Enhanced aqueous solubility of long wavelength voltage-sensitive dyes by covalent attachment of polyethylene glycol†

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Long wavelength voltage-sensitive dyes (VSDs) called Pittsburgh (PGH) dyes were recently synthesized by coupling various heterocyclic groups to a styryl-thiophene intermediate forming extended, partially rigid chromophores. Unlike most styryl VSDs, dyes with a sulfonic acid anchor directly attached to the chromophore showed no solvatochromic absorption shifts. The limited water solubility of many long wavelength VSDs requires the use of surfactants to transport the dye through physiological saline solutions and effectively label biological membranes. Here, we tested the chemical substitution of the sulfonic acid moiety with polyethyleneglycol (PEG) chains, ranging from MW 750 to 5000, to overcome the poor solubility of VSDs while retaining their properties as VSDs. The chemical synthesis of PGH dyes and their PEG derivatives are described. The PEG derivatives were soluble in aqueous solutions (>1 mM) and still reported membrane potential changes. In frog and mouse hearts, the voltage sensitivity ($\Delta F/F$ per action potential) and spectral properties of PEG dyes were the same as the sulfonated analogues. Thus, the solubility of VSDs can be considerably improved with small polyethyleneglycol chains and can provide an effective approach to improve staining of excitable tissues and optical recordings of membrane potential.

Introduction

Two- and three-dimensional mapping of electrical activity through changes in voltage-sensitive dye (VSD) fluorescence has greatly increased our understanding of the function of nerve networks and of cardiac electrophysiology in normal and pathological states.[1–5] Effective VSDs are amphiphilic molecules that contain a water soluble moiety and a lipophilic region. To measure rapid changes in cellular membrane potential, the sensor portions of these dye molecules associate with the hydrophobic interior of the plasma membrane where the electric field gradient is the highest. At the same time, the dyes carry charged groups to facilitate transfer of the dyes to the target tissues and to anchor the dyes at cell surfaces, minimising diffusion of the VSDs to other membranes within the cell.[6] Effective imaging deep within tissues requires VSDs with high molar extinction coefficients at red wavelengths and near-IR emission with high quantum yields to reduce light scattering and intrinsic tissue absorption and fluorescence.[5,7,8]

Styryl dyes are a class of fluorescent VSDs that are widely used to measure membrane potentials because of their ability to follow voltage changes on a millisecond timescale.[2,5,9,10] Their structures consist of an electron-rich aminophenyl group linked to a quaternized nitrogen heterocycle by a conjugated polymethine chain. Many styryl dyes, including the series of RH and JPW dyes, have been synthesised resulting in a wide range of excitation and emission wavelengths and a variety of voltage responses.[4,5,11,12] VSDs that have high quantum yields and produce a large change in fractional fluorescence ($\Delta F/F$) are desirable because they generate adequate signals at lower dye concentrations. Recently, Salama *et al.* reported a series of long-wavelength VSDs designed to report cardiac action potentials (APs) from deeper layers of the heart by incorporating a thienyl group in the polymethine bridge to maintain the rigidity of the styryl chromophore while extending the fluorescence wavelengths.[3]

Staining of target tissues requires transport of the dye to the plasma membrane and T-tubules in sufficient quantities for voltage measurements. Most styryl VSDs require an additional reagent such as DMSO, Pluronics or cyclodextrins for transport through the aqueous medium to the tissues.[8,13] Labelling conditions are determined for each dye, which can be a time-consuming, trial and error process.[3] Attachment of an uncharged hydrophilic anchoring group to the dye structure may eliminate the need for such agents. Poly(ethylene glycol)s (PEGs) are commonly used to improve the water solubility of small molecule pharmaceuticals and exhibit water solubility, high mobility in solution, lack of toxicity, lack of immunogenicity, and are readily cleared from the body.[14] Covalent linkage of PEGs to voltage dyes will greatly simplify protocols for probe delivery to excitable tissue.[12]

In this report, we present the synthesis and spectral characterization of a set of styryl dyes which fluoresce at near infrared wavelengths. The series, PGH-1 through PGH-8, demonstrates the effect of different heterocycles on the spectral properties of the dyes. Analogues of two VSDs were synthesised and coupled to PEG groups of varying chain lengths (MW 750–5000) to increase their water solubility. The enhanced solubility of PEG derivatives

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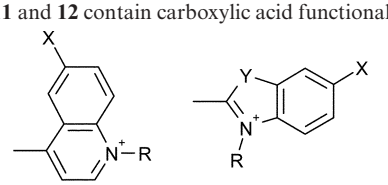
facilitated delivery to target tissues while retaining their spectral properties and voltage sensitivity ($\Delta F/F$ per action potential) in cardiac tissues.

Results

Sulfonated dyes

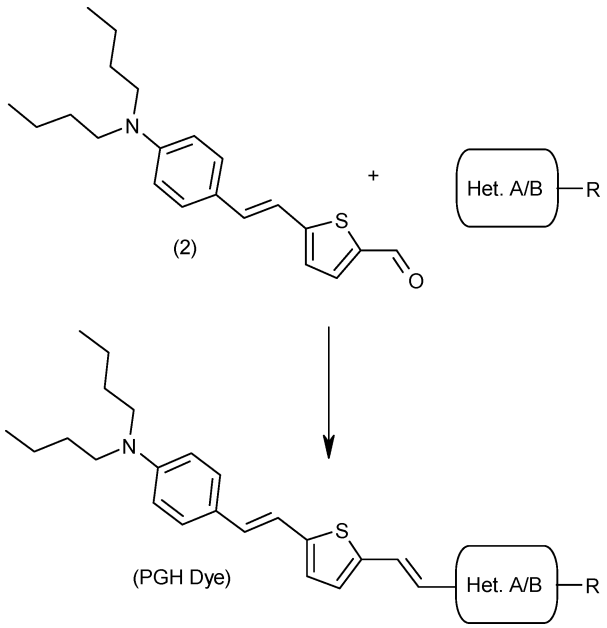
VSDs were synthesised from heterocycles A or B quaternised with sulfonic acid anchors or carboxylic acid groups (Table 1), and a common styrene-thienyl aldehyde intermediate as shown in Scheme 1. The structures of PGH-1 through PGH-10 are listed in Table 2. Briefly, styrene (**1**) was made from dibutylamino-benzaldehyde *via* Wittig reaction and then coupled with bromothiophene-carboxaldehyde *via* a Heck reaction to yield the styryl-thiophene intermediate (**2**).[15] Other syntheses of these intermediates have been reported;[16,17] however, the methods used here require minimal purification of the styrene and fewer steps to prepare the aldehyde. Wuskell *et al.* recently reported the synthesis of VSDs with similar chromophores utilizing quaternary ammonium anchors.[8,18] The sulfonic acid anchors used here in dyes PGH-1 to PGH-8 produced zwitterionic dyes with the positive charge of the chromophore delocalized between the nitrogen atoms. The benzoxazole dye, PGH-5, was produced; however, the dye rapidly decomposed after chromatographic purification upon drying under mild conditions.

Table 1 Structures of heterocyclic intermediates used in dye syntheses. Heterocycles (A) are lepidines (**3**, **10** and **11**); or (B) are indolenines (**4**, **6**, **8** and **12**); benzothiazole (**5**); benzoxazole (**7**); and quinaldine (**9**). Intermediates **11** and **12** contain carboxylic acid functional groups



Het. A Het. B

Intermediate	Het.	X	Y	R
3	A	H	—	$C_4H_8SO_3$-
4	B	H	CMe_2	$C_4H_8SO_3$-
5	B	H	S	$C_4H_8SO_3$-
6	B	SO_3K	CMe_2	$C_4H_8SO_3$-
7	B	H	O	$C_4H_8SO_3$-
8	B	SO_3K	CMe_2	C_2H_5
9	B	OMe	CH=CH	$C_4H_8SO_3$-
10	A	OMe	—	$C_4H_8SO_3$-
11	A	H	—	$C_5H_{10}CO_2H$
12	B	SO_3K	CMe_2	$C_5H_{10}CO_2H$



Scheme 1 Synthesis of voltage sensitive dyes described in this report.

The spectral properties of these new dyes, PGH-1 to PGH-8, were characterized in alcohols of different polarity to verify the solvatochromic sensitivity of the various heterocycles. The absorption maxima and extinction coefficients are shown in Table 3, while the emission maxima and relative fluorescence intensities are given in Table 4. The observed increased Stokes shift with increasing solvent polarity is typical for styryl VSDs.[19] For two of the dyes, PGH-4 and PGH-6, the increased Stokes shift was asymmetrical; the absorption maxima did not change with solvent polarity. These anomalous dyes have a sulfonic acid

Table 2 Key to substitutions used in Scheme 1. Heterocycles A and B are shown in Table 1

Dye	Het.	X	Y	R
PGH-1	A	H	—	$C_4H_8SO_3$-
PGH-2	B	H	CMe_2	$C_4H_8SO_3$-
PGH-3	B	H	S	$C_4H_8SO_3$-
PGH-4	B	SO_3K	CMe_2	$C_4H_8SO_3$-
PGH-5[a]	B	H	O	$C_4H_8SO_3$-
PGH-6	B	SO_3K	CMe_2	C_2H_5
PGH-7	B	OMe	CH=CH	$C_4H_8SO_3$-
PGH-8	A	OMe	—	$C_4H_8SO_3$-
PGH-9	A	H	—	$C_5H_{10}CO_2H$
PGH-10	B	SO_3K	CMe_2	$C_5H_{10}CO_2H$
PGH-9-PEG750	A	H	—	$C_5H_{10}CONH[C_2H_4O]_{\sim 17}Me$
PGH-9-PEG2000	A	H	—	$C_5H_{10}CONH[C_2H_4O]_{\sim 45}Me$
PGH-9-PEG5000	A	H	—	$C_5H_{10}CONH[C_2H_4O]_{\sim 113}Me$
Bis-PGH-9-PEG3400[b]	A	H	—	$\{C_5H_{10}CONH[C_2H_4O]_{\sim 37}CH_2\}_2$
PGH-10-PEG5000	B	SO_3K	CMe_2	$C_5H_{10}CONH[C_2H_4O]_{\sim 113}Me$

[a] Decomposed after purification. [b] Contains two PGH-9 dyes with both R substitutions being either end of the same bis-functional PEG3400 linker.

Table 3 Absorption properties of VSDs in various solvents

	Methanol		Ethanol		2-Propanol		n-Butanol		n-Octanol	
Dye	A_{max}/nm	ε/M^{-1} cm^{-1}	A_{max}/nm	ε/M^{-1} cm^{-1}	A_{max}/nm	ε/M^{-1} cm^{-1}	A_{max}/nm	ε/M^{-1} cm^{-1}	A_{max}/nm	ε/M^{-1} cm^{-1}
1	601	24900	608	24800	612	24300	619	24800	620	24700
2	664	32200	673	31500	670	33800	678	35200	678	33300
3	614	47400	620	46800	626	45750	630	47100	636	43000
4	686	53600	690	55200	680	53900	688	60600	686	55800
6	690	62500	690	67600	690	65800	696	52900	692	45500
7	587	42500	594	42100	594	43100	604	42000	606	43400
8	588	28400	598	34100	596	30300	602	32000	602	32000

Table 4 Emission properties of VSDs in various solvents. $\lambda_{EX} = 610$ nm

	Methanol		Ethanol		2-Propanol		n-Butanol		n-Octanol	
Dye	λ_{max}/nm	Fl. rel. int.	λ_{max}/nm	Fl. rel. int.	λ_{max}/nm	Fl. rel. int.	λ_{max}/nm	Fl. rel. int.	λ_{max}/nm	Fl. rel. int.
1	1025	107	966	300	934	714	900	795	868	2859
2	935	487	914	968	887	1855	890	1882	855	4392
3	928	707	901	1489	890	2621	888	2534	848	6626
4	940	341	923	832	888	1947	888	2046	849	2174
6	940	341	927	707	892	1397	893	486	861	1607
7	952	187	888	496	870	1121	866	1370	835	4062
8	959	108	960	351	940	686	898	930	866	2283

anchor directly attached to the chromophore. This difference in absorption behaviour is illustrated in Fig. 1. PGH-1 shows a wavelength shift with similar extinction values, while PGH-6 gives an absorbance difference with similar wavelength maxima.

A dye that exhibits a large $\Delta F/F$ response also must be soluble in solvents or buffers that are compatible with physiological conditions of excitable tissues. Great care must be taken to avoid the precipitation of dye or the formation of dye particles in the capillaries which would block flow and render the organ ischemic. The solubilities of selected dyes were tested by diluting a known concentration of dye in DMSO into various solvents. At micro-molar concentrations, the dyes remained soluble in alcohol solvents as well as aqueous buffers, and even buffered saline solutions. However, effective labelling of tissues and intact organs with a VSD is typically achieved by perfusing the tissue with a bolus injection of dye at 1–2 mM resulting in a homogeneous and intense VSD labelling on the membranes of the tissue. Fig. 2 shows the excitation and emission spectra of PGH-1 in cardiac

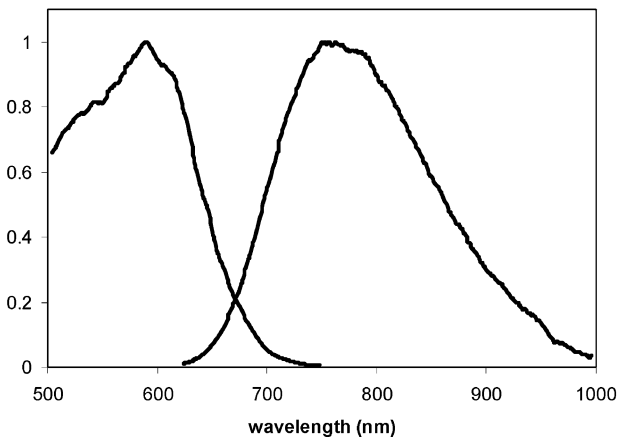


Fig. 2 Excitation–emission curve pair of PGH-1 in frog cardiac tissue. Parameters: $\lambda_{EM} = 875$ nm for excitation scan, $\lambda_{EX} = 600$ nm for emission scan.

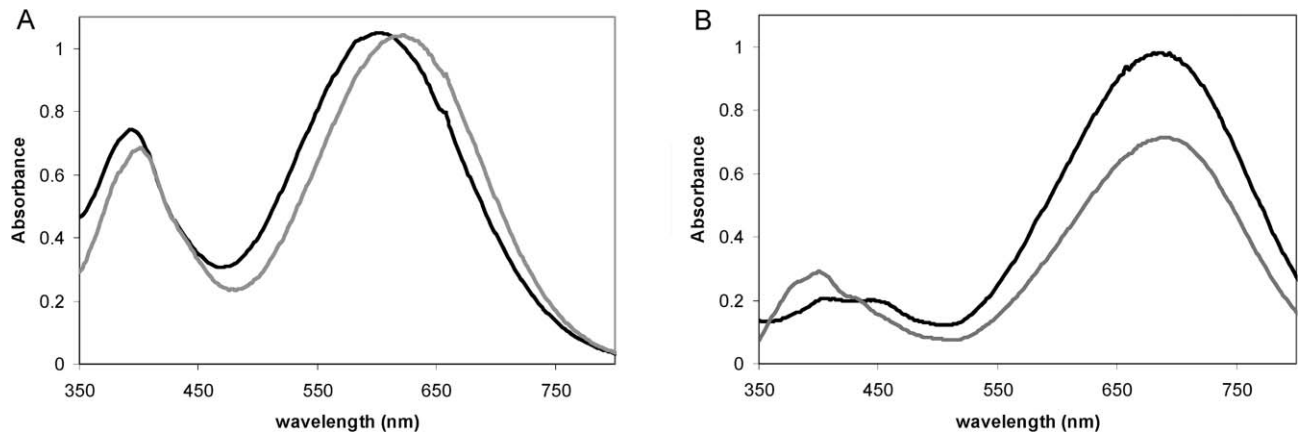


Fig. 1 Absorption spectra of PGH-1 (A) and PGH-6 (B) in methanol (black lines) and octanol (gray lines).

tissue. PGH dyes have desirable spectral properties in tissue but in order to achieve sufficient loading, DMSO and Pluronic L-64 are needed to keep the dyes from coming out of solution as they course through the vascular bed.[3] The search for alternative methods to solubilise the dyes without extraneous surfactants led to the development of PEG derivatives.

PEG derivatives

VSDs with functional groups (PGH-9 and PGH-10) were synthesized as in Scheme 1. The highly water-soluble PEG derivatives were prepared from different molecular weight amino-methoxy-PEGs (mPEG-NH_2) using standard methods and are listed in Table 2.[14,20] The combination of the hydrophilic polymer and the hydrophobic styryl dye made purification of these PEG dyes less straightforward than that of the sulfonated dyes. On acidic alumina, the blue chromophore irreversibly disappeared for PGH-9 derivatives of mPEG molecular weights 2000 to 5000. These larger PEG-derivatives were successfully purified by C_2-reversed phase chromatography with water–methanol gradients containing 1% acetic acid. After purification, the PGH-9 derivatives had an increased intensity of the short wavelength band (~400 nm) relative to long wavelength band (~600 nm). Table 5 shows a comparison of A_{short}/A_{long} of the PEG-dye derivatives, their corresponding parent dyes (carboxylic acids PGH-9 and PGH-10) and the sulfonate analogues (PGH-1 and PGH-6). When compared with PGH-1 (containing no PEG group), the ratio of A_{short}/A_{long} increased as the molecular weight of PEG increased, ranging from 1.4 to 2.7 for PEG750 and PEG5000 derivatives respectively. This increased absorbance ratio could not be reversed. Syntheses of two PGH-9 PEG-derivatives were repeated with closer stoichiometric control. Product isolation by precipitation from ether (PGH-9-PEG5000) or recovery by rotary evaporation (PGH-9-PEG750) yielded sufficiently pure material (single component by TLC), eliminating the need for chromatographic purification. The A_{short}/A_{long} increase seen previously was not observed with these resynthesized preparations and the ratio (0.6) was the same as underivatized PGH-9. Mass spectral analyses confirmed that the PGH-9 PEG-derivatives were intact. This enhanced short wavelength absorption was not observed with PGH-10-PEG5000 following chromatographic purification.

The mass profile of the PEG derivatives showed polydispersity as evidenced by a distribution of mass spectral peaks spaced 44 m/z units apart. The products showed polydispersity similar to the starting mPEG-amine reagents, but shifted by approximately the mass of the dye.[14] A marked change in the TLC migration of the products from the starting materials, with no change in spectral properties, gave further evidence of dye–PEG conjugation.

Table 5 Absorbance ratios (short/long) were calculated for PGH-1, PGH-6 and PEG derivatives of PGH-9 and PGH-10 in MeOH

	Short $\lambda_{Max.}$	Long $\lambda_{Max.}$	A_{short}/A_{long}
Lepidine dyes:			
PGH-1	392	601	0.71
PGH-9	398	606	0.56
PGH-9-PEG750 (LC)	396	596	1.39
PGH-9-PEG2000 (LC)	390	590	1.99
PGH-9-PEG3400 (LC)	390	588	1.44
PGH-9-PEG5000 (LC)	390	588	2.71
PGH-9-PEG750 (No LC)	398	610	0.58
PGH-9-PEG5000 (No LC)	398	608	0.60
Indolenine dyes:			
PGH-6	406	690	0.21
PGH-10	410	692	0.22
PGH-10-PEG5000	405	691	0.23

Table 6 Percentage aqueous solubility of voltage dyes and PEG derivatives

	Solubility of stock solutions	
Dye	Water	PBS
PGH-1	40.7%	7.3%
PGH-9-PEG750	63.7%	69.0%
PGH-9-PEG5000	77.5%	83.2%
PGH-6	0%[a]	0%[a]
PGH-10-PEG5000	71.4%	78.9%

[a] Solution was colourless.

The PEG dye derivatives had much higher water solubility than the sulfonated dyes. The aqueous solubilities of PGH-1, PGH-6, PGH-9-PEG750, PGH-9-PEG5000 and PGH-10-PEG5000 are shown in Table 6. With the aid of DMSO, PGH-1 showed limited solubility in water and saline; however, PGH-6 was essentially insoluble. Each of the PEG dye derivatives showed high water solubility without the need for DMSO; the relative aqueous solubility of PGH-9-PEG5000 was approximately 20% higher than that of PGH-9-PEG750 in both water and PBS. The fluorescence properties of PGH-10-PEG5000 and the four PGH-9 PEG-derivatives were similar to those of PGH-6 and PGH-1, respectively.

PEG derivatives of PGH dyes had similar spectral, voltage-sensitive responses and retention in cardiac tissues indicating that these dyes provide similar voltage sensing and tissue retention as their non-PEGylated analogues with the added advantage of being highly water soluble. Fig. 3 illustrates action potentials recorded from mouse hearts stained with PGH-1 and its PEG analogues. Action potentials recorded from PGH-1 delivered with a pH 6 aqueous stock solution (A) were similar to those recorded with PGH-1 delivered from a DMSO plus Pluronic stock solution (B). Similar results were obtained with PGH-9-PEG750 derivative delivered from Ringer's stock solution in the absence of Pluronic (C). PGH-9-PEG750 (D) shows the same inversion of the action potential signal as observed with PGH-1 when the excitation wavelength was shifted from 540 to 690 nm.[3] PGH-9-PEG2000 (E) had similar spectral response characteristics and exhibited action potential signals with similar signal to noise (S/N) ratio compared to the PGH-9-PEG750 derivative. In contrast, the PEG-5000 derivative yielded considerably lower S/N ratio compared to the other dyes, most likely due to reduced insertion in the membrane (F).

Discussion

For a dye to function satisfactorily as a potentiometric probe, it must exhibit the following essential properties: the dye must get to target tissue and cells; must interact in the proper orientation to sense membrane potentials across the plasma membrane; should exhibit large and specific optical changes that vary only with membrane potential changes; should induce minimal chemical- and photo-toxicity to the cells and should be optically and

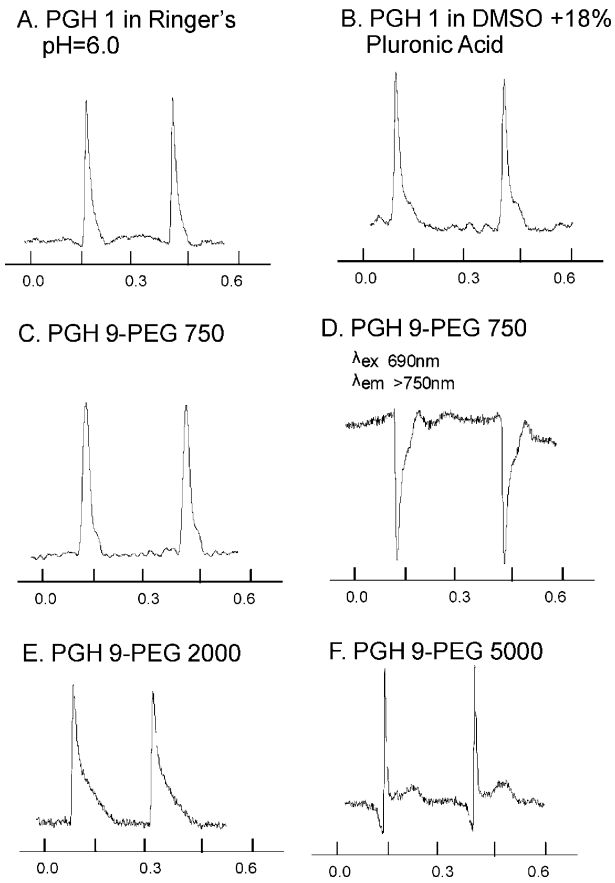


Fig. 3 Comparison of mouse ventricular action potentials recorded with PGH-1 and PGH-9 PEG derivatives. Excitation at 540 ± 25 nm and emission monitored >650 nm (except D). A: PGH-1 in Ringer's solution at pH 6. B: PGH-1 in a stock solution of 18% Pluronic L64 and DMSO. C: PGH-9-PEG750 in Ringer's at pH 7.4. D: PGH-9-PEG750 stained as C, excited at 690 ± 25 nm and the emission >750 nm. E: PGH-9-PEG2000 in Ringer's stock solution at pH 7.0. F: PGH-9-PEG5000 in Ringer's stock solution at pH 7.0.

spatially stable for long-lasting experiments.[4,6,9] The voltage sensor component of the dye should lie in the membrane where the electric field and charge movements are most significant during electrical activity. Molecular design features include (1) extended conjugation for long wavelength absorption while maintaining photostability; (2) various nitrogen heterocycles contributing to the long wavelength absorption and providing spectral sensitivity to voltage changes; (3) hydrophobic groups for stable partitioning into the lipid bilayer; and (4) a hydrophilic group making the dye membrane impermeant and facilitating tissue staining.[5,21] Through the structural modifications of the dyes, we have reached near IR excitation wavelengths with increased brightness and improved the delivery of the dyes to the tissues.

Increasing the absorption wavelength is typically done by extending the conjugated system. Incorporation of a thienyl unit in the conjugated system extended the wavelength and was effective in expanding conjugation of donor–acceptor molecules providing increased photostability and greater oscillator strength.[17,22] Extension of fluorescence to the near infra-red range improves penetration of the excitation and transmission of emitted light through thick muscle tissue. Intrinsic chromophores in tissue are avoided and Rayleigh scattering is reduced at the longer wavelengths. Far-red absorption was achieved with PGH-2, PGH-4 and PGH-6 dyes.

The length of the alkyl chains on the styryl portion of the dye can determine the depth of penetration into the lipid bilayer and the rate of dye washout.[13] Because of their effectiveness in other dyes, butyl groups were selected to maximize tissue retention without an excessive compromise on water solubility.[12] The depth of chromophore penetration into the lipid bilayer also increases with the number of carbons between it and the covalently linked anchor.[13] The type of hydrophilic group is significant. Loew and coworkers developed a family of styryl dyes which excite at 600–650 nm and emit >750 nm, but have restricted application for cardiac tissue studies.[8,23] While these dyes have improved spectral ranges, they exhibit limited photostability and low S/N ratio signals. The hydrophilic anchor of these dyes is the quaternary ammonium group, which can be drawn through the plasma membranes of cells with large negative membrane potential and thus is not effective for anchoring the dyes at the cell surface.[3] We have used sulfonic acid counter ions to anchor our dyes to the membrane surface.

The choice of the heterocycle can impact the quantum yield and voltage sensitivity of the dye within the membrane.[5] In this study, styryl dyes, PGH-1 through PGH-8, were synthesized from lepidine, quinoline, indolenine, benzothiazole and benzoxazole heterocycles. While most of the heterocycles gave absorption maxima near 600 nm, the indolenine dyes (PGH-2, PGH-4 and PGH-6) showed maxima near 690 nm. Unlike most styryl VSDs, the indolenine dyes with the sulfonic acid group attached directly to the chromophore (PGH-4 and PGH-6) did not show absorbance solvatochromism. The close proximity of the anchor to the aromatic system may maintain a polar environment for the chromophore. The sulfonate group will effectively orient the hydroxyl groups of the solvent molecules near the chromophore, even in octanol. While PGH-1 yielded larger $\Delta F/F$ signals in heart, PGH-6 also was effective as a VSD, even though it shows minimal absorption wavelength solvatochromism.[3]

The surfactant-like properties of PGH-9 PEG derivatives presented additional challenges for purification. Column purified PGH-9 PEG derivatives showed an increased A_{short}/A_{long} ratio while alternate synthesis of these derivatives, and the PGH-10 derivative, showed the same ratios as the parent dyes. As the hydrophobic voltage dye core and the highly water soluble PEG group became more dissimilar (as with increasing molecular weight of PEG group), the A_{short}/A_{long} ratio increased. Interactions between the PEG groups, the hydrophobic dye moiety and the chromatographic stationary phase may have induced these spectral changes. A shift in absorption maximum from 600 nm to 400 nm, while maintaining an intact dye structure, can be caused by a disruption of the π electron system. Encapsulation by the lengthy PEG group could stabilize a folded conformation of the dye. Johnsson *et al.* incubated other types of PEG derivatives at 60–70 °C for 3 h to obtain clear solutions.[24] Heating had no effect on the absorption spectra of our voltage dye-PEGs. PEG lipids with molecular weight >1000 form spherical micelles because of the steric hindrance of the larger molecular weight PEG groups.[24] Because of the duality inherent in their structure, these new VSD PEG derivatives may behave like PEG lipids. Practical considerations such as concentration may be necessary in

applications where critical micellar concentration and formation of spherical or lamellar aggregates are factors.[24]

Conjugation of PEG groups to VSDs significantly improved water solubility and simplified delivery to excitable tissues. Derivatives with a longer PEG group (MW 5000) allowed easier isolation from reaction mixtures using precipitation with ether. While derivatives with larger PEG groups are easier to synthesise, the 2 mM stock concentrations of these materials approach 1% solute concentration. At these concentrations, the viscosity of the solution is high and can be problematic in applications where this is a concern. Derivatives with a shorter PEG group (MW 750) could not be isolated by precipitation, thus making product recovery more arduous. However, the shorter PEG dyes labelled the tissues better in mouse hearts than longer PEG dyes, and did not cause ischemia. The difference in the relative aqueous solubilities of PGH-9-PEG750 and PEG-9-PEG5000 (Table 5) shows that the length of the PEG chain is also an important consideration. The attachment of PEG groups to styryl VSDs, regardless of their lengths, has made it possible to deliver these dyes to tissues without the need for DMSO or Pluronics. The simplified delivery method greatly improves the productivity of excitable membrane experimentation and may prove to be particularly advantageous in neurobiology and nerve networks.

Conclusion

The use of voltage sensitive dyes has been valuable for basic research and medical physiology of excitable tissues like heart and nerve networks. Improvements in the sensitivity of the probes significantly enhance the data quality and range of possible experiments. Furthermore, advantages can be obtained through improved dye delivery and imaging of excitable cells from deeper layers in heart and brain tissues. In developing new methods, we have uncovered a better probe and enhanced membrane staining using water-soluble VSDs. These developments provide incremental improvements that may help us venture into new paradigms that can produce major increments in dye performance.

Experimental

Chemical syntheses

Full synthetic and characterization details are available as electronic supplementary information (ESI†).

Synthesis of voltage dyes

Briefly, fluorescent dyes (PGH-1 through PGH-10) were synthesized by the following general method. Equi-molar quantities of anilino-thiophene-carboxaldehyde (**2**) in ethanol (~0.25 M) and quaternized heterocyclic intermediate (**3** through **12**) dissolved in methanol (~0.25 M) were stirred at room temperature. Quinoline and lepidine intermediates (**3**, **9**, **10** and **11**) required the addition of 1% (v/v) piperidine to facilitate the reaction. At various times a sample of the mixture was diluted in ethanol and measured by UV/Vis spectroscopy. Reaction progress was monitored by appearance of the product dye (Table 3) and decrease of the aldehyde (A_{444}). If significant starting aldehyde remained unreacted, additional heterocycle was added and the reaction continued. When the A_{dye}/A_{444} ratio remained constant, the reaction was concentrated to dryness by rotary evaporation. Dyes were purified by either normal or reversed phase column chromatography. Refer to ESI† for details.

Synthesis of PEG derivatives

Voltage dyes derivatised with PEG groups were synthesised from PGH-9 and PGH-10 by conversion of carboxylic acid functional groups to the highly reactive succinimidyl ester using 1.5 molar equivalents of *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate (TSTU) in DMF containing 1% diisopropylethylamine. mPEG-amines were then added to the reactive NHS esters in 1.1 molar excess and the progress of the reaction monitored by TLC. PEG derivatives were isolated by rotary evaporation or precipitation with ethyl ether. Additional purification for some of the PEG derivatives was done by column chromatography. Refer to ESI† for details.

Spectral analysis of dyes

Absorption spectra and extinction coefficients were determined using a Hewlett Packard Spectrophotometer (8452A Diode Array) or a Perkin Elmer Lambda 45 Spectrophotometer. Corrected excitation and emission spectra were obtained using a PTI QuantaMaster fluorescence system configured with double grating excitation and emission monochromators and a Hamamatsu R5108 photomultiplier tube cooled with dry ice and run at 1100 V in photon-counting mode. Data acquisition and analysis was done with Felix32 software. Fluorescence samples were prepared by 1 : 10 dilution of the dye solution used for absorption spectroscopy in an appropriate solvent. Excitation wavelength was set 10–20 nm below the absorption maximum of the dye (for lepidine-type dyes at 600 nm; for indolenine-type dyes at 675 nm). Real-time correction software was used. Extinction coefficients presented in the synthesis methods (refer to ESI†) were calculated from samples typically prepared by dissolving 1.00 mg of dye in 100.0 ml of methanol or ethanol with the aid of sonication. Dye samples in DMSO (1.00 mg ml^{-1}) were diluted into methanol or ethanol to produce an optical density of approximately 1.0 AU. The same volume of DMSO stock solution was diluted into solvents for measurement of absorption spectra. Further dilutions of these solutions were made into corresponding solvents yielding optical densities of approximately 0.1 AU for measurement of fluorescence spectra.

Solubility of dyes and PEG derivatives

Dye solids were dissolved to make 2 mM solutions in water and PBS. While PEG derivatives dissolved directly, PGH-1 and PGH-6 required initial dissolution in 10 μl of DMSO followed by dilution into aqueous solvents (1000 μl). After brief vortex mixing and 1 h of sonication, samples were filtered through 0.22 μm filters then diluted into methanol for concentration determination by absorption spectroscopy.

Tissue labeling experiments

Frog hearts were isolated and perfused in a Langendorff apparatus and stained with a bolus of concentrated dye solution (20 μl of 1 mg ml^{-1} dye in DMSO plus or minus Pluronic) until it was

noticeably stained.[3,6] The heart was placed in a 10 mm × 10 mm cuvette and right-angle fluorescence measurements were taken from re-emergent light through the tissue. Emission spectrum parameters: excitation 600 nm, emission 620–1000 nm; excitation spectrum parameters: emission 875 nm, excitation 500–865 nm, Fluorolog 3 Jobin-Yvon, Hariba, Inc.

Mouse ventricular action potential experiments

Mouse hearts were isolated and perfused in a Langendorff apparatus, with Ringer's solution containing (in mM): NaCl 136, KCl 5.4, $MgCl_2$ 1, NaH_2PO_4 0.33, $CaCl_2$ 2.5, HEPES 10, glucose 10; pH 7.4. Hearts were placed in a chamber designed to reduce contraction artefacts, with temperature controlled to 37 ± 1 °C *via* a feedback control, as previously described.[25] Hearts were stained with a VSD by adding a bolus of 30 µl dye from a stock solution of 1 mM dye dissolved in different solvents. The amount of VSD delivered to each heart was thus kept constant to compare the different dyes through the S/N ratio of their optical action potentials (APs) when the dye was excited at 540 ± 25 nm and emission with a long pass filter at 650 nm. All animal experiments complied with the University of Pittsburgh's Animal Care and Usage Committee and the National Institute of Health.

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