Journal of Fluorescence, Vol. 13, No. 1, January 2003 (© 2003)

Polystyrene Microspheres in Tissue-Simulating Phantoms Can Collisionally Quench Fluorescence

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Received June 3, 2002; revised August 20, 2002; accepted August 26, 2002

Tissue-simulating phantoms that replicate intrinsic optical properties in a controlled manner are useful for quantitative studies of photon transport in turbid biological media. In such phantoms, polystyrene microspheres are often used to simulate tissue optical scattering. Here, we report that using polystyrene microspheres in fluorescent tissue-simulating phantoms can reduce fluorophore quantum yield via collisional quenching. Fluorescence lifetime spectroscopy was employed to characterize quenching in phantoms consisting of a fluorescein dye and polystyrene microspheres (scattering coefficients $\mu_s \sim 100\text{-}600 cm^{-1}$). For this range of tissue-simulating phantoms, analysis using the Stern-Volmer equation revealed that collisional quenching by polystyrene microspheres accounted for a decrease in fluorescence intensity of 6-17% relative to the intrinsic intensity value when no microspheres (quenchers) were present. The intensity decrease from quenching is independent of additional, anticipated losses arising from optical scattering associated with the microspheres. These results suggest that quantitative fluorescence measurements in studies employing such phantoms may be influenced by collisional quenching.

KEY WORDS: Collisional quenching; tissue phantoms; time-resolved fluorescence lifetime spectroscopy; polystyrene microspheres; fluorescein dye.

INTRODUCTION

Methods of fluorescence spectroscopy and imaging are being investigated for potential applications to clinical biomedicine due to their ability to noninvasively probe biological tissues during a variety of diagnostic and therapeutic procedures [1–3]. Critical to the success of such methods will be the quantitative understanding of how optical signals propagate in turbid biological tissues, for example, by considering light-tissue interactions to be governed by radiative transport [3–5]. Optical tissue phantoms are artificial media designed to have independently controlled optical properties (e.g., absorption coefficient, scattering coefficient (μ_s), fluorophore quantum yield, and lifetime (τ_0) to experimentally simulate light propagation in biological tissues [6–8]. Such phantoms are widely used in controlled studies of light propagation within turbid media to quantify optical measurements in relation to theoretical predictions or to test biomedical optical instrumentation prior to clinical studies [3,9–14].

A variety of materials can be used to create tissue-simulating phantoms. For example, India ink and methylene blue are commonly used as absorbers [15,16]; polystyrene microspheres, silicon dioxide, and intralipid are often used as scatterers [10,17]; and many laser dyes, including rhodamine and fluorescein, are used as fluorophores [13,18]. Such phantom media are frequently used in liquid solution, but they may be cast into solid form by embedding the materials in a matrix of agarose [6,7].

Here, we report that using polystyrene microspheres

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1053-0509/03/0100–0105/0

as an optical scatterer in a fluorescent tissue phantom containing fluorescein dye can reduce the fluorophore quantum yield via collisional quenching. This reduction in fluorophore quantum yield will manifest itself as a shortened fluorophore lifetime and as a decreased remitted fluorescence intensity [19]. However, in a typical experiment using such a turbid tissue phantom for steady-state fluorescence intensity measurements, the effects of collisional quenching would be difficult to isolate from expected intensity losses arising from optical scattering associated with the microspheres, unless simultaneous measurements of fluorophore lifetimes were also performed [19]. Here, we describe time-resolved fluorescence measurements relating observed decreases in lifetimes in such tissue phantoms with increasing polystyrene microsphere (quencher) concentrations. We then discuss implications of these results to quantitative fluorescence studies using phantoms with polystyrene microspheres to control optical scattering.

EXPERIMENTAL AND RESULTS

Tissue-simulating phantoms were prepared by mixing solutions of fluorescein dye (Cat. No. F6377; Sigma-Aldrich, St. Louis, MO) dissolved in deionized (DI) water with suspensions of monodisperse 1-μm-diameter polystyrene microspheres (Cat. No. 07310; Polysciences, Warrington, PA) [14] and were held at room temperature (~20°C) for all measurements. Two sets of phantom media were prepared for the study. The first set comprised a series of five phantoms with fixed fluorophore concentration $\sim 1 \times 10^{-5}$ M and increasing concentrations of polystyrene microspheres: 10%, 15%, 22%, 33%, and 50 ± 1% by volume. The second set comprised a series of five phantoms with higher fixed fluorophore concentration $\sim 8 \times 10^{-5}$ M and increasing concentrations of polystyrene microspheres: 25%, 40%, 50%, 57%, and 63 ± 1% by volume. It was determined experimentally that both concentrations of fluorescein used (a) were devoid of artifacts arising from reabsorption (inner filter) effects [19] and (b) generated fluorescence signal strengths comparable to those emanating from tissue in vivo [20]. For both phantom sets, polystyrene microsphere concentrations were chosen to give optical scattering coefficients ($\mu_s \sim 100$–600 cm^{-1}) similar to the values reported for the optical scattering of a variety of biological tissues [11,14,21].

Time-resolved fluorescence measurements were obtained using a fluorescence lifetime spectrometer (FLS), described previously in detail [20]. Briefly, the FLS delivered excitation light at 337.1 nm from a pulsed nitrogen laser (VSL-337; Laser Science Inc., Franklin, MA) to the phantom medium via a fiber-optic probe. The remitted fluorescence decay was collected by the same fiber, filtered by a bandpass filter centered at 550 ± 10 nm to reject excitation light, and directed to an avalanche photodiode (APD) (C5658; Hamamatsu, Bridgewater, NJ) for detection. The transient response from the APD was digitized on a 1-GHz oscilloscope (TDS-680C; Tektronix, Willsonville, OR) to obtain the time-resolved fluorescence decay, which was then analyzed to remove the instrument response and extract the fluorescence lifetime τ of the fluorophore species [14,20]. For each phantom set, the intrinsic fluorophore lifetime, τ_0, was taken as the lifetime measured from a solution of fluorescein in DI water with no scatterers (quenchers) present.

Figure 1 shows the measured variation in fluorophore lifetime τ relative to the intrinsic lifetime τ_0 for the two sets of phantom media with fixed fluorescein dye concentrations: 1×10^{-5} M (circles) and 8×10^{-5} M (triangles). For the phantom set with lower fluorophore concentration (1×10^{-5} M), the measured lifetime τ clearly decreased with increasing polystyrene microsphere (quencher) concentration, indicating that the polystyrene microspheres were acting to collisionally quench the fluorescein fluorescence. In that phantom set, the measured decrease in lifetime τ relative to the intrinsic

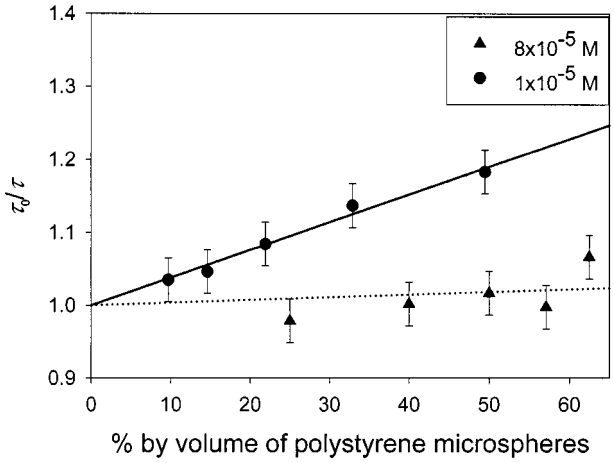

Fig. 1. Measured variation in fluorophore lifetime τ relative to the intrinsic lifetime τ_0 and versus microsphere (quencher) concentration for the two sets of phantom media with fixed fluorescein dye concentrations: 1×10^{-5} M (circles) and 8×10^{-5} M (triangles). The error bars associated with the plotted lifetimes reflect the standard deviation of three repeated measurements for each phantom medium. Each plotted data set was fit to a straight line that was analyzed using the Stern-Volmer equation describing collisional quenching. The slope of each line determined the Stern-Volmer quenching constant K_D for each phantom set: $K_D \sim 0.38$ (%vol)$^{-1}$ for fluorescein concentration 1×10^{-5} M (solid line) and $K_D \sim 0.02$ (%vol)$^{-1}$ for fluorescein concentration 8×10^{-5} M (dotted line).

value τ_0 ranged from 6% at the lowest microsphere concentration to 17% at the highest microsphere concentration. For the phantom set with higher fluorophore concentration (8×10^{-5} M), the measured lifetime decreased only slightly with increasing microsphere concentration. Each data set plotted in Figure 1 was fit to a straight line that was analyzed using the Stern-Volmer equation describing collisional quenching [19]

$$\frac{\tau_0}{\tau} = 1 + K_D[Q]$$

where K_D is the Stern-Volmer quenching constant and [Q] is the quencher (microsphere) concentration.

DISCUSSION AND CONCLUSIONS

Fluorescence quenching is defined as any physical mechanism resulting in a decrease of fluorescence intensity from a sample [19]. Collisional quenching of fluorescence refers to a process whereby an excited fluorophore molecule is de-excited nonradiatively to its ground state due to physical interactions (collisions) with some agent (quencher). Collisional quenching therefore reduces fluorophore quantum yield (the ratio of emitted fluorescence photons to absorbed excitation photons), resulting in a decrease in emitted fluorescence intensity, as well as a reduction in fluorophore excited-state lifetime. Because steady-state fluorescence intensity measurements will detect decreased fluorescence intensities from a variety of quenching mechanisms (e.g., static quenching or losses due to scattering), decreases in fluorescence lifetimes (which are insensitive to other forms of quenching) are considered to provide distinct proof of collisional quenching in a sample [19]. Figure 1 clearly demonstrates that the presence of 1-μm-diameter polystyrene microspheres reduced the quantum yield of 1×10^{-5} M fluorescein via collisional quenching, resulting in a fluorescence lifetime shortened by as much as 17% of the intrinsic lifetime when the microsphere (quencher) concentration was ~50% by volume ($\mu_s \sim 500$ cm^{-1}).

On increasing fluorescein concentration to $\sim 8 \times 10^{-5}$ M, the effects of collisional quenching were considerably reduced, with the fluorophore lifetime remaining nearly constant and independent of the amount of quencher (microsphere) present. This may be understood physically by considering that the quencher (~1-μm-diameter microsphere) is much larger than a fluorescein molecule (~20 Å), indicating that quenching should be ultimately limited (saturated) by the number of fluorescein molecules contacting the surface of a single microsphere (~10^6). For each phantom studied, we calculated the number of fluorescein molecules per unit microsphere (η_{ps}). At fluorescein concentration 1×10^{-5} M, where collisional quenching was manifest, η_{ps} ranged between $\sim 1.7 \times 10^6$ to 2.5×10^{-5}, approximately equal to or well below the saturation limit estimated above. However, at higher fluorescein concentration 8×10^{-5} M, η_{ps} ranged between 9.5×10^6 and 1.8×10^6, well above the saturation limit, thereby limiting the efficiency of collisional quenching in this phantom set. Thus, preparing tissue-simulating phantoms of fluorescein dye and polystyrene microspheres with η_{ps} values above the estimated saturation limit may reduce the effects of collisional quenching detected at lower η_{ps} values. If fluorophore concentration is increased to achieve this goal, samples should be carefully checked for other quenching artifacts arising from reabsorption (inner filter) effects [19].

Because collisional quenching reduces fluorophore quantum yield, fluorescence intensities F will decrease (relative to values obtained in the absence of quencher) in exact relation to decreases in fluorophore lifetime τ: i.e., $\tau_0/\tau = F_0/F$ [19]. Thus, for the range of 1×10^{-5} M fluorescein tissue-simulating phantoms studied here, collisional quenching by polystyrene microspheres accounted for a decrease of fluorescence intensity of 6–17% relative to the intensity value when no microspheres (quenchers) were present. The fluorescence intensity decrease caused by collisional quenching is independent of additional, anticipated losses arising from optical scattering associated with the microspheres, and accounted for 10–17% of the total detected loss in fluorescence in these tissue-simulating phantoms.

Although the present study identified the ability of polystyrene microspheres to collisionally quench fluorescence from fluorescein molecules, we note that the microspheres may have a similar effect on other commonly used tissue phantom fluorophores. For example, in a previous study employing polystyrene microspheres in fluorescent tissue phantoms using three standard laser dyes 3,3′-diethylthiatricarbocyanine iodide (DTTCI), IR-125, and IR-140, fluorophore lifetimes measured in the presence of microspheres decreased by 20%, 7%, and 18%, respectively, relative to the intrinsic fluorophore lifetimes obtained in the absence of microspheres [17]. The report suggested that these lifetime decreases might be attributed to varying amounts of water in the polystyrene microsphere suspensions used. Our present results suggest the possibility that collisional quenching by polystyrene microspheres might account for the decreases in fluorophore lifetimes detected in that previous study.

The results discussed here suggest that fluorescence measurements on tissue-simulating phantoms that use polystyrene microspheres to control optical scattering may be significantly affected by collisional quenching. The effects due to collisional quenching may be difficult to isolate in steady-state fluorescence intensity measurements on such turbid phantoms, because a decrease in detected fluorescence intensity is expected due to optical scattering alone. Measurements of fluorophore lifetimes versus polystyrene microsphere concentration, as presented here, provide a useful method of testing for collisional quenching in tissue-simulating phantoms prior to using the phantoms for quantitative fluorescence studies.

ACKNOWLEDGMENTS

This work was supported by American Cancer Society Institutional Research Grant IRG-82-003-17 (M.-A.M.), National Science Foundation Grant BES-9977992 (M.-A.M.), and The Whitaker Foundation (M.-A.M.).

REFERENCES

1. R. Richards-Kortum and E. Sevick-Muraca (1996) Quantitative optical spectroscopy for tissue diagnosis. *Annu. Rev. Phys. Chem.* **47**, 555–606.
2. G. Wagnieres, W. Star, and B. Wilson (1998) In vivo fluorescence spectroscopy and imaging for oncological applications. *Photochem. Photobiol.* **68**, 603–632.
3. B. B. Das, L. Feng, and R. R. Alfano (1997) Time-resolved fluorescence and photon migration studies in biomedical and model random media. *Rep. Progr. Phys.* **60**, 227–292.
4. S. Chandrasekhar (1960) *Radiative Transfer.* Dover, NY
5. A. J. Welch and M. J. C. van-Gemert (1995) *Optical-Thermal Response of Laser-Irradiated Tissue*. Plenum Press, New York.
6. A. J. Durkin, S. Jaikumar, and R. Richards-Kortum (1993) Optically dilute, absorbing, and turbid phantoms for fluorescence spectroscopy of homogeneous and inhomogeneous samples. *Appl. Spectrosc.* **47**, 2114–2121.
7. J. C. Hebden, D. J. Hall, M. Firbank, and D. T. Delpy (1995) Time-resolved optical imaging of a solid tissue-equivalent phantom. *Appl. Optics* **34**, 8038–8047.
8. K. Rinzema, L. H. P. Murrer, and W. M. Star (1998) Direct experimental verification of light transport theory in an optical phantom. *J. Optical Soc. Am.* **15**, 2078–2088.
9. A. E. Cerussi, J. S. Maier, S. Fantini, M. A. Franceschini, W. W. Mantulin, and E. Gratton (1997) Experimental verification of a theory for the time-resolved fluorescence spectroscopy of thick tissues. *Appl. Optics* **36**, 116–124.
10. J. R. Mourant, T. Fuselier, J. Boyer, T. Johnson, and I. Bigio (1997) Predictions and measurements of scattering and absorption over broad wavelength ranges in tissue phantoms. *Appl. Optics* **36**, 949–957.
11. A. Sefkow, M. Bree, and M.-A. Mycek (2001) A method for measuring cellular optical absorption and scattering evaluated using dilute cell suspension phantoms. *Appl. Spectrosc.* **55**, 1495–1501.
12. M. Patterson, B. Chance, and B. Wilson (1989) Time resolved reflectance and transmittance for the non-invasive measurement of tissue optical properties. *Appl. Optics* **28**, 2331–2336.
13. E. M. Sevick-Muraca, J. S. Reynolds, T. L. Troy, G. Lopez, and D. Y. Paithankar (1998) *Adv. Optical Biopsy Optical Mammogr.* **838**, 46–57.
14. K. Vishwanath, B. W. Pogue, and M.-A. Mycek (2002) Quantitative fluorescence lifetime spectroscopy in turbid media: Comparison of theoretical, experimental and computational methods. *Phys. Med. Biol.* **47**.
15. S. A. Ramakrishna and K. D. Rao (2000) Estimation of light transport parameters in biological media using coherent backscattering. *Pramana J. Phys.* **54**, 255–267.
16. S. J. Madsen, M. S. Patterson, and B. C. Wilson (1992) The use of India ink as an optical absorber in tissue-stimulating phantoms. *Phys. Med. Biol.* **37**, 985–993.
17. C. L. Hutchinson, T. L. Troy, and E. M. Sevick Muraca (1996) Fluorescence-lifetime determination in tissues or other scattering media from measurement of excitation and emission kinetics. *Appl. Optics* **35**, 2325–2332.
18. L. Wang, D. Liu, N. He, S. L. Jacques, and S. L. Thomsen (1996) Biological laser action. *Appl. Optics* **35**, 1775–1779.
19. J. R. Lakowicz (1999) *Principles of Fluorescence Spectroscopy.* 2nd ed. Kluwer Academic/Plenum, New York.
20. J. D. Pitts and M.-A. Mycek (2001) Design and development of a rapid acquisition laser-based fluorometer with simultaneous spectral and temporal resolution. *Rev. Sci. Instrum.* **72**, 3061–3072.
21. W.-F. Cheong, S. Prahl, and S. Welch. (1990) A review of the optical properties of biological tissues. *IEEE J. Quantum Electr.* **26**, 2166–2185.