Published in final edited form as:

Proc SPIE Int Soc Opt Eng. 2019 February ; 10862: . doi:10.1117/12.2507296.

Investigation of Oxazine and Rhodamine Derivatives as Peripheral Nerve Tissue Targeting Contrast Agent for *In Vivo* Fluorescence Imaging

Lei G. Wang¹, Connor W. Barth¹, Jason R. Combs¹, Antonio R. Montañó¹, Summer L. Gibbs^{1,2,3,§}

¹Biomedical Engineering Department, Oregon Health & Science University, Portland, OR 97201

²Knight Cancer Institute, Oregon Health & Science University, Portland, OR 97201

³OHSU Center for Spatial Systems Biomedicine, Oregon Health & Science University, Portland, OR 97201

Abstract

Accidental nerve transection or injury is a significant morbidity associated with many surgical interventions, resulting in persistent postsurgical numbness, chronic pain, and/or paralysis. Nerve-sparing can be a difficult task due to patient-to-patient variability and the difficulty of nerve visualization in the operating room. Fluorescence image-guided surgery to aid in the precise visualization of vital nerve structures in real time during surgery could greatly improve patient outcomes. To date, all nerve-specific contrast agents emit in the visible range. Developing a near-infrared (NIR) nerve-specific fluorophore is poised to be a challenging task, as a NIR fluorophore must have enough “double-bonds” to reach the NIR imaging window, contradicting the requirement that a nerve-specific agent must have a relatively low molecular weight to cross the blood-nerve-barrier (BNB). Herein we report our efforts to investigate the molecular characteristics for the nerve-specific oxazine fluorophores, as well as their structurally analogous rhodamine fluorophores. Specifically, optical properties, physicochemical properties and their *in vivo* nerve specificity were evaluated herein.

Keywords

Fluorophore; Oxazine; Rhodamine; Fluorescence Imaging; Contrast Agent; Image-Guided Surgery; Nerve

1. INTRODUCTION

Over 300 million surgical procedures are performed annually worldwide.¹ Accidental nerve transection or injury is a major morbidity associated with many surgical interventions, resulting in persistent postsurgical numbness, chronic pain, and/or paralysis.² Nerve sparing can be a difficult task due to patient-patient variability and the difficulty of nerve

§ To whom all correspondence should be addressed: Summer L. Gibbs, Ph.D. Oregon Health & Science University, Collaborative Life Sciences Building, 2730 SW Moody Ave, Mail Code: CL3SG, Portland, OR 97201. gibbonss@ohsu.edu, Phone: 503494-8940.

visualization in the operating room. Currently, nerve detection in the operating room is largely completed through electromyographical monitoring or direct visualization under white light.³ Fluorescence image-guided surgery to aid in the precise visualization of vital nerve structures in real time during surgery could greatly improve^{4–8} patient outcomes. However, no clinically approved nerve-specific contrast agent exists, where small fluorescent molecules with inherent tissue-specificity without the requirement of conjugation to any targeting agent are particularly attractive. Contrast agents that fall within the near-infrared (NIR) window (650–900 nm) are highly desirable for fluorescence image-guided surgery because absorbance, scattering, and autofluorescence are all at local minima, making tissue light penetration maximal in this range.^{9–11}

Currently, a NIR nerve-specific fluorophore does not exist. This is a particularly challenging problem because a nerve-specific contrast agent must have a relatively low molecular weight to cross the blood-nerve barrier. Complicating this requirement is the fact that a NIR fluorophore must have a sufficient number of “double bonds” (i.e. degree of conjugation) to reach NIR wavelengths, by definition increasing molecular weight. Oxazine 4 has the longest emission maximum (635 nm maximum) among all the fluorophores that have been reported to highlight peripheral nerves.¹² Interestingly, with high structural similarity to Oxazine 4, Oxazine 1 has NIR emission (670 nm maximum), but only provides pan-tissue fluorescence with no nerve-specificity following *in vivo* administration. Thus, a comprehensive understanding of the molecular characteristics for nerve-specific contrast agent would be beneficial to the development of a NIR nerve-specific contrast agent.

In this work, the nerve-specific fluorophore Oxazine 4, the NIR Oxazine fluorophore Oxazine 1, as well as their structurally analogous Rhodamine fluorophores were selected as base compounds to investigate their physicochemical properties in correlation to their nerve-specificity (Fig. 1). Even though Rhodamines are generally blue-shifted in comparison to Oxazine fluorophores, functionalization of the benzoic acid in the Rhodamine provide an easy route to modify the overall charge. Furthermore, xanthene-based fluorophores have been reported featuring inherent pancreas tissue affinity in the murine models, and such tissue-specificity was accomplished through a systematic modification of the chromophore with an emphasis on pharmacological properties.¹³ The study described herein focuses on the fundamental physicochemical property comparison between the Oxazine and Rhodamine fluorophores, including charge, dipole moment, lipophilicity, molecular weight, number of hydrogen bond donors and acceptors, number of rotatable bonds, and polar surface area. Physicochemical property calculations revealed that overall charge and lipophilicity could be the key factors affecting a small fluorescent molecule’s nerve-specificity.

2. MATERIALS & METHODS

2.1 Chemistry

Oxazine 1 perchlorate and Oxazine 4 perchlorate were obtained from Exciton (Lockbourne, OH). Rhodamine B and Rhodamine 6G were purchased from Sigma-Aldrich (St. Louis, MO). Rhodamine 19 and Rhodamine B ethyl ester (Rhodamine B EE) were synthesized according to published literature protocols.¹⁴ Analytical thin layer chromatography (TLC) was performed on ready-to-use plates with silica gel 60 (32–63 μ m, EMD Millipore,

Burlington, MA). Flash chromatography 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).

2.1.1 Synthesis of (Z)-2-(6-(ethylamino)-3-(ethyliminio)-2,7-dimethyl-3#-xanthen-9-yl)benzoate (Rhodamine 19).—Rhodamine 19 was prepared following a slightly modified literature protocol by Chen *et. al.*¹⁴ Under nitrogen gas, Rhodamine 6G (1 g, 2.25 mmol) was dissolved in a solution of 2M sodium hydroxide (NaOH, 20 mL) and ethanol (5 mL). The resulting reaction mixture was then heated to 80 °C and stirred overnight. After cooling to room temperature, the ethanol was removed under reduced pressure. The aqueous solution was neutralized with 2M hydrochloric acid (HCl). The resulting precipitate was filtered and air dried to give pure product (753 mg, 81%) as a light maroon solid. HRMS (ESI-TOF) *m/z*: [M + H]⁺ + Calculated for C₂₆H₂₇N₂O₃ 415.2016; found 415.2041.

2.1.2 Synthesis of N-(6-(diethylamino)-9-(2-(ethoxycarbonyl)phenyl)-3#-xanthen-3-ylidene)-N-ethylethanaminium (Rhodamine B EE).—Rhodamine B EE was prepared using Fisher's esterification reaction protocol. Rhodamine B (1 g, 2.25 mmol) was dissolved in 50 mL ethanol, and chilled in an ice bath. Concentrated sulfuric acid (H₂SO₄, 2.5 mL) was carefully added, and the resulting reaction mixture was stirred overnight at reflux. After cooling to room temperature, the ethanol was removed under reduced pressure. The product was purified by recrystallization, and a dark red solid was obtained (792 mg, 75%). HRMS (ESI-TOF) *m/z*: [M + H]⁺ + Calculated for C₃₀H₃₅N₂O₃ 471.2642; found 471.2678.

2.2 HPLC-MS characterization

The mass-to-charge ratio (*m/z*) of Rhodamine B EE and Rhodamine 19 were characterized by LCMS (Agilent Technologies). Each sample was injected (10 µL in a 1:1 ratio of acetonitrile:water) onto a C18 column (Poroshell 120, 4.6 × 50 mm, 2.7 micron, Agilent Technologies), and eluted with a solvent system of A (water, 0.1% formic acid) and B (acetonitrile, 0.1% formic acid) at 0.4 mL/min, from A/B = 90/10 to 5/95 over 10 min and maintained at A/B = 5/95 for additional 5 min. Ions were detected in positive ion mode by setting the capillary voltage at 4 kV and gas temperature at 350 °C.

2.3 Absorption and fluorescence characterization

Fluorophores were solubilized in phosphate buffered saline (PBS), pH 7.4 with 10% dimethyl sulfoxide (DMSO) at a concentration of 10 µM. Absorbance and fluorescence spectra were collected using a SpectraMax M5 spectrometer (Molecular Devices, Sunnyvale, CA) at room temperature in a 1-cm quartz cuvette. All absorbance spectra were reference corrected. Extinction coefficients were calculated using Beer's Law plots of absorbance versus concentration. Fluorescence spectra were collected with the excitation wavelength 30–50 nm below the corresponding maximum absorbance wavelength.

2.4 Physicochemical property calculations

Physicochemical properties including the partition coefficients at pH 7.4 (LogD), the number of hydrogen bond donors and acceptors, the polar surface area, the number of rotatable bonds, and dipole moment were calculated using Marvin and JChem calculator plugins (ChemAxon, Budapest, Hungary).

2.5 Animals

Approval for the use of all animals in this study was obtained from the Institutional Animal Care and Use Committee (IACUC) at Oregon Health and Science University (OHSU). Male CD-1 mice weighing 22–24g were purchased from Charles River Laboratories (Wilmington, MA). Prior to surgery, animals were anaesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine (Patterson Veterinary, Devens, MA) administered intraperitoneally (IP). At the completion of all studies, mice were euthanized using inhalation of carbon dioxide followed by cervical dislocation.

2.6 Fluorescence imaging system

A custom-built small animal imaging system capable of real-time color and fluorescence imaging was used to acquire *in vivo* murine images.¹⁵ The imaging system consisted of a QImaging EXi Blue monochrome camera (Surrey, British Columbia, CA) for fluorescence detection with a removable Bayer filter for collection of co-registered color and fluorescence images. A PhotoFluor II light source was focused onto the surgical field through a liquid light guide and used unfiltered for white light illumination. For fluorescence excitation, the PhotoFluor II was filtered with 545 ± 12.5 nm or a 620 ± 30 nm bandpass excitation filter for Rhodamine or Oxazine fluorophores, respectively. The resulting fluorescence was collected with a 605 ± 35 nm or a 700 ± 37.5 nm bandpass emission filter for Rhodamine or Oxazine fluorophores, respectively. All filters were obtained from Chroma Technology (Bellows Falls, VT). Camera and light source positions were held constant throughout the course of all imaging studies, allowing quantitative comparison of *in vivo* fluorescence intensities.

2.7 *In vivo* murine nerve tissue staining

Contrast agents were solubilized for *in vivo* use in the previously described co-solvent formulation.¹⁶ The previously optimized staining procedure was utilized for direct administration.¹⁷ The sciatic nerves were surgically exposed by removal of overlying adipose and muscle tissues. The fluorophore was formulated at 125 μ M in the co-solvent formulation and 100 μ L was incubated on the exposed sciatic nerve for 5 minutes. The fluorophore containing solution was removed and the area was irrigated with saline nine times, followed by a five-minute incubation with blank formulation and then irrigation with saline nine additional times to remove any unbound fluorophore. Images were acquired 30 minutes following completion of staining. Unstained nerve sites were used for all control images to quantify auto-fluorescence. Each fluorophore was screened in n=3 mice.

2.8 Image Analysis

Custom written MatLab 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 assessment 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) 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 Chemical synthesis

Oxazine 4 has been previously reported to show peripheral nerve tissue specificity,¹² and was selected as the positive nerve-specific base compound, while Oxazine 1 was selected as the negative base compound. Both oxazines are cationic fluorophores in the physiological pH range. Replacing the central nitrogen atom with an *o*-toluate moiety afforded Rhodamine 6G and Rhodamine B EE which are structurally analogous to Oxazines 4 and 1, respectively, with an overall net charge of positive one. To investigate the impact of the net charge of the fluorophore on a molecule's nerve-specificity, additional structural analogs Rhodamine 19 and Rhodamine B were also selected, with overall net neutral charge at physiological pH. Oxazine 1, Oxazine 4, Rhodamine 6G and Rhodamine B were purchased and used as manufactured. Rhodamine B EE was prepared from Rhodamine B following Fisher's esterification protocol with an overall yield of 75%. Rhodamine 19 was synthesized from the hydrolysis reaction of Rhodamine 6G under base-catalyzed conditions with an overall yield of 81% (Scheme 1).

3.2 Optical and physicochemical properties comparison

The absorbance and fluorescence emission spectra of the Oxazine and Rhodamine fluorophores were collected in aqueous buffer containing 10% DMSO (Fig. 2). Their extinction coefficients ranged from 49,614 to 93,221 M⁻¹cm⁻¹ (Table 1). Both Oxazines 1 and 4 displayed broader absorbance peaks compared to all the rhodamines included in this study, with Oxazine 1 having the longest absorbance/emission wavelength at 655/671 nm, and Rhodamine 19 the shortest at 522/555 nm. At pH 7.4, Rhodamine 19 and Rhodamine B exhibited as their neutral/zwitterionic forms. Esterification of the benzoic acid moiety from the Rhodamine 19 and Rhodamine B afforded Rhodamine 6G and Rhodamine B EE with overall net charge going from neutral to positive one, and red shifted absorbance/emission by 5/15 nm and 6/33 nm, respectively. Stokes shifts ranged from 8 to 29 nm.

Physicochemical properties of all six compounds were calculated and evaluated to assess viability of candidate fluorophores for *in vivo* imaging according to the Lipinski and Veber rules (Table 1).^{18–19} Overall, their molecular weights were between 296 and 472 g/mol (Lipinski rule: <500 g/mol) and LogD values ranged from 2.48 to 7.67 (Lipinski rule: <5). Fluorophores had 0–2 hydrogen bond donors (Lipinski rule: <5) and 3–4 hydrogen bond

acceptors (Lipinski rule: <10) present. Calculated polar surface areas ranged from 28 to 74 Å² (Veber rule: <140 Å²).

3.2 *In vivo* nerve-specificity comparison

As a preliminary nerve-specificity test, a previously established direct/topical administration protocol was used to obtain tissue fluorescence intensities and to determine the nerve contrast. Nerves and surrounding tissues were surgically exposed, followed by local staining with direct contact of the contrast agent. This technique allowed assessment of nerve-specificity of a particular fluorescence imaging agent without the requirement of BNB penetration. Following direct administration, fluorescence images of exposed nerve, muscle, cut muscle, and adipose tissue were acquired (Fig. 3A and 3B). Tissue fluorescence intensities were quantified (Fig. 3C), and used to generate nerve-to-muscle (N/M) and nerve-to-adipose (N/A) contrast ratios as a metric of nerve contrast (Fig. 3D).

Overall, Rhodamine B showed the highest fluorescence signal in the nerve tissue, followed by Oxazine 4, while Rhodamine 19 provided the lowest nerve tissue intensity (Fig. 3C). Equilibria between the open quinoid and closed lactone form in the rhodamine scaffolds are commonly used for molecular sensing,²⁰ where the closed form is non-fluorescent and only absorbs in the ultraviolet wavelengths, as compared to fluorescent open quinoid form. Low tissue fluorescence intensities of Rhodamine 19 could be attributed to this equilibrium leaning towards the closed lactone form as the predominant species in the tissues. Nerve contrast comparison revealed that Rhodamine B had the highest averaged N/M at 4.27, followed by Oxazine 4 (N/M, 2.64). Interestingly, Oxazine 4 provided highest nerve contrast at 3.51, followed by Rhodamine B (N/A, 2.87) when adipose was used as the background tissue. The remaining four fluorophores (Oxazine 1, Rhodamine 6G, Rhodamine 19, and Rhodamine B) showed pan-tissue fluorescence without specific nerve contrast (Fig. 3D). In an effort to correlate nerve-specificity with the calculated physicochemical properties, we noted a general trend where the partition coefficient (lipophilicity) could potentially be the key factor controlling these fluorophore's nerve-specificity. Specifically, the LogD values for nerve-specific Oxazine 4 and Rhodamine B both fall within the 3–5 range, while the LogD values for the remaining molecules were all >5 . However, Rhodamine 19 was the exception to this rule. Moreover, following direct administration, Rhodamine 6G and Rhodamine B EE showed no nerve-specificity, similarly to the observed tissue staining pattern of Oxazine 1. Both rhodamines possessed an overall net charge of positive one, while the nerve-specific Rhodamine B had an overall net neutral/zwitterionic charge. The charge correlation to nerve-specificity of a particular fluorophore could be specific to certain fluorophore classes (e.g. specific to oxazine or rhodamine classes).

4. CONCLUSION

The mechanism of nerve tissue uptake and the biomolecular target of these nerve-specific fluorophores are currently unknown. However, it appears that the partition coefficient (LogD) and overall charge are crucial for nerve-tissue targeting and rapid off-target tissue clearance. The work presented here lays a foundation to direct further studies towards a more comprehensive understanding of the molecular characteristics and biomolecular targets

of these contrast agent, and to provide design considerations for the NIR tissue-specific contrast agent development. This study points to the particular importance of the overall net charge for tissue-specific uptake that has not been realized in the previous inherent tissue targeting agent development studies.

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Scheme 1.
Synthesis of Rhodamine 19 and Rhodamine B EE.

Figure 1.
Chemical structures of the Oxazine and Rhodamine fluorophores.

Figure 2.

Absorbance (*left*) and emission spectra (*right*) of the Oxazine and Rhodamine fluorophores in pH 7.4 buffer. Fluorophore concentration was 10^{-6} M in an aqueous solution contain 10% DMSO. Fluorophores were excited at 30–50 nm below the corresponding maximum absorbance wavelength. Absorbance and emission values were normalized to the maximum value for each curve and displayed as a percentage of the maximum.

Figure 3.

Direct administration of the Oxazine and Rhodamine fluorophores. Representative fluorescence images of the (A) Oxazine and (B) Rhodamine fluorophore stained tissues compared to unstained control tissue. (C) Quantified tissue fluorescence intensity and (D) Signal-to-background ratio comparison following direct administration of the Oxazine and Rhodamine fluorophores. All images are representative of data collected for n=6 nerve sites per fluorophore. All quantified data is presented as mean $\pm$ standard deviation.

Table 1.

Tabulated spectral and physicochemical properties of the Oxazine and Rhodamine fluorophores.

Compound ID	Max Abs (nm)	Max Em (nm)	Extinction coefficient ($M^{-1}cm^{-1}$)	Molecular weight (g/mol)	LogD (pH7.4)	Number of H bond donor/acceptor	Polar surface area ($\text{\AA}^2$)	Number of Rotatable bonds	Dipole moment (Debye)
Oxazine 1	655	671	93211	324.45	5.21	0/3	27.84	5	8.92
Oxazine 4	618	634	77156	296.39	4.26	2/3	47.59	3	8.81
Rhodamine 6G	527	556	73200	443.57	6.71	2/3	59.92	6	14.01
Rhodamine 19	522	550	71661	414.51	2.48	2/4	73.75	4	29.87
Rhodamine B EE	560	585	62488	471.62	7.67	0/3	41.78	8	13.38
Rhodamine B	544	552	49614	442.56	3.43	0/4	55.61	6	29.14