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Fluorescence studies of a local anesthetic–phospholipid interaction

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Abstract

Steady-state and time-resolved fluorescence data are reported for the local anesthetic dibucaine in the absence and presence of phospholipid vesicles. These vesicles were comprised of dimyristylphosphatidyl choline and ~ 10% dimyristylphosphatidyl glycerol. Solute quenching studies show the bound drug to be protected from collision with iodide ion. The fluorescence lifetime of dibucaine is not significantly changed upon binding to vesicles. The fluorescence anisotropy of dibucaine increases upon association with the vesicles. Anisotropy decay measurements show that the rotational correlation time, ϕ, of bound dibucaine is increased about one hundred fold over that for free dibucaine. This indicates that the rotational motion of bound dibucaine is slowed by its interaction with the phospholipids. However, we find no evidence that the rotational motion of bound dibucaine is anisotropic.

Keywords: Dibucaine; Phospholipids; Unilamellar vesicles; Fluorescence quenching; Fluorescence lifetime; Fluorescence anisotropy decay

1. Introduction

A preceding manuscript [1] reports studies of the thermodynamics of the interaction of the cationic, amphipathic local anesthetic, dibucaine, with model phospholipid membranes. It was shown that there is weak intrinsic affinity of dibucaine for phospholipid vesicles and that interaction is greatly influences by electrostatic interactions, which include the charge repulsion between neighboring bound dibucaine molecules and the attraction between cationic dibucaine and negatively charged polar head groups (i.e., phosphatidylglycerol). The present study takes advantage of the intrinsic fluorescence properties of dibucaine to further investigate the nature of the dibucaine–phospholipid complexes. Specifi-

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Abbreviations used: α_i, pre-exponential factor in intensity decay law; DMPC, (α-L-dimyristylphosphatidylcholine; DMPG, α-L-dimyristylphosphatidylglycerol; f_i, fractional intensity of ith component to the steady-state fluorescence spectrum; F_0 and F, steady-state fluorescence intensity in the absence and presence of quencher; g_i, fractional degree of depolarization due to the ith rotational process; k_q, solute quenching rate constant; K_i, Stern–Volmer quenching constant; ONS, *N*-octadecyl-naphthyl-2-amino-6-sulfonic acid; POPOP,; ϕ_i, rotational correlation time for ith rotational process; τ_i, ith fluorescence decay time; [Q], solute quencher concentration; r, fluorescence anisotropy, r_0, limiting fluorescence anisotropy in the absence of motion.

cally, we have used steady-state and time-resolved fluorescence measurements to obtain information about the topography (i.e., degree of solvent exposure) and rotational dynamics of the bound drug molecules. The preceding thermodynamics study provides a guide for selecting conditions in which most of the dibucaine molecules will be associated with vesicles (a condition usually achieved by adding 10–20% DMPG to DMPC vesicles to impart an attractive negative surface potential).

Various fluorescence techniques have been used to study the interaction of fluorescent probes, drugs, antibiotics, and ionophores with bilayers [2–19]. Among these techniques have included solute quenching of the bound probe, resonance energy transfer from the probe in question to a second probe of known location in a bilayer, and time resolved intensity and anisotropy decay measurements [6,7,11,15–19]. Many of the referenced studies involve fluorescence probes that have a very high affinity for the membrane bilayer or which have a much higher fluorescence intensity in the bound state, so that the fluorescence contribution from molecules free in solution can be neglected. For molecules such as dibucaine, which are amphipathic, have only moderate affinity for membranes, and fluoresce in the aqueous state, it is important to assess the degree of binding (as we have done in the preceding study) and to recognize the fluorescence contribution from free molecules.

The fluorescence properties of aqueous dibucaine has been well characterized by Vanderkooi, Lin and coworkers [20–22]. Mertz and Lin [21] have reported a fluorescence study of dibucaine–detergent micelle complexes.

To characterize the topography of the dibucaine–phospholipid complexes, we have used the solute quencher, KI, to probe the accessibility of the bound drug. Also, we have exploited the ability of dibucaine to act as a quencher of the fluorescence of the lipid probe, *N*-octadecylnaphthyl-2-amino-6-sulfonic acid (ONS), embedded in vesicles' bilayers, in order to determine whether dibucaine permeates vesicles. Finally we have performed time-resolved fluorescence intensity and anisotropy decay measurements to characterize the rotational motion of the bound fluorescent drug.

2. Experimental section

2.1 Materials

L-α-Dimyristylphosphatidylcholine (DMPC) and L-α-dimyristylphosphatidylglycerol (DMPG), dibucaine-HCl, and Fisk–Subbarow reducing agent were obtained from Sigma Chemical Co. Single lamellar phospholipid vesicles were prepared using the procedure of Barenholz and coworkers [24]. The lipid phosphate concentration was determined using the method of Bartlett [25]. *N*-Octadecylnaphthyl-2-amino-6-sulfonic acid (ONS) was obtained from INC Pharmaceuticals, Inc. 1,4-Bis(5-phenyloxazol-2-yl)-*O*-benzene (POPOP), and methyl viologen dichloride were obtained from Aldrich Chemical Company, Inc. The rest of the reagents, ammonium molybdate, sulfuric acid, sodium acetate, and KI (recrystallized from 1:10 water:ethanol), were analytical grade reagents. Distilled deionized water was used in all experiments.

2.2 Methods

Fluorescence quenching measurements were made by adding aliquots of a concentrated solution of quencher (i.e., 5 *M* KI) to about 2 ml of a sample contained in a fluorescence cell. Steady-state fluorescence measurements were made with either a Perkin-Elmer MPF44A or a SLM fluorometer. With the latter instrument, interference filters were used to isolate the excitation wavelength and the emission was observed through band pass filters. Temperature of the samples was controlled using a thermoregulated cell holder. Data were analyzed by fitting (by using a non-linear least squares program) with the following general form of the Stern–Volmer equation [26].

$$\frac{F}{F_0} = \sum_i \frac{f_i}{(1 + K_i[\mathrm{Q}])\exp(V_i[\mathrm{Q}])} \qquad (1)$$

where K_i and V_i are the dynamic and static quenching constants for component i, [Q] is the quencher concentration, and F_0 and F are the fluorescence in the absence and presence of quencher, respectively. The quenching rate constant, k_q, is calculated as $k_q = K_i/\tau_i$, where τ_i is the fluorescence lifetime (see below).

Measurements of steady-state fluorescence anisotropy were usually made with the above Perkin-Elmer fluorometer. Correction for the photomultiplier response to vertically and horizontally polarized light was made in the calculation of anisotropy, r, values. For the measurement of the anisotropy excitation spectrum of dibucaine in vitrified solvent (propylene glycol at −50°C), a SLM fluorometer was used with a quartz finger dewar.

Time-resolved fluorescence lifetime measurements were made with an ISS phase/modulation fluorometer, equipped with a 20 W argon ion source. The laser line at 351.5 nm was used for excitation and emission was collected through a 450 nm band pass filter. A pockels cell modulator was used to modulate the excitation beam in the range of 5–200 MHz. Temperature was controlled with a thermoregulated cell holder. A sample of POPOP in ethanol was used as a reference ($\tau = 1.35$ ns). Phase/modulation data were analyzed in terms of the following intensity (I) decay law

$$I(t) = I_0 \sum \alpha_i \, e^{-t/\tau_i} \qquad (2)$$

where τ_i is the fluorescence lifetime and α_i the normalized amplitude for component i. Data were analyzed using either software from ISS Inc. or GLOBALS Unlimited [27]. In some cases the baseline subtraction was performed in the fluorescence lifetime measurements, to correct for possible contributions from scattered light from the phospholipid vesicles. This baseline subtraction was found not to make a significant difference in the lifetime parameters.

Differential polarized phase/modulation (anisotropy decay) data were obtained using the same instrument. Data were analyzed using the following anisotropy, r, decay law

$$r(t) = r_0 \sum g_i \, e^{-t/\phi_i} \qquad (3)$$

where ϕ_i and $g_i r_0$ are the rotational correlation time and amplitude for component i. Again, a baseline subtraction procedure was used.

3. Results and discussion

3.1 Topography of bound dibucaine

Several fluorescence measurements were made to assess the solvent exposure of vesicle's bound dibucaine.

The fluorescence spectrum of dibucaine is blue-shifted from 410 nm to 390 nm upon binding to the DMPC:DMPG (90:10) sonicated vesicles at pH 5.0, 35°C. (The λ_{max} of bound dibucaine was estimated from measurement in the presence of 5×10^{-3} M phospholipid.) The λ_{max} shift is similar to that reported for the binding of other fluorescent drugs to phospholipid vesicles [26] and indicates that the bound quinoline ring of dibucaine experiences an environment that has a lower polarity than the aqueous medium.

Solute quenching by KI was measured for dibucaine in the absence and presence of 1×10^{-3} M DMPC:DMPG (80:20) at 35°C. As shown in Fig. 1, I^- is a very effective quencher of free dibucaine. Analysis of these data with eq. (1) gave a dynamic and static quenching constant of 48 and 3.3 M^{-1}, respectively (see Table 1). From the fluorescence lifetime of 3.3 ns for free dibucaine (see next section), the quenching rate constant, k_q, is calculated to be 14.5×10^9 M^{-1} s^{-1}, which is somewhat larger than expected for a diffusion limited reaction. We suspect that this larger value is due to an attraction between the anionic quencher and cationic fluorophore. In the presence of 1×10^{-3} M phospholipid, quenching by KI is greatly reduced. This amount of phospholipid is not enough to bind all of the dibucaine (see Fig. 4). Therefore, we fitted eq. (1) to these data with the assumption that there is a free dibucaine component and that it has a dynamic quenching constant, K_1, of 48^{-1}. With this assumption, analysis yielded a K_2 (for bound dibucaine) of 2.2 M^{-1}, with $f_1 = 0.55$ (free) and $f_2 = 0.45$ (bound). Thus, upon binding to the vesicles, the I^- quenching constant is reduced by a factor of over 20 fold. Later we show that the fluores-

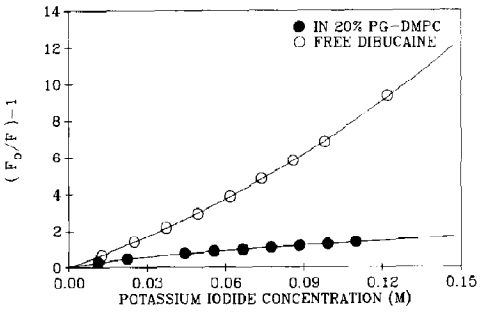

Fig. 1. Stern–Volmer plots of fluorescence quenching of dibucaine by potassium iodide at pH 5 (0.05 *M* sodium acetate) and 35°C. The solid lines are theoretical fits of eq. (1) with the parameters listed in Table 1.

cence lifetime of bound dibucaine is decreased only slightly from the value for free dibucaine. The decrease in dynamic quenching constant reflects a decrease in the quenching rate constant, k_q, when dibucaine is bound. Since the vesicle has a negative surface charge, due to the presence of DMPG, an electrostatic, as well as steric burial, may contribute to this reduction in k_q.

A question regarding the topography of bound dibucaine is whether the drug binds only to the outer monolayer of the vesicle or whether it also crosses the bilayer and binds to the inner monolayer. (Our thermodynamic studies in the previous manuscript are unable to directly address this question; we determined binding isotherms data,

Table 1

Stern–Volmer parameters for the fluorescence quenching of free and bound dibucaine by KI [a]

Condition	K_1 (M^{-1})	f_1	K_s (M^{-1})	f_2
Without lipid	48.3±0.5 (3.3) [b]	1.0	–	–
With lipid	48 [c] (1.5) [b]	0.55	2.2±0.1 (0.44) [b]	0.45

[a] Conditions, pH 5, 35°C. Data in the absence and presence of 1×10^{-3} *M* DMPC:DMPG (80:20).

[b] Static quenching constants, included to improve the fits of eq. (1). Fits were obtained by a non-linear least squares analysis; the parameters listed give a minimum sum of squares of residuals.

[c] Value fixed to be approximately the same as in the absence of lipid.

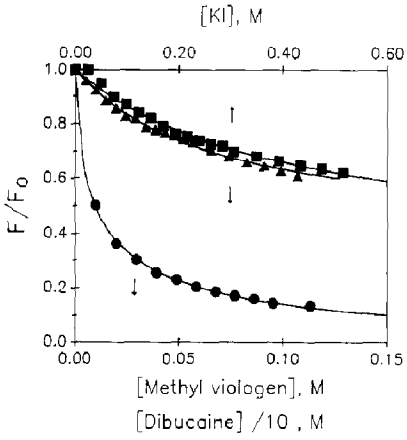

Fig. 2. Fluorescence of ONS in DMPC vesicles as a function of added dibucaine (●), KI (■) and methylviologen dichloride (▲). Note the appropriate abscissa for each quencher; the concentrations of dibucaine are 1/10th the values on the abscissa. The solid lines are a fit of the Stern–Volmer (actually its reciprocal) with the following parameters: methyl viologen, $K_1 = 15.0\pm0.3\ M^{-1}$, $f_1 = 0.61$, $K_2 = 0$, $f_2 = 0.39$. KI, $K_1 = 2.76\ M^{-1}$, $f_1 = 0.61$ (assumed), $K_2 = 0.14\ M^{-1}$, $f_2 = 0.39$; dibucaine, $K_1 = 2400\ M^{-1}$, $f_1 = 0.61$ (assumed), $K_2 = 238\ M^{-1}$, $f_2 = 0.039$.

that is, the moles of bound drug per mole of phospholipid, with no distinction between external and internal binding sites.) To try to experimentally determine whether dibucaine binds to the internal monolayer, we studied the ability of dibucaine to quench the fluorescence of ONS, a fatty acid analog which can be incorporated into both monolayers of a vesicle. As shown in Fig. 2, the fluorescence of ONS (mixed with lipid prior to sonication) in DMPC vesicles is almost completely quenched by the addition of dibucaine. For comparison, the addition of two other quenchers, KI and methyl viologen dichloride (a dicatonic bipyridine), quenches only about half of the fluorescence, in both cases. An analysis of the data for methyl viologen with Stern–Volmer equation (1) indicates that 39% (i.e., $f_1 = 0.39$) of the fluorescence of vesicle-associated ONS is completely inaccessible ($K_2 = 0$) to this quencher. A similar analysis of the KI data is consistent with a 39% unquenchable ONS component (although a free floating non-linear least squares analysis of equation 1 will not recover this f_1 value). The value of $f_2 = 0.39$ is approximately

the fraction of ONS molecules that is expected to exist in the inner monolayer of a small sonicated vesicle, assuming a random distribution of ONS between the monolayers. The fact that 100% of the fluorescence of ONS is quenched by dibucaine indicates that it must come into contact with ONS on both the outside and inside of the vesicle. (Note that there is not sufficient spectral overlap for dibucaine the act as an energy transfer quencher; quenching by dibucaine must involve close contact.) This observation does not prove that dibucaine binds to the inner monolayer, but it is consistent with dibucaine being able to penetrate the bilayer and to exist inside the vesicles.

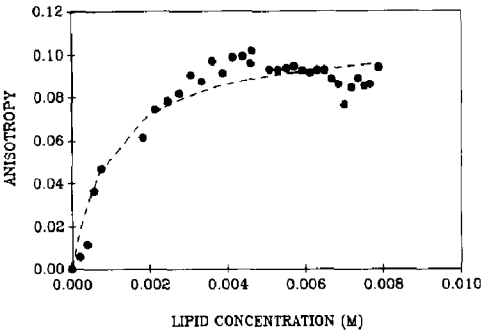

Fig. 4. Fluorescence anisotropy of dibucaine as a function of added 10%DMPG/90%DMPC at pH 5 (0.05 *M* sodium acetate) and 35°C. The solid line is the theoretical fit with a Langmuir binding isotherm.

3.2 Dynamics of bound dibucaine

Information about the rotational dynamics of bound dibucaine can be obtained from fluorescence anisotropy studies. First we show in Fig. 3 the excitation anisotropy spectrum of dibucaine in a vitrified solvent. This shows that at wavelengths above 320 nm the steady-state fluorescence anisotropy of immobilized dibucaine approaches the maximum value of 0.4. Between 280–300 nm the anisotropy of dibucaine is negative. This indicates the existence of a higher energy electronic transition that contributes to the absorption in this region and that has an orientation that is nearly orthogonal to the lowest energy electronic transition. The spectrum shows that excitation above 320 nm will primarily populate the lowest S_1 state.

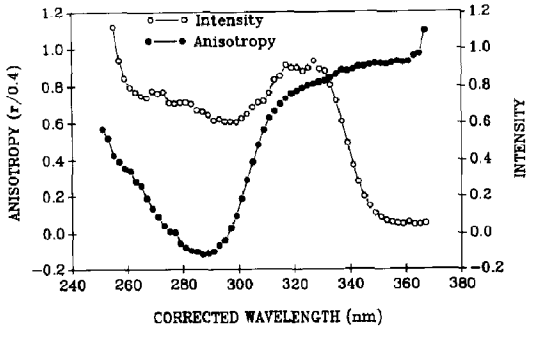

Fig. 3. Fluorescence anisotropy (•) and emission intensity (○) of dibucaine as a function of excitation wavelength (in propylene glycol at −50°C). The excitation spectrum was corrected using Rhodamine B as quantum counter. Note that the ordinate values have been divided by the theoretical maximum of 0.4.

In aqueous solution the steady-state fluorescence anisotropy of free dibucaine is essentially zero, due to its rapid rotational motion. The addition of DMPC : DMPG (90 : 10) vesicles causes an increase in the anisotropy of dibucaine, as shown in Fig. 4, as expected due to the binding process and to the hindrance of motion of the bound state.

Time-resolved anisotropy decay data can more clearly reveal details of the rotational motion of bound dibucaine. Before presenting such data we first report intensity decay data for dibucaine in the absence and presence of DMPC:DMPG (90:10). The decay of free dibucaine is a mono-exponential with $\tau = 3.33$ ns, as shown in Fig. 5. In the presence of 1×10^{-3} *M*, 2×10^{-3} *M*, and 3.8×10^{-3} *M* phospholipid, the decay still appears to be a mono-exponential and the apparent fluorescence lifetime shortens slightly (i.e., lifetimes are 2.96 ns, 2.79 ns, and 3.05 ns in the presence of the above three phospholipid concentrations).

Differential polarized phase/modulation (anisotropy decay) data are shown in Fig. 6 for free and bound dibucaine. (For both the intensity and anisotropy decay data, a baseline correction routine was employed to subtract the small contribution from light scattered by the vesicle suspensions.) Using the above fluorescence lifetime pa-

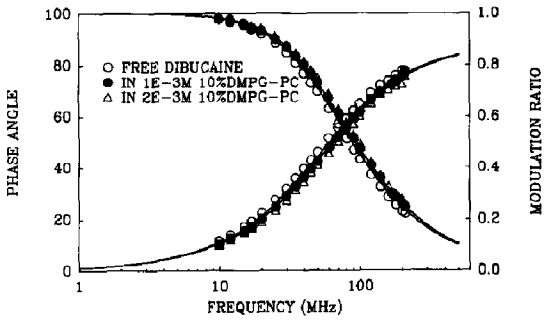

Fig. 5. Phase/modulation fluorescence lifetime data for dibucaine at pH 5 (0.05 *M* sodium acetate) and 35°C. The solid lines are the theoretical fits to eq. (2) to a mono-exponential decay law. The descending curves are modulation ratios and the ascending curves are phase angles.

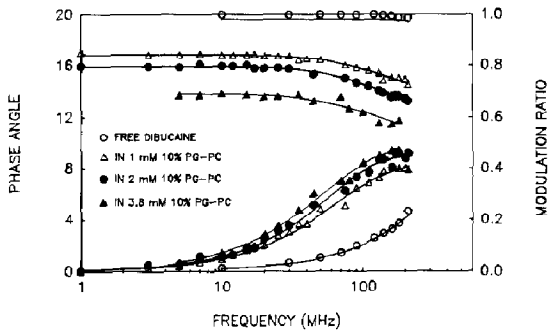

Fig. 6. Differential polarized phase/modulation anisotropy decay for dibucaine as it binds to 10%DMPG/90%DMPC at pH 5 (0.05 *M* sodium acetate) and 35°C. The solid lines are the theoretical fits to eq. (3). The descending curves are modulation ratios and the ascending curves are phase angles.

rameters, the anisotropy decay data were analyzed using eq. (3) to obtain the rotational correlation times, ϕ_i, and $r_0 g_i$ values in Table 2. Two rotational correlation times are needed for the fits. We have fixed the short ϕ value to be that for free dibucaine, since the degree of saturation of dibucaine by the vesicles should be approximately 50%, 65%, and 82% at 1×10^{-3} *M*, 2×10^{-3} *M*, and 3.8×10^{-3} *M* DMPC:DMPG (90:10), respectively. The analysis shows the ϕ value for the bound component to be between 2–5 ns. A global analysis of the data sets was performed for a three component model. The resulting fitting parameters are given in Table 2. The bound drug is described as having a long rotational correlation time of 4.6 ns and a short rotational correlation time of 0.73 ns.

The longest rotational correlation time, ϕ_3, is about 100× longer than that for rotation of dibucaine in aqueous solution. This indicates some restriction of the motion of dibucaine when it is bound to the vesicles. However, this ϕ_3 value is much smaller than the value expected for overall rotation of a small unilamellar vesicle. The absence of such a very long rotational correlation time indicates that the rotational motion of bound dibucaine is essentially isotropic. If the rotational motion were anisotropic with respect to the surface of the bilayer, then a very long ϕ (or, in other words, a non-zero r_∞) would have been expected, as, for example, is the case for probes such as diphenylhexatriene, which reflect the or-

Table 2

Anisotropy decay parameters for free and membrane bound dibucaine [a]

[Phospholipid] (m*M*)	ϕ_1 (ns)	$r_0 g_1$	ϕ_2 (ns)	$r_0 g_2$	ϕ_3 (ns)	$r_0 g_3$	χ^2
0	0.06	0.35					2.6
1.0	⟨0.06⟩	0.312	2.36	0.115			0.9
2.0	⟨0.06⟩	0.24	2.29	0.160			0.6
3.8	⟨0.06⟩	0.47	4.77	0.195			4.8
Global analysis							
1.0	⟨0.06⟩	0.087	0.733	0.094	4.59	0.063	0.61
	⟨0.06⟩	0.024	0.733	0.111	4.59	0.094	
	⟨0.06⟩	0.396	0.733	0.020	4.59	0.193	

[a] For data obtained at 35°C, pH 5.0. The phospholipid was DMPC:DMPG (90:10). The values of ϕ_1 in angled brackets were fixed in the analysis. The global analysis was performed over three data sets.

der of the fatty acid chains of the phospholipids [7,9,16]. Thus, the rotational motion of dibucaine is restricted in an isotropic manner, which suggests that a bound dibucaine molecule does not have a strongly preferred orientation with respect to the bilayer surface.

Cationic drugs such as dibucaine are thought to interact with phospholipid bilayers at the polar head group region, with some intercalation into the bilayer. There is little direct, structural data for the interaction of dibucaine with bilayers. A previous ^{1}H-NMR nuclear Overhauser effect measurement by Kuroda and Fujiwara [29] with this drug was interpreted in terms of the interaction of the protonated ammonium group of dibucaine with the anionic phosphate group of a phospholipid and the wedging of the quinoline ring between the upper portions of the fatty acid chains. Our observations, that bound dibucaine (a) has a reduced accessibility to solvent and solute quenchers, due to either steric or electrostatic shielding, and (b) has an increased rotational correlation time, are consistent with this model, with the caveat that the quinoline ring can make sufficient rotational excursions on its fluorescence time scale so that it is less ordered than the phospholipids.

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References

1 S. Barghouthi, R. Puri and M.R. Eftink, Biophys. Chem. 46 (1992) 1–11.
2 P. Wooley, Biophys. Chem. 10 (1979) 289–303.
3 C.L. Bashford, B. Chance, J.C. Smith and T. Yoshida, Biophys. J. 25 (1979) 63–85.
4 R. Goldman, T. Facchinetti, D. Bush, A. Raz and M. Shinitzky, Biochim. Biophys. Acta 512 (1978) 254–289.
5 S. Kawato, K.J. Kinosita and A. Ikegami, Biochemistry 16 (1977) 2319–2324.
6 E.A. Haigh, K.A. Thulborn and W.H. Sawyer, Biochemistry 18 (1979) 3525–3532.
7 F.G. Prendergast, R.P. Haugland and P.J. Callahan, Biochemistry 20 (1981) 7333–7338.
8 W.K. Surewicz and W. Leyko, Biochim. Biophys. Acta 643 (1981) 387–397.
9 R.F. Kauffman, C.J. Chapman and D.R. Pfeiffer, Biochemistry 22 (1983) 3985–3992.
10 A. Theretz, J. Teissie and J.-F. Tocanne, Eur. J. Biochem. 1942 (1984) 113–119.
11 E.A. Griffin, J.M. Vanderkooi, G. Maniara and M. Erecinska, Biochemistry 25 (1986) 7875–7880.
12 P. Chatelain, J. Ferreira, R. Laurel and J.M. Roysschaert, Biochem. Pharmacol. 35 (1986) 3007–3013.
13 A. Chattopadhyay and E. London, Biochemistry 26 (1987) 39–45.
14 M.T. Allen, L. Miola and D.G. Whitten, J. Am. Chem. Soc 110 (1988) 3198–3206.
15 C.E. Kung and J.K. Reed, Biochemistry 25 (1986) 6114–6121.
16 H. Szmacinski, R. Jayaweera, H. Cherek and J.R. Lakowicz, Biophys. Chem. 27 (1987) 233–241.
17 T. Parasassui, G. De Stasio, A. d'Ubaldo and E. Gratton, Biophys. J. 57 (1990) 1179–1186.
18 G.E. Dobretsov, N.K. Kurek, V.N. Machov, T.I. Syrejshchikova and M.N. Yakimenko, J. Biochem. Biophys. Methods 19 (1989) 259–274.
19 B.W. Williams and C.D. Stubbs, Biochemistry 27 (1988) 7994–7999.
20 G. Vanderkooi, Photochem. Photobiol. 39 (1984) 755–762.
21 C.T. Lin, M. Malak, G. Vanderkooi and W.R. Mason, Photochem. Photobiol. 45 (1987) 749–755.
22 C.T. Lin, L.N. Williamson, Y.G. Chyan and A.D.S. Margues, Photochem. Photobiol. 48 (1988) 733–740.
23 C.J. Mertz and C.T. Lin, Photochem. Photobiol. 53 (1991) 307–316.
24 Y. Barenholz, D. Gibbes, B.J. Litman, J. Goll, T.E. Thompson and F.D. Carlson, Biochemistry 16 (1977) 2806–2810.
25 G.R. Bartlett, J. Biol. Chem. 234 (1959) 466–468.
26 M.R. Eftink, in: Fluorescence in biochemistry and cell biology, ed. G. Dewey (Plenum Press, New York, 1991) pp. 1–41.
27 J.M. Beechem, E. Gratton, M. Ameloot, J.R. Knutson and L. Brand, in: Topics in fluorescence spectroscopy, Vol. 2, ed. J.R. Lakowicz (Plenum Press, New York, 1991) pp. 241–305.
28 T. Guilmin, E. Goormaghtigh, R. Brasseur, J. Caspers and J.M. Ruysschaert, Biochim. Biophys. Acta 685 (1982) 169–176.
29 Y. Kuroda and Y. Fujiwara, Biochim. Biophys. Acta 903 (1987) 395–410.