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In Vivo Nerve-Specificity of Rhodamines and Si-rhodamines

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Abstract

Accidental nerve damage or transection of vital nerve structures remains an unfortunate reality that is often associated with surgery. Despite the existence of nerve-sparing techniques, the success of such procedures is not only complicated by anatomical variance across patients but is also highly dependent on a surgeon's first-hand experience that is acquired over numerous procedures through trial and error, often with highly variable success rates. Fluorescent small molecules, such as rhodamines and fluoresceins have proven incredibly useful for biological imaging in the life sciences, and they appeared to have potential in illuminating vital nerve structures during surgical procedures. In order to make use of the current clinically relevant imaging systems and to provide surgeons with fluorescent contrast largely free from the interference of hemoglobin and water, it was first necessary to spectrally tune known fluorescent scaffolds towards near infrared (NIR) wavelengths. To determine whether the well-documented Si-substitution strategy could be applied towards developing a NIR fluorophore that retained nerve-specific properties of candidate molecules, an *in vivo* comparison was made between two compounds previously shown to highlight nervous structures – TMR and Rhodamine B – and their Si-substituted derivatives.

Keywords

Nerve-specific Fluorophores; Contrast agent; Fluorescent imaging; Image-guided surgery

1. INTRODUCTION

Recent statistics approximate the volume of surgical procedures occurring annually at more than 300 million worldwide, with millions more needed in lower- and middle-income countries and an even greater demand projected as the population increases.¹ Many such procedures are required not only to save lives but to prevent permanent, life-altering disability. Accidental nerve damage or transection of vital nerve structures leading to

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chronic post-surgical pain, loss of sensation, or paralysis is an unfortunate reality that is often associated with many potentially life-saving procedures.²

While nerve-sparing techniques exist, the success of such procedures is often dependent on a surgeon's first-hand experience that must be acquired through trial and error over numerous procedures with highly variable rates of success.^{3, 4} Furthermore, nerves are by nature translucent and evasive structures that are often difficult, if not impossible to visualize adequately. This is because intraoperatively, a surgeon's only means of navigating these structures are currently limited to white light visualization, neuroanatomical knowledge, and electromyographical monitoring,⁵ leaving substantial room for methodologies that improve both nerve detection and visualization to improve surgical outcomes for patients and surgeons.

Fluorophores like rhodamine and fluorescein, among others have been extensively utilized in the life sciences as sensors allowing one to visualize intracellular activities and environmental conditions.^{6–10}

However, the utility of these compounds as intraoperative nerve-highlighting contrast agents for fluorescence-guided surgery (FGS) has not been as extensively explored.^{4, 11–13} Among the many derivatives belonging to this class of fluorescent molecules, those with the greatest potential for clinical translation are the ones with excitation and emission in the near-infrared (NIR) region between 650 and 900 nm, where tissue absorbance, light-scattering and autofluorescence are all at their local minima.¹⁴ Furthermore, current clinically relevant FGS instrumentation is designed to accommodate and visualize FDA-approved NIR contrast agents (e.g., methylene blue and indocyanine green) and as such, a synergistic integration of technology could better facilitate the process of clinical translation. For a fluorophore to provide surgeons with optimal contrast in the context of a nerve-sparing surgical procedure however, it must not only emit in the NIR range, but selectively bind to nerves as well, providing contrast against surrounding tissues including muscle, adipose, fascia, etc.

A well-documented strategy to structurally induce bathochromic shifts of existing fluorescent xanthene molecules has been demonstrated in fluorophores like rhodamines, fluoresceins, and rhodols. Selective substitution of a silicon (Si) or other heteroatom in place of the bridging oxygen of these structures has been shown to induce bathochromic shifts of ca. 90 nm relative to their parent structural analogues.^{15–19} The study detailed herein represents an effort to investigate whether such a strategy may be applied towards spectrally tuning potential fluorescent candidates to be used as nerve-specific contrast agents for FGS. A comparison was made to determine whether two previously reported compounds, Si-TMR and Si-rhodamine B, would maintain the nerve-specific properties previously observed in structurally analogous rhodamine derivatives.^{20, 21}

2. MATERIALS & METHODS

2.1 Chemistry

All fluorophores used in this study, with the exception of rhodamine B, were synthesized according to previously reported synthetic routes.^{19, 22} Rhodamine B was purchased from

Sigma-Aldrich (St. Louis, MO). All analytical thin layer chromatography (TLC) was performed on ready-to-use plates with silica gel 60 (32–63 μm , EMD Millipore, Burlington, MA) and all flash chromatographic purification was performed by column chromatography using silica gel (Sorbent Technologies Inc., Norcross, GA). High-resolution mass spectra (HRMS) were measured on an Agilent 6244 time-of-flight tandem liquid chromatography mass spectroscopy (LCMS) instrument with a diode array detector VL+ (Agilent Technologies, Santa Clara, CA).

2.2 HPLC-MS characterization

Mass-to-charge ratios of all compounds presented were confirmed by LCMS (Agilent Technologies). Samples were dissolved in a 1:1 mixture of acetonitrile:water and injected (10 μL) onto a C18 column (Poroshell 120, 4.6×50 mm, 2.7 micron, Agilent Technologies). Elution by a gradient of water (A) and acetonitrile (B), both containing 0.1% formic acid, proceeded from A/B = 90/10 to 5/95 over 10 min and maintained at A/B = 5/95 for an additional 5 min at a rate of 0.4 mL/min. Detection was accomplished through positive ionization, with capillary voltage set at 4 kV, and gas temperature of 350 $^{\circ}\text{C}$.

2.3 Optical characterization

Optical samples of all fluorophores were prepared in phosphate buffered saline (PBS), pH 7.4 with 10% dimethyl sulfoxide (DMSO) at 10 μM concentration. Absorbance and fluorescence spectra were collected using a SpectraMax M5 spectrophotometer (Molecular Devices, Sunnyvale, CA) with a 1-cm quartz cuvette at ambient temperature. Absorbance spectra were reference corrected and attenuation coefficients calculated using Beer's Law. Fluorescence spectra were collected using excitation wavelengths ca. 30–50 nm below the corresponding maximum absorbance wavelength.

2.4 Calculation of physicochemical properties

Physicochemical property calculations were completed using Marvin and JChem calculator plugins (ChemAxon, Budapest, Hungary).

2.5 Animals

All animals used for this study were obtained under a protocol approval of the Institutional Animal Care and Use Committee (IACUC) at Oregon Health and Science University (OHSU). Animals used for this study were male CD-1 mice, weighing between approximately 22 and 24 g (Charles River Laboratories, Wilmington, MA). Animals were anaesthetized using 100 mg/kg ketamine and 10 mg/kg xylazine administered intraperitoneally. Mice were euthanized at the end of each study via inhalation of carbon dioxide followed by cervical dislocation in accordance with guidelines laid out by IACUC.

2.6 Fluorescence imaging system

Fluorescent images were collected using a custom imaging system comprised of a QImaging EXi Blue monochrome camera equipped with a Bayer filter, capturing both color and fluorescence images. A PhotoFluor II excitation source was focused onto the surgical site by way of a liquid light guide using unfiltered white light for color images and 545 ± 12.5 nm

or 620 ± 30 nm excitation bandpass filters for fluorescent excitation of rhodamine or Si-rhodamine fluorophores, respectively. Fluorescence was then captured using either a 605 ± 35 nm or 700 ± 37.5 nm bandpass emission filter for the rhodamine or Si-rhodamine fluorophores, respectively.

2.7 Direct administration

Fluorophores were suspended in a co-solvent formulation detailed previously.¹¹ Suspended compounds were applied directly to surgically exposed nervous tissue in an optimized manner detailed previously, allowed to sit in place for 5 minutes, and removed with gauze followed by a nine-fold irrigation of the nerve site with PBS. This was supplemented by a second incubation using a blank co-solvent solution for another 5 minutes that was removed and followed with an irrigation procedure analogous to the first.²³

2.8 Image processing

Custom written MatLab (The Math Works, Natick, MA) code was used to analyze the tissue specific fluorescence where regions of interest (ROIs) were selected on the nerve, muscle, cut-muscle and adipose tissues using the white light images, but blinded to the fluorescence images. These ROIs were then analyzed on the co-registered, matched fluorescence images permitting quantification of the mean tissue intensities as well as the nerve to muscle (N/M), nerve to cut-muscle (N/CM), and nerve to adipose (N/A) tissue ratios. Fluorescence intensity measurements were divided by the exposure time to obtain normalized intensity per second measurements. Mean nerve to background tissue ratios (N/M, N/CM, and N/A) were calculated for each fluorophore.

3. RESULTS & DISCUSSION

3.1 Chemistry

Previous reports have demonstrated that a dramatic bathochromic shift of ca. 90 nm in emission relative to its parent fluorophore can be elicited through replacement of the bridging oxygen at position 10 in the rhodamine structure with a silicon atom.^{9, 21–24} This strategy has been successfully applied in rhodamine, fluorescein, and rhodol scaffolds with notably larger shifts observed in some cases upon replacement of a sulfur atom in the form of a sulfone.²⁴ These observations have been attributed to the difference in the energy gap between the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO), where the additionally electropositive inductive effect of the dimethylsilyl group relative to oxygen accounts for an increase in the HOMO.¹⁵ It has also been observed that where the $p^*-\pi^*$ interaction between the bridging oxygen and adjacent carbons effectively raises the LUMO energy in rhodamine, the analogous interaction between the empty $3d^*$ and π^* orbitals in Si-rhodamines serves to lower the LUMO energy, enabling a smaller energy gap to fluorescence emission.²⁴ Herein, we examined differences between tetramethylrhodamine (TMR), silicon (Si) TMR (Si-TMR), Rhodamine B and Si-Rhodamine B (Figure 1).

3.2 Comparison of optical and physicochemical properties

Maximum absorbance and emission values ranged from 544 to 648 nm and from 552 to 668 nm, respectively, with Stokes' shifts ranging from 8 to 25 nm. A wide range of extinction coefficients were observed, from 5,293 to 83,638 M⁻¹cm⁻¹. While both of the ethylated rhodamines (i.e., rhodamine B and Si-rhodamine B) gave broad spectra, rhodamine B gave a particularly broad spectrum relative to its methylated counterpart, TMR. However, the spectral profile of Si-TMR was remarkably similar to its oxygen-containing analog, but with a 90 nm bathochromic shift in fluorescence emission and a 95 nm shift in absorbance (Figure 2). Both compounds obtained from the Si-substitution strategy showed lower molar attenuation coefficients and lower fluorescent intensities relative to their parent compounds; this effect was especially pronounced in aqueous media. In particular, Si-rhodamine B had an observed molar attenuation coefficient that was more than ten times less than its structural analog, rhodamine B (Table 1).

To assess the potential of each compound for clinical translation, physicochemical properties were calculated and analyzed according to the guidelines given by Lipinski and Veber.^{25, 26} Molecular weight of the compounds ranged from 386 to 484 g/mol and thus fell within an acceptable range (Lipinski rule < 500 g/mol). Similarly, LogD values ranged from -0.53 to 3.43, well within the recommended range (Lipinski rule < 5). None of the fluorophores contained hydrogen bond donors (Lipinski rule <5) but had between 3–4 hydrogen bond acceptors (Lipinski rule <10). Polar surface area was maintained across rhodamine and Si-rhodamine variants and ranged from 46.38 to 55.61 Å² (Veber rule <140 Å²).

3.3 Comparison of nerve-specificity

To compare nerve-specific properties of the fluorophores, a previously optimized method of direct administration was used wherein a solution of the fluorophore in co-solvent was applied topically to a surgically exposed area of nerve tissue (Figs. 3A, 3B).²³ Images collected of stained nerve sites were used to quantify fluorescence intensity and to determine the selective affinity of each compound for nerve tissue by calculating nerve-to-adipose, nerve-to-muscle, and nerve-to-cut muscle contrast ratios (Fig. 3C).

Differences in measured optical properties were further apparent during *in vivo* murine tissue staining studies, where TMR and rhodamine B gave significantly higher fluorescence intensity compared to their Si-derivatives that were two orders of magnitude dimmer on average (Fig. 3C). Nerve-specificity was also compromised in the Si-derivatives, with nerve-to-muscle, nerve-to-adipose, and nerve-to-cut muscle contrast ratios comparable to autofluorescence. Despite a low nerve-to-cut muscle ratio of ca. 1.9, TMR gave average nerve-to-muscle and nerve-to-adipose signal-to-background ratios of 5.8 and 4.7, respectively. Rhodamine B had lower nerve-to-muscle and cut muscle ratios but gave a better than average nerve-to-adipose contrast ratio of 5.3.

It had been previously speculated that compound lipophilicity was a potential factor contributing to nerve-specificity, noting that nerve-specific Rhodamine B and Oxazine 4 both had LogD values between 3–5, while the remaining pan-fluorescent compounds had values greater than 5.²¹ Although the calculated LogD value of TMR, 2.19, is just outside

this range, it was noteworthy that Si-rhodamine B and Si-TMR had much smaller values of 0.84 and -0.53 , respectively. This may suggest not only an upper limit of 5 in accordance with Lipinski's rules, but also a lower limit less than two may be optimal for maintaining nerve-specificity.

4. CONCLUSION

The mechanism behind selective nerve tissue uptake as well as the location and character of the binding site targeted by nerve-specific fluorophores like TMR and rhodamine B remain unknown. Although the strategy of Si-substitution is a viable option for shifting fluorescent emission further towards the clinically relevant NIR region through selective tuning of molecular orbital interactions, it does not appear to be one that can be applied to designing nerve-specific fluorophores. Previously, it was noted that the lipophilicity (LogD) of some nerve-specific fluorophores fell within an approximate range of 3–5, with several pan fluorescent compounds possessing LogD values greater than 5.²¹ Here the loss of nerve specificity outside this range was notably observed to extend to compounds with LogD values less than 2. While this study further highlights the difficulty involved in designing NIR nerve-specific fluorophores as well as the need for further study into their biomolecular target, it also eliminates potential candidates by demonstrating the dramatic effect observed from such a seemingly small structural modification and serves to corroborate previously observed trends suggesting an important role lipophilicity might play in facilitating tissue uptake.

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TMR **Si-TMR** **Rhodamine B** **Si-Rhodamine B**

Figure 1:
Chemical structures of the rhodamine and Si-rhodamine derivatives.

Figure 2:

Absorbance (A & C) and emission (B & D) spectra of the rhodamine fluorophores (A & B) and their Si-substituted derivatives (C & D). Fluorophores were dissolved in PBS (pH 7.4) containing 10% DMSO and were excited at wavelengths ca. 15–50 nm shorter than the corresponding wavelength of maximum absorbance. Absorbance and emission values were normalized to the maximum value for each curve.

Fig. 3: Representative fluorescence images of tissue stained with (A) rhodamine fluorophores TMR and Rhodamine B, and (B) Si-rhodamine fluorophores Si-TMR and Si-rhodamine collected following direct administration along with unstained control images for comparison, and (C) quantified tissue fluorescence intensity and signal-to-background ratios of nerve contrast relative to muscle, adipose, and cut muscle tissue following direct administration of rhodamine and Si-rhodamine derivative fluorophores.

Table 1:

Physicochemical and optical properties of the rhodamine fluorophores and Si-containing derivatives

Compound I.D	Max Abs. (nm)	Max Em. (nm)	Attenuation coefficient (M ⁻¹ cm ⁻¹)	Molecular weight (g/ mol)	LogD (pH7.4)	H-bond donors/ acceptors	Polar surface area (Å ²)	Rotatable bonds	Dipole moment (Debye)
TMR	550	575	83638	386.45	2.19	0/4	55.61	2	30.34
Si-TMR	645	665	23179	428.61	-0.53	0/3	46.38	3	24.62
Rhodamine B	544	552	62488	442.56	3.43	0/4	55.61	7	29.14
Si-Rhodamine B	648	668	5293	484.72	0.84	0/3	46.38	6	23.08