Journal of Fluorescence, Vol. 13, No. 6, November 2003 (©2003)

Selective Dequenching by Photobleaching Increases Fluorescence Probe Visibility

K. E. Roberts,[1,2] A. K. O'Keeffe,[1] C. J. Lloyd,[1] and D. J. Clarke[1]

Received August 31, 2003; accepted September 15, 2003

Self-quenching properties of fluorescent dyes have been developed to improve the sensitivity of fluorescent measurement. Photobleaching of Calcein at a concentration greater than the critical, self-quenching concentration actually increased fluorescence, whereas at lower concentrations photobleaching decreased fluorescence, enhancing signal to noise by almost 4000. The photobleaching-dequenching principle has been demonstrated in giant liposomes encapsulating Calcein at higher quenched concentrations. Upon photobleaching background fluorescence was reduced and the liposome fluorescence increased. Liposomes invisible in the presence of background fluorescence became visible upon photobleaching. Fluorescent lifetime was unaffected by photobleaching, whereas the lifetime decreased significantly upon dilution, allowing distinction between photobleached fluorescence particularly upon dequenching. The principle may be suited to improving fluorescence imaging and resolving fluorescent probes in particle-based assays.

KEY WORDS: Self-quenching; photobleaching; fluorescent lifetime; calcein; liposomes.

INTRODUCTION

Particularly where fluorescence is used for image contrast and where small volumes or particles are analysed, background autofluorescence of the sample or materials used to hold the sample continues to be a significant problem.

Fluorescent dyes also suffer from quenching, from dissolved gases, solvent and other compounds in the sample, and many dyes undergo self-quenching at higher concentrations. Self- or proximity-quenching is often avoided in solutions of dyes by use of concentrations less than those where self-quenching affects fluorescence. This is an important aspect of the standardisation of fluorescence assays. However, when multiple fluorophores bind at high local concentrations or where they are attached or encapsulated in a particle within a few nanometers of each other they usually begin to quench one another [1]. Such quenching occurs mostly in dyes which have a small Stokes shift where their absorbance and emission spectra overlap, and is greater for dyes with a broad emission peak.

As high sensitivity detectors are now commonplace, background fluorescence is the most significant practical problem affecting detection of small single particles or molecules. The contribution of background fluorescence from the sample or solution may be reduced by the use of small detection volumes because the signal from a single molecule or particle is independent of the volume, whereas the relative fluorescence from the suspending background solution is proportional to the detection volume [2]. Detection volume often cannot be made insignificantly small and further problems arise when close to the diffraction limit. Consequently, background fluorescence often remains the major factor affecting detection limits. Spectral filters are also a useful means of reducing the contribution of background fluorescence, and fluorescence lifetime measurements are also an effective temporal filter. In some cases, it has been shown that the contribution of unbound

[1] School of Pharmacy and Pharmaceutical Sciences, University of Manchester, Manchester, United Kingdom.
[2] To whom correspondence should be addressed at Green 2 Tutors Flat, Owens Park, 293 Wilmslow Road, Fallowfield, Manchester M14 6HD, United Kingdom. E-mail: karen.e.roberts@man.ac.uk.

1053-0509/03/1100-0513/0 © 2003 Plenum Publishing Corporation

fluorescence, including unbound fluorescence may be reduced by first bleaching the buffers and materials used in the analysis [2] which has been reported for use in flow cytometry [3]. Bleaching of reagents may not always be effective, particularly when the sample has significant autofluorescence and when significant fluorescence remains unbound or leaks from the probe.

We demonstrate, using Calcein as a model, the enhancement of the sensitivity of fluorescent detection by the use of photobleaching to reduce the self-quenching and increase the fluorescence of the probe relative to background fluorescence, which is further enhanced by fluorescent lifetime measurement.

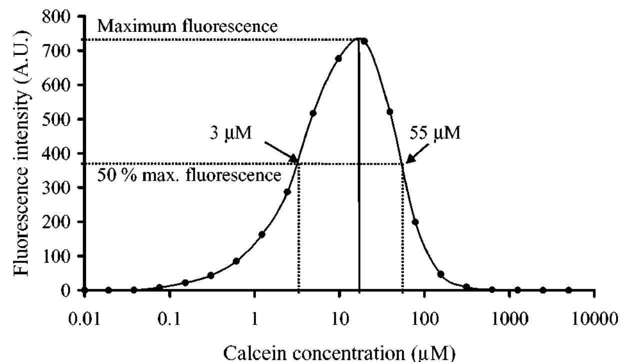

Fig. 1. Self-quenching of calcein fluorescence, indicating the concentrations (3 and 55.2 μM) used in subsequent photobleaching studies.

EXPERIMENTAL

Calcein, phosphatidyl choline, cholesterol, phosphatidyl glycerol, triolein and sucrose were used as purchased from Sigma (Dorset, UK). All other reagents were of analytical reagent grade.

Dilutions of Calcein were prepared in Phosphate Buffered Saline (PBS) buffer solution pH 7.4 from 100 mM stock solutions (unless stated otherwise).

Measurements of quenching were performed using a Perkin Elmer Luminescence Spectrometer LS50-B, with excitation (490 nm) and emission (520 nm) slits set at 5 nm. Photobleaching was undertaken using either a Spectra-Physics argon ion laser (488 nm), or a 0.23 J guided Xenon flashlamp (Oriel Instruments, Surrey, UK).

Fluorescence was recorded at the same wavelengths using a Hitachi F-2000 spectrophotometer, with excitation slits set at 10 nm and emission slits set at 20 nm. Following such UV exposure, the cuvette was inverted gently to minimise introduction of air bubbles when the fluorescence spectrum was collected immediately. Fluorescence lifetime measurements were carried out using IBH (Glasgow, UK) instrumentation. Samples were excited using an IBH NanoLED (peak wavelength of 490 nm) with a >530 nm filter in the emission path, and a neutral density (ND) filter in the excitation path (both Edmund Industrial Optics, York, UK). Time-correlated single photon counting (TCSPC) lifetime data was collected and DAS 6 analysis software (IBH) was used to determine lifetimes.

Calcein (100 mM)-encapsulating liposomes were prepared based on the method described by Akashi *et al.* [4]. Solutions (10 mL) of lipids were prepared separately in chloroform and diethyl ether, each containing 10 mg phosphatidylcholine, 4 mg cholesterol, 5 mg phosphatidylglycerol and 2 mg triolein (2.2 μL, liquid dispensed at room temperature). Three aliquots of (each 1 mL) of 100 mM Calcein in PBS, pH 7.4 were added to the lipid solution in chloroform with manual swirling between each addition, when the mixture was vortex-mixed (Vortex Genie2, Scientific Industries, N.Y. USA) horizontally at top speed for 45 s to form an emulsion. Lipids in diethyl ether (0.5 mL) were then added to 25 mM calcein in PBS, pH 7.4 (6 mL), and similarly vortex-mixed for 15 s. Emulsion in chloroform (1 mL) was then added to the ether mixture and immediately followed by similar vortex mixing for a further 10 s. The double emulsion was then transferred to a flat-bottomed (8 cm diameter) 250 mL conical flask, and shaken gently in a thermocirculating water bath at 37°C whilst passing oxygen-free nitrogen over the gas space for 45 min at a flow rate of between 1 and 2 litres per minute. The calcein-containing liposomes were diluted with PBS (5 mL), divided into aliquots (1 mL) and centrifuged (Denver Instruments microcentrifuge) at 1,000 G for 20 min at room temperature. Each pellet was resuspended in PBS pH 7.4 (1 mL), followed by further similar centrifugation at 1,000 G for 20 min. This was repeated until calcein was not visually apparent in the supernatant.

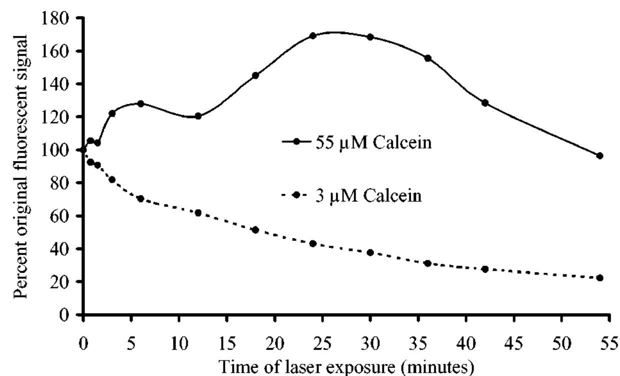

Fig. 2a. Laser photobleaching of the selected calcein solutions (3 and 55 μM, see text for details).

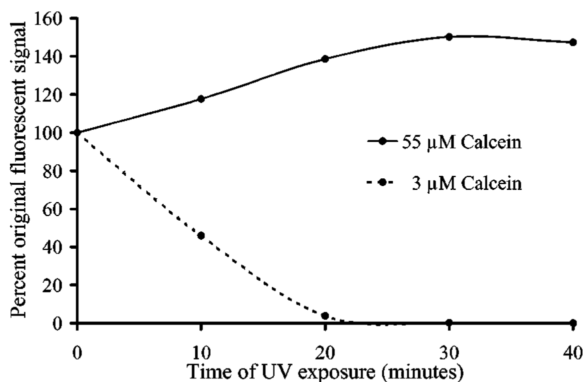

Fig. 2b. Xenon lamp photobleaching of the selected calcein solutions (3 and 55 μM, see text for details).

Microscopic studies of the liposomes were undertaken using a confocal microscope comprising of a BioRad MRC1024 MP confocal scanning head mounted on a Nikon Eclipse TE300 fluorescence microscope fitted with a Nikon Plan APO 60x oil immersion lens, with an American Laser Corporation krypton/argon ion laser as the excitation source, operating at a wavelength of 488 nm and 0.3% power. The system was computer-controlled using BioRad LaserSharp software running on a Dell Pentium 2 (450 MHz) PC. Gain was set at 1000, iris set at 2.2. For photobleaching, the same laser was used, but at higher (3%) power.

RESULTS AND DISCUSSION

The fluorescence of calcein solutions increased until a critical concentration (19 μM), when followed a decrease in fluorescence intensity (Fig. 1) due to self-quenching. In order to study photobleaching above and below the critical concentration for quenching, calcein solutions at 3 μM and 55 μM were chosen, each demonstrating approx. 50% of the maximum fluorescence. Samples (2 mL) of each were exposed in a fluorescence cuvette (1 cm path length) to irradiation from the laser (488 nm) (Fig. 2a) and the xenon flash lamp (Fig. 2b).

After 24 min laser exposure, the fluorescence of 55 μM calcein was approximately 170% of its original level. After the same length of time, however, the fluorescence of the 3 μM calcein solution had fallen to approximately 40% of its original level. A similar effect occurred when using the xenon lamp, after 20 min, the fluorescence of the 3 μM calcein solution had fallen to approximately 4% of its original level, whereas the 55 μM calcein solution has correspondingly increased to approximately 140% of the original level. It would appear that, when solutions of self-quenched calcein are exposed to photobleaching conditions, their fluorescence actually increases.

Self-quenched calcein was then incorporated into particles, using giant liposomes to allow microscopic examination following exposure to photobleaching conditions. From the photographs (Fig. 3a), photobleaching of relatively high background calcein (2 μM) fluorescence can be clearly seen, when fluorescence of the liposome particles remain visible. In order to observe the increase in fluorescence of the self-quenched calcein liposome particles upon exposure to photobleaching conditions, related studies were performed in the absence of background fluorescence. Upon photobleaching of calcein liposomes suspended in PBS, fluorescence of those exposed to laser irradiation clearly increased (viz. encircled region of photographs in Fig. 3b). Prior to laser exposure, the liposome particles were barely visible (left, Fig. 3b), whereas laser

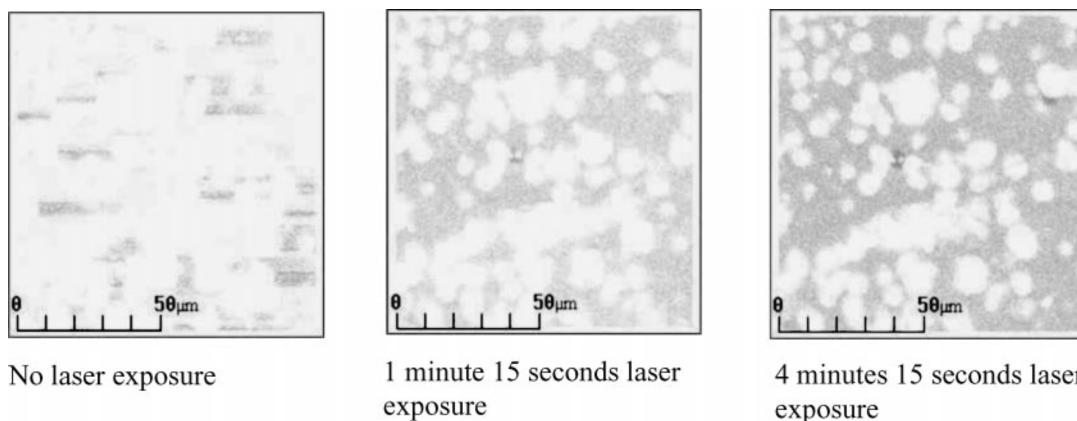

Fig. 3a. Microscopy of laser photobleaching of background fluorescence (2 μM calcein) to reveal calcein liposomes.

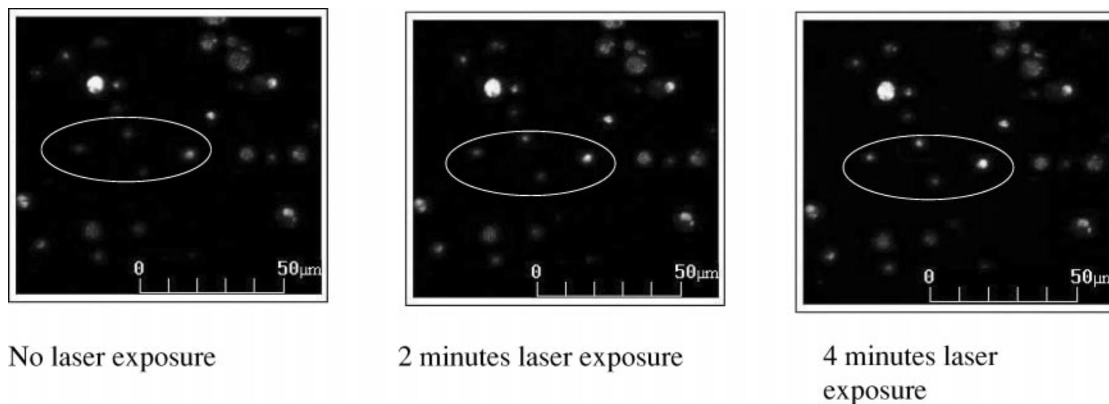

Fig. 3b. Microscopic observation of increased calcein liposome fluorescence upon photobleaching.

exposure led to much increased visibility of the liposome particles (right, Fig. 3b).

The fluorescence lifetimes of calcein solutions show sharp increases (Fig. 4) corresponding to the self-quenching concentrations (compare Fig. 3). However, following UV exposure and photobleaching of various concentrations of calcein, there was no significant change in fluorescence lifetime measured (Fig. 4).

Usually when a fluorophore is in the presence of a quencher the fluorescent lifetime decreases. At high concentrations of calcein the measured lifetime of the solution was longer than that at lower concentrations. Although the lifetime of more concentrated calcein solutions becomes longer, it is well known that there is no significant shift in the maximum emission wavelength. While self-quenching resulting from re-absorption of emitted photons by neighbouring fluorophores would be more probable at higher concentrations of calcein, photobleaching of calcein appeared to have no effect on its lifetime. If photons were being reabsorbed and re-emitted prior to detection in the case of the photobleached solutions, then the lifetime might be expected to be longer. However, this does not appear be the case. If photobleached calcein fluorophores have entered a trapped state, then one or more radiationless energy transition may occur [5]. This would account for the lack of effect of photobleaching on the fluorescent lifetime of calcein, as well as the difference in fluorescence intensity observed. Whatever the mechanism, it is apparent that compartments of photobleached self-quenched calcein (e.g. in the liposome particles) may be distinguished from photobleached calcein in background solution at lower concentration on the basis of measurement of fluorescent lifetime.

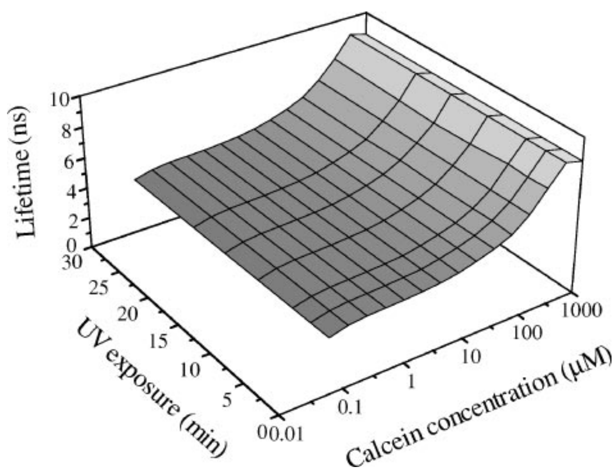

Fig. 4. Fluorescence lifetime of Xenon lamp photobleached calcein solutions.

CONCLUSION

By encapsulating self-quenched fluorescent dye, the signal to noise ratio can be significantly enhanced through photobleaching of the entire sample prior to measurement, thereby reducing the background fluorescence level to a minimum and increasing the particle-entrapped fluorescence. In the case of giant liposomes containing calcein at self-quenching concentrations, the ratio of increased fluorescence of the particles and photobleached suspending medium could approach the order of 4000 following photobleaching. This observation could be useful for improving the sensitivity of particle-based assays, and for improving the visibility of fluorescence probes encapsulated or binding at high local concentrations in microscopic studies.

Fluorescent lifetime measurement also appeared to distinguish between photobleached and non-

photobleached samples, suggesting such measurements could be further used to resolve the difference between fluorescence probes upon photobleaching. These studies are not inconsistent with calcein self-quenching involving a radiationless process, as opposed to reabsorption of emitted photons.

ACKNOWLEDGMENTS

This work was supported by ManPharm Ltd., Multiplex Photonics Ltd., and a BBSRC postgraduate studentship.

REFERENCES

1. R. Y. Tsien and A. Waggoner (2001). In J. B. Pawley (Ed.), *Handbook of Biological Confocal Microscopy*, Plenum Press, New York. pp. 267–279.
2. W. P. Ambrose, P. M. Goodwin, J. H. Jett, A. Van Orden, J. H. Werner, and R. A. Keller, (1999). Single molecule fluorescence spectroscopy at ambient temperature. *Chem. Rev.* **99**, 2929–2956.
3. R. L. Affleck, W. P. Ambrose, J. D. Demas, P. M. Goodwin, J. A. Schecker, M. Wu, and R. A. Keller (1996). Reduction of luminescent background in ultrasensitive fluorescence detection by photobleaching. *Anal. Chem.* **68**, 2270–2276.
4. K. Akashi, H. Miyata, H. Itoh, and K. Kinosita Jr. (1996). Preparation of giant liposomes in physiological conditions and their characterisation under an optical microscope. *Biophys. J.* **71**, 3242–3250.
5. J. B. Birks (1969). *Photophysics of Aromatic Molecules*, Wiley, London, UK.