 s (program time limit). For the ratio application, which continuously irradiates the sample, fluorescence intensity readings were acquired every 1 s for 99,999 s total. The excitation wavelength was set at 470 nm and the emission wavelength was set at 540 nm. The 515 nm cutoff filter was used to limit stray light into the detector. The excitation slit width was set at 15 nm and the emission slit width was adjusted so that the signal was around 500 intensity units (out of 1000 total). A baseline value of ∼500 (fluorescence intensity) was established with buffer solution. The buffer was then replaced with sugar solution and the fluorescence intensity was monitored over time.

RESULTS AND DISCUSSION

Our initial studies with viologen boronic acid quenchers employed ortho benzyl boronic acid derivatives because they had previously been shown to be good glucose receptors [23]. In the present study we introduce two new viologens, a water soluble derivative, 4,4′-*N*,*N*′-bis-(benzyl-3-boronic acid)-dipyridinium dibromide, *m*-BBV (**4**) and a polymerizable derivative, **12**. The synthesis and evaluation of compound **12** was reported earlier [31]. Compound **4** was synthesized as described in Scheme 1.

The ability of compound **4** to quench HPTS (**5**) was then evaluated (Fig. 1a), and glucose response studies were carried out with this quencher/dye combination (Fig. 1b). The results showed **4** is equally as effective as *o*-BBV in quenching HPTS and giving glucose response. The Stern-Volmer quenching constants were determined to be $K_s = 8749\ M^{-1}$ and $V = 1549\ M^{-1}$ (for *o*-BBV $K_s = 16{,}000\ M^{-1}$ and $V = 21{,}000\ M^{-1}$); the binding constant was $K = 50$ (for *o*-BBV $K = 43\ M^{-1}$).

Pyranine is a well-known dye extensively studied for sensor applications [34–39], especially by Wolfbeis and collaborators [40–43]. The interest in using HPTS in sensors is warranted by its desirable combination of properties. Pyranine is highly stable, water soluble, resistant to photobleaching, has a long-wave excitation maximum, and a large Stokes shift [40]. It is susceptible to quenching, and the electron transfer process between



Scheme 1. Synthesis of 4,4′-*N*,*N*′-bis-(benzyl-3-boronic acid)-dipyri-dinium dibromide (*m*-BBV) (**4**).

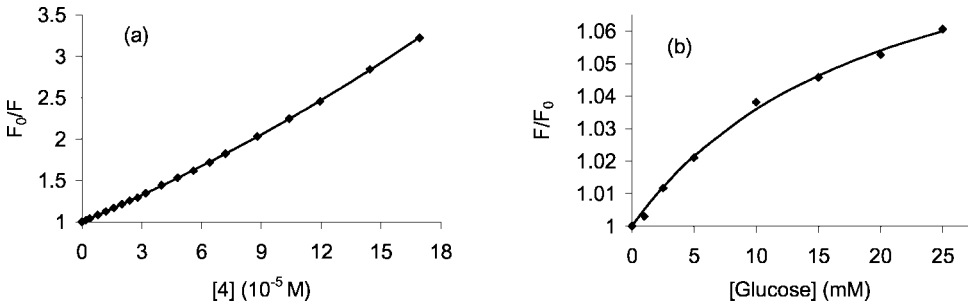
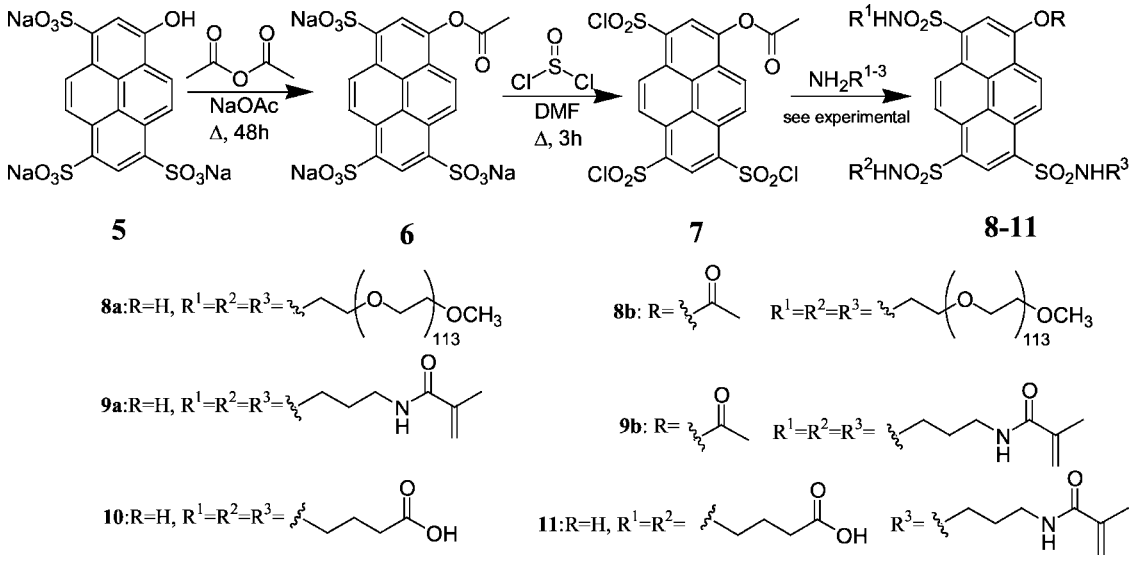


Fig. 1. (a) Stern-Volmer Plot of **5** (4×10^{-6} M) showing the quenching efficiency of **4** Eq. (1), $\lambda_{ex} = 470$ nm and $\lambda_{em} = 510$ nm; (b) Relative fluorescence intensity of **5** (4×10^{-6} M) with **4** (9×10^{-5} M) and increasing [glucose] Eq. (3).

HPTS and methylviologen has been studied in detail [44]. For these reasons we chose to work with HPTS as well as a number of its sulfonamide derivatives including water soluble model compounds, monomers, and polymers (Scheme 2).

The various water soluble dyes can be used in solution studies, but to be useful in a sensing device, the dye/quencher combination must be immobilized in a polymer or confined in a membrane in order to be isolated from the body. Pyranine (**5**) lacks polymerizable groups and has a relatively small size making it impractical to be confined by a membrane. Additionally, the λ_{max} (435 nm) and the pK_a (7.3) for **5** [40] make it unsuitable for use under physiological conditions. It has been shown that conversion of the sulfonic acid groups in **5** to sulfonamides results in both a decrease in the pK_a and a shift to a greater excitation λ_{max} (465 nm) [41]. This reduces signal fluctuations caused by slight pH changes. The red shift in absorbance makes the dye excitable by a visible light source, such as a commercially available blue LED.

Though HPTS is commonly used in aqueous solution, it has also been immobilized by a variety of techniques. For example, it has been bonded to a water insoluble polyamine by direct reaction of the latter with a sulfonyl chloride intermediate [42]. Pyranine also forms water insoluble complexes with cationic polyelectrolytes [38]. Finally, it can be rendered water insoluble by amidation with a hydrophobic amine [43] or by forming a water insoluble salt with a hydrophobic quaternary ammonium counterion [39].



Scheme 2. HPTS trisulfonamide synthesis.

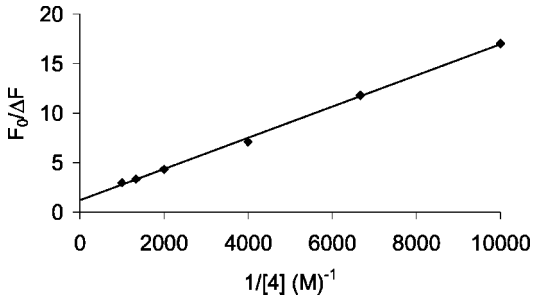
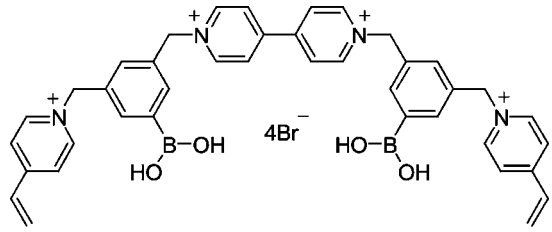


Fig. 2. Stern-Volmer Plot of **8** (1×10^{-5} M) showing the quenching efficiency of **4** Eq. (2), $\lambda_{ex} = 470$ nm and $\lambda_{em} = 540$ nm.

Because we needed to incorporate both dye and quencher in specific ratios as well as insure complete conversion to a hydrophilic trisulfonamide that would be resistant to leaching, none of these procedures was deemed suitable for our purposes. We chose instead to make a soluble PEG derivative **8** that could be immobilized in a semi-interpenetrating polymer network (semi-IPN) or a trisulfonamide monomer **9** that could be copolymerized in a hydrogel. Compound **8** is a large water-soluble dye molecule that can be trapped into an IPN or used as a soluble dye derivative in solution studies. HPTS derivative **8** in conjunction with quencher **4** in solution showed a decrease in the quenching ability ($K_s = 750$ M^{-1}) when compared to HPTS ($K_s = 8749$ M^{-1}) (Figs. 1 and 2). The IPN hydrogel also gave a weak glucose signal [25]. Further work on this approach was abandoned when we observed that in some cases **8** appeared to be leaching out of IPN hydrogels.

We then shifted our attention to immobilization of the sensing components through covalent bonds in a hydrogel matrix. This required the preparation of appropriate monomers, then combining these monomers with PEGDMA and HEMA to form a hydrogel. HPTS derivative **9** (Scheme 2) was selected as the polymerizable dye derivative. The replacement of a boronic acid group in **4** with a polymerizable group resulted in a much weaker glucose signal suggesting that it was essential to have two boronic acid groups along with a polymerizable group. This led to the synthesis of the tetracationic viologen **12** [31].



12

Stern-Volmer quenching studies and glucose response studies were carried out with viologen salt **12** and HPTS (**5**) in solution (Fig. 3). The Stern Volmer quenching constants were determined to be $K_s = 130{,}000$ M^{-1} and $V = 31{,}000$ M^{-1}, and the binding constant was $K = 157$ M^{-1}. A thin film hydrogel (**14**) containing **12** and **9** was formed and studied under dynamic conditions. In this experiment pH 7.4 PBS solution was circulated through a cell in which the hydrogel was mounted. A high flow rate was utilized to insure that there were no boundary effects. The emission of the hydrogel was monitored at 540 nm. The solution was switched to pH 7.4 PBS containing increasing amounts of glucose. This resulted in an increase in fluorescence emission with increasing glucose concentration (Fig. 4). The circulating solution was then switched to pH 7.4 PBS containing decreasing amounts of glucose, and the fluorescence emission decreased accordingly, showing reversibility of the system under dynamic conditions.

The results with both solution measurements and with the hydrogel **14** demonstrated the importance of ionic

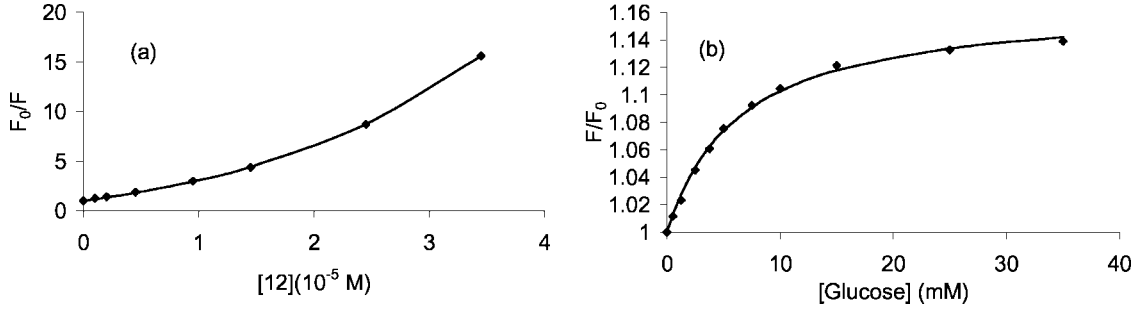


Fig. 3. (a) Stern-Volmer Plot of **5** (4×10^{-6} M) showing the quenching efficiency of **12** Eq. (1), $\lambda_{ex} = 470$ nm and $\lambda_{em} = 510$ nm; (b) Relative fluorescence intensity of **5** (4×10^{-6} M) with **12** (4×10^{-5} M) and increasing [glucose] Eq. (3).

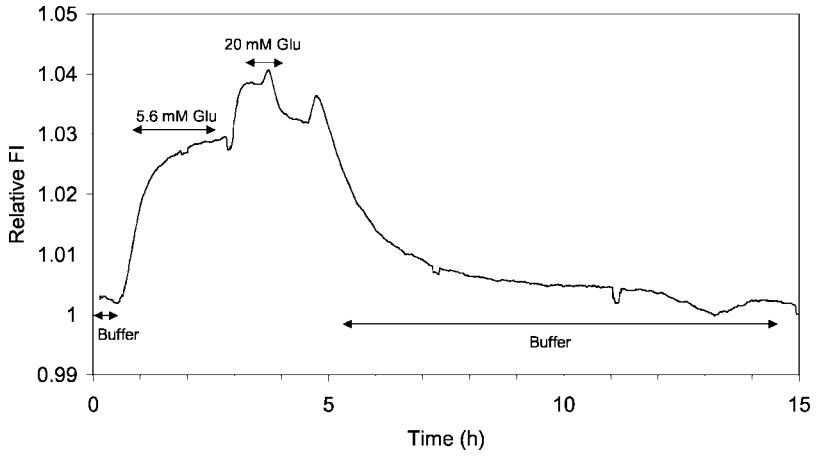
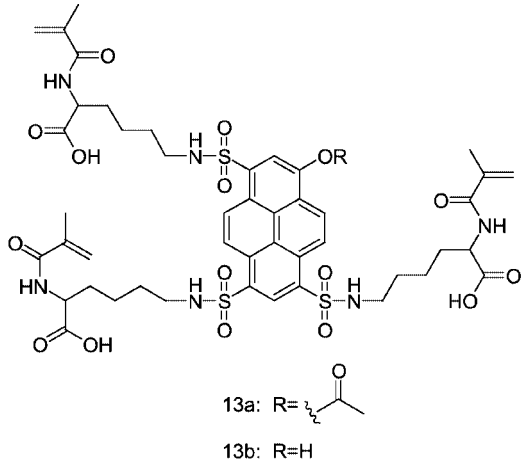


Fig. 4. Continuous glucose sensing profile of **14**, a 0.002″ hydrogel, at 22°C. $\lambda_{ex} = 470$ nm and $\lambda_{em} = 540$ nm.

interactions, and led us to reevaluate the experiments carried out with **4** and **8**. It appears that the reduced quenching of **8** was a consequence of the lack of anionic groups on this dye. To test this hypothesis a water-soluble model compound **10** (Scheme 2) was synthesized, because measurements in aqueous solution could not be carried out with water insoluble **9**. Derivative **10** is a trisulfonamide like **9**, but in its neutralized form, carries three negative charges like **5**. Quenching and glucose response studies were run with viologen **4** and dye **10**. The results in Fig. 5 show that with the anionic HPTS derivative **10** the quenching is more effective compared to **8**; in fact it is comparable to **5** itself (Fig. 5a). In addition, there is a broad range for glucose response, with a fairly linear response over the biologically significant range (Fig. 5b). The Stern Volmer quenching constants were determined to be $K_s = 12{,}500\ M^{-1}$ and $V = 660\ M^{-1}$, and the binding constant was $K = 7\ M^{-1}$.

This exciting result prompted us to synthesize a polymerizable analog. We first attempted to substitute HPTS with two acid groups and one double bond, but the product (**11**) was a complex mixture. When incorporated into a hydrogel polymer, part of the dye was found to leach out over time so this approach was not pursued further.

Instead we chose to synthesize an HPTS derivative that was similar to **9**, but also carries three carboxylic acid groups. This derivative **13** was successfully prepared in five steps [31]. The monomer **13b** was initially characterized by fluorescence measurements in solution using both **4** and **12** (Figs. 6 and 7 respectively). Glucose response was similar to that observed for **5** and **10** suggesting that the hypothesis concerning the negative charges on the dye was plausible. The Stern Volmer quenching constants for **4** with **13** were determined to be $K_s = 3880\ M^{-1}$ and $V = 0\ M^{-1}$, and the binding constant was $K = 51\ M^{-1}$. The Stern Volmer quenching constants for **12** with **13** were determined to be $K_s = 12{,}000\ M^{-1}$ and $V = 130\ M^{-1}$, and the binding constant was $K = 275\ M^{-1}$.



Monomers **12** and **13a** were then used to make a thin film hydrogel **15**. In these hydrogel studies we were interested in demonstrating continuous glucose sensing with reversible response, and were not concerned with response times. Response times are largely dependent on hydrogel thickness and water content and studies are continuing on the effects of binding kinetics. We were pleased to see that the enhanced performance seen in the solution studies carried over to the hydrogel studies (Fig. 8).

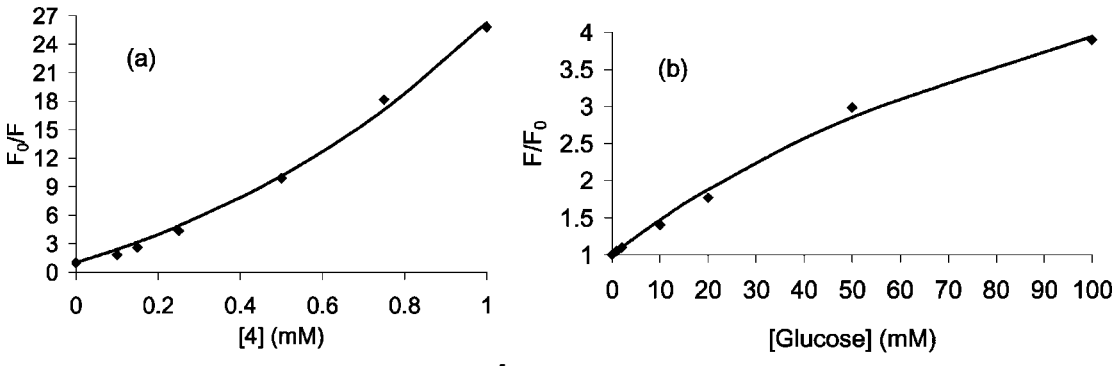


Fig. 5. (a) Stern-Volmer Plot of **10** (1×10^{-5} M) showing the quenching efficiency of **4** Eq. (1), $\lambda_{ex} = 470$ nm and $\lambda_{em} = 515$ nm; (b) Relative fluorescence intensity of **10** (1×10^{-5} M) with **4** (1 mM) and increasing [glucose] Eq. (3).

The sensing polymer made from anionic dye derivative **13** has a higher percent recovery upon addition of glucose (38% for 20 mM) when compared to the sensing polymer made with **9** (4% for 20 mM) using the same quencher monomer **12**. A stepwise increase in glucose concentration led to an increase in fluorescent emission, and a stepwise decrease in glucose concentration led to a decrease in fluorescent signal. This sequence was repeated numerous times, demonstrating the reversibility of this sensing system.

An important question to be addressed by this study was whether the sensing components behave the same, whether in solution or immobilized in a hydrogel. A comparison of the glucose response for the combination **12** and **13** show that this is clearly the case (Fig. 9).

As Fig. 9 shows, the glucose response curves nearly match. This suggests that there is a considerable degree of functional group mobility in the hydrogel, allowing them to behave much the same as the monomers in solution. In fact, the binding constants calculated from these curves suggests that glucose binding is somewhat enhanced by incorporating the monomers into a polymer.

A summary of the quenching and binding constants for solution studies is given in Table I. Assuming that the binding constant is a measure of the reactivity of the boronic acid with glucose, several inferences can be drawn from the data in Table I.

First the number of cationic groups on the quencher affects glucose binding as well as quenching and fluorescence recovery. The more surprising observation is that the dye also affects glucose binding even though dye and receptor are not directly bonded together. What seems likely is that ground state complex formation changes the inherent reactivity of the boronic acid by neutralizing the adjacent positive charges. This is not unreasonable since we hypothesize that binding should be

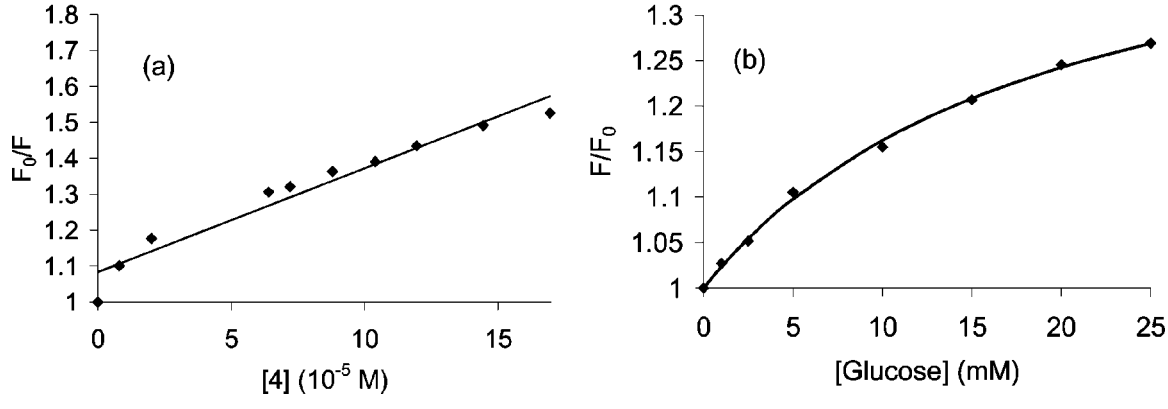


Fig. 6. (a) Stern-Volmer Plot of **13b** (4×10^{-6} M) showing the quenching efficiency of **4** Eq. (1), $\lambda_{ex} = 470$ nm and $\lambda_{em} = 540$ nm; (b) Relative fluorescence intensity of **13b** (4×10^{-6} M) with **4** (9×10^{-5} M) and increasing [glucose] Eq. (3).

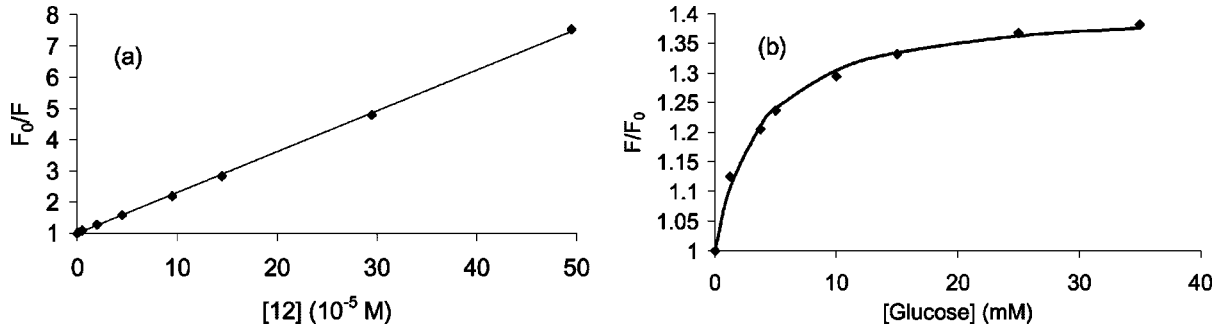


Fig. 7. (a) Stern-Volmer Plot of **13b** (4×10^{-6} M) showing the quenching efficiency of **12** Eq. (1), $\lambda_{ex} = 470$ nm and $\lambda_{em} = 540$ nm; (b) Relative fluorescence intensity of **13b** (4×10^{-6} M) with **12** (4×10^{-5} M) and increasing [glucose] Eq. (3).

enhanced by the presence of the positive charge near the boron.

A comparison of the data in Table I also shows that fluorescence recovery is highly dependent on dye with **5** giving much lower values. This result suggests that the complex between **5** and the quenchers is stronger than that of **13**. At a pH of 7.4, **5** carries four negative charges directly on the pyrene ring core, which would favor strong complex formation with the viologens. In the case of **4**, it is probably a 2:1 complex; for **12**, it is more likely a 1:1 complex. Formation of a negatively charged boronate may not be sufficient to completely dissociate these complexes.

In contrast, **13** has three carboxyl groups that are several nanometers removed from the aromatic core. In addition the carboxyl groups may not be completely ionized. The net result is that the ionic environment around the pyrene ring is more diffuse, resulting in weaker complexes. The fact that substantially higher fluorescence recoveries are seen with **13** supports this contention.

CONCLUSION

We have shown that the number of anionic groups on pyranine derivatives used in our glucose sensing system is an important variable. When combined with cationic bipyridinium salts having boronic acid saccharide receptors, trisulfonamide HPTS derivatives with anionic groups in the side chains had superior fluorescence recovery upon addition of glucose. This is seen both in solution studies and when the sensing components are incorporated into hydrogel polymer films. We believe our system involves a bimolecular dye/quencher complex that is non-fluorescent. When the quencher binds to glucose the complex dissociates resulting in the relative increase in fluorescence. Since the binding of glucose in these systems is reversible, the fluorescence signal is modulated by glucose. We are actively pursuing the optimization of glucose transport and fluorescence recovery over the biologically significant range in the hydrogel matrix.

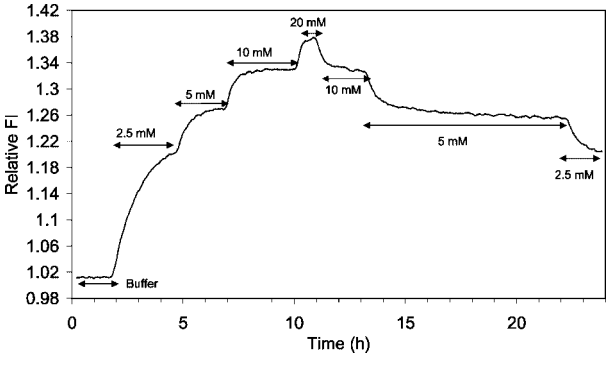


Fig. 8. Continuous glucose sensing profile of **15**, a 0.002″ hydrogel at 22°C. $\lambda_{ex} = 470$ nm and $\lambda_{em} = 540$ nm.

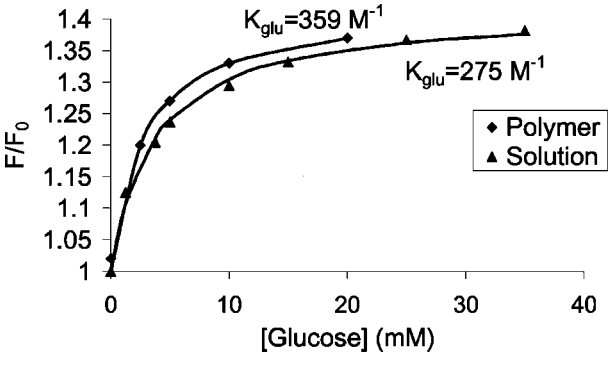


Fig. 9. Comparison of glucose response curves for **12/13** in solution and in a hydrogel polymer **15**.

Table I. Comparison of Charge Interaction Between Dyes and Quenchers

Quencher	Dye	K_s $(M)^{-1}$	V $(M)^{-1}$	K $(M)^{-1}$	% Fluorescence recovery[a]
4	5	8,749	1,549	50	6
4	13	3,880	−0	51	25
12	5	130,000	31,000	157	12
12	13	12,000	130	275	35

[a]Fluorescence recovery for 20 mM glucose.

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