Pergamon

Journal of Pharmaceutical & Biomedical Analysis, Vol. 12, No. 3, pp. 307–312, 1994

0731-7085/94 $6.00 + 0.00

Effects of surfactants on the spectral behaviour of calcein

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Abstract: The spectral behaviour of calcein, a water-soluble self-quenching molecule that is often used as a marker in biomedical analysis, can be considerably affected by the presence of surfactants. The aim of this work was to investigate the spectral properties of calcein under conditions that are particularly significant in liposome studies. For this purpose the fluorescence and absorbance of this dye were determined in solutions of ionic and non-ionic surfactants (cetyltrimethylammonium bromide, sodium dodecyl sulphate and Triton X-100), at different concentrations below and above their critical micelle concentrations (c.m.c.) as well as in the presence of phospholipids in the form of liposomes and/or mixed micelles. Cationic surfactants induced changes in λ_{max}, absorbance and fluorescence but these changes were less noticeable in the phospholipid dispersions. The anionic and the non-ionic surfactants induced mainly changes in fluorescence intensity.

Keywords: *Calcein; surfactants; phospholipids; fluorescence changes; absorbance changes; liposomes; mixed micelles.*

Introduction

Quantitative determinations of fluorescent probes with self-quenching properties are often used for the evaluation of stability and entrapment capacity of liposomes [1, 2]. Often these measurements are carried out in the presence of surfactants that can be used for different purposes, such as vesicle preparation [3], release of the probes from the aggregated structures and complete rupture of liposomes [4–6]; furthermore, surfactants can be included in cosmetic formulations containing liposomes.

It has already been pointed out that the spectral behaviour of fluorescent dyes can be affected by the presence of surfactants [7, 8] and the observed effects have been related to the interaction between micelles and dye molecules [9].

This paper reports the fluorescence and absorbance behaviour of calcein [10], a water-soluble self-quenching molecule often used as a marker in liposome studies [11] and also for investigations on membrane properties [12]. Experiments were performed in the presence of: ionic and non-ionic surfactants at different concentrations (below and above their c.m.c.); phospholipids in the form of vesicles; and phospholipids and surfactants (vesicles and mixed micelles).

Materials and Methods

Crystalline calcein was purchased from Sigma. Enriched soya phosphatidylcholine (90% pure; phospholipon 90, Nattermann Phospholipids GmbH; P90) was employed for vesicle preparations because this product leads to liposomes that were previously studied [13] and that are sufficiently stable without the addition of other substances (e.g. cholesterol) that can interfere with the determinations. Triton X-100, sodium dodecyl sulphate (SDS), cetyltrimethylammonium bromide (CTAB), all other surfactants and products used for the present investigation were of analytical grade.

A HEPES buffer solution (pH 7.5; 10^{-3} M), prepared with freshly distilled and de-aerated water, was used as the solvent. In most cases, measurements were repeated in distilled water for an appropriate comparison.

The absorbance was determined with a Perkin Elmer Lambda 3A spectrophotometer equipped with 10-mm quartz cells. Fluor-

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escence measurements were carried out by means of a Perkin Elmer LS5 spectrofluorometer whose signal was appropriately attenuated with a filter in order to use the same sample employed for absorbance determinations. Calcein absorbance and fluorescence were determined at a fixed dye concentration in order to avoid the variations of the absorption spectrum that are related to its concentration; therefore the value of 10^{-5} M (i.e. just before self-quenching) was chosen [14]. The turbidity was evaluated with the same LS5 instrument, by setting both excitation and emission wavelengths at 600 nm. Sonication was performed with a Soniprep 150 apparatus (MSE, Crowley) equipped with an exponential probe, operating at 23 kHz and an amplitude of 6 μm.

Vesicles containing calcein were prepared according to the following procedure. An exactly weighed amount of P90 was completely dissolved in 4–5 ml of methanol. The solvent was evaporated under vacuum to form a thin film of phospholipids; calcein (10^{-5} M) in HEPES buffer was then added and the mixture was gently shaken for 1 h. Sonication was finally carried out, under a nitrogen stream, for 40 min (8 times for 5 min). The temperature was maintained at 15–20°C by means of a water-bath. Liposome dispersions were then diluted with the 10^{-5} M calcein solution containing the calculated amount of surfactant to obtain, as previously described [13], the final phospholipid concentration of 0.3 mg ml^{-1}. The same procedure was carried out for the preparation of the reference liposome dispersions without calcein.

It has been pointed out [14] that sonication of phospholipid dispersions leads mainly to small unilamellar vesicles (SUV, 10–100 nm), but according to the aim of this study, neither actual liposome sizes nor their loading capacity were evaluated.

The c.m.c. of surfactants was determined from measurements of Sudan III solubility at 25°C in HEPES or in water solutions containing increasing amounts of the detergents. Dye concentrations were determined from the absorbance value of the tested solutions at the appropriate wavelength. The same experiments were performed in the presence of P90.

All reported data represent the mean values obtained from at least three separate experiments that always presented good reproducibility.

Results and Discussion

A preliminary determination of the c.m.c. for CTAB, SDS and Triton X-100 indicated that for the non-ionic surfactant its value is the same in water, in HEPES and in the presence of P90. Both anionic and cationic surfactants showed, as expected, lower c.m.c. values in the buffer solution as well as in the phospholipid dispersion (Table 1). Moreover, in the presence of phospholipids the term 'critical micellar concentration' is to be considered less significant because of the presence of liposomes and/or mixed micelles, according to the surfactant concentration, as discussed below.

Turbidity variations were measured in order to study the solubilization of phospholipid vesicles by the tested surfactants (Figs 1–3). After an initial increase, the turbidity decreased gradually and almost linearly. The increase of turbidity observed at low surfactant concentrations was interpreted as an incorporation, without disruption, of surfactant monomers by the liposomes; the further addition of surfactant led to a decrease of turbidity related to the fragmentation of the vesicle structure and finally to the formation of mixed micelles [5, 13, 17]. For all tested surfactants complete disintegration of liposome structures occurred well above their c.m.c.; concentrations of about 10 times the c.m.c. were needed for CTAB and SDS,

Table 1
The c.m.c. of surfactants determined under different conditions

Type of surfactant	c.m.c. in water* (mM)	c.m.c. in HEPES (mM)	c.m.c. in HEPES and P90 (0.3 mg ml^{-1}) (mM)
CTAB	1.0	0.2	0.2
SDS	8.0	6.0	1.0
Triton X-100	0.25	0.25	0.25

*These values are in good agreement with those reported in the literature [8, 15].

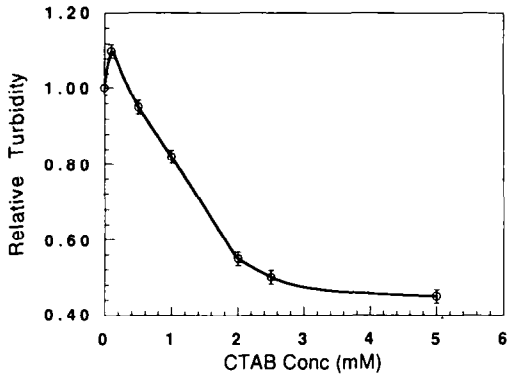

Figure 1
Effect of increasing CTAB concentration on the turbidity of liposome dispersions. Turbidity changes are expressed as the ratio of the value observed in the presence of surfactant to that of the reference (i.e. without surfactant).

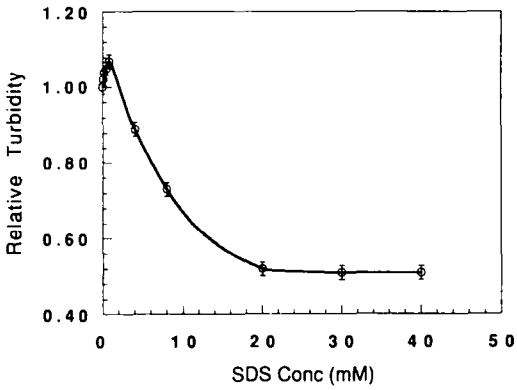

Figure 2
Effect of increasing SDS concentration on the turbidity of liposome dispersions. Turbidity changes are expressed as the ratio of the value observed in the presence of surfactant to that of the reference.

whereas in the case of Triton X-100 a concentration 100 times above the c.m.c. must be attained in order to have only mixed micelles.

Cationic surfactant

The absorption spectrum of calcein was significantly affected by the presence of CTAB. The range of concentrations that was investigated (0.1–5 mM) was limited by CTAB solubility. Figure 4 indicates how the addition of this cationic surfactant caused a bathochromic shift of the absorbance maximum (λ_{max}) that reached about 13 and 7 nm, in HEPES and in water, respectively. This shift remained almost constant above the surfactant c.m.c. It is interesting that a similar shift of λ_{max} can also be obtained, without surfactant, by increasing the dye concentration in solution, i.e. by affecting its dissociation [14].

Besides this variation of the maxima, a slight decrease (never more than 4%) of absorbance values, determined at the corresponding λ_{max}, was detected only below the c.m.c. values, whereas the absorbance remained constant at higher concentrations.

For fluorescence measurements the observed shift in λ_{max} led to an increase of the wavelength corresponding to the maximum of emission (from 516 up to 527 nm) and, at the same time, the fluorescence intensity considerably decreased with a minimum corresponding to c.m.c. This decreased fluorescence was observed using either an excitation wavelength corresponding to the absorption maximum previously evaluated, as reported in Fig. 5, or the fixed value of 491 nm.

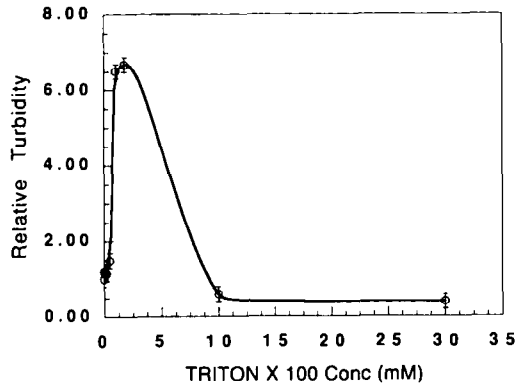

Figure 3
Effect of increasing Triton X-100 concentration on the turbidity of liposome dispersions. Turbidity changes are expressed as the ratio of the value observed in the presence of surfactant to that of the reference.

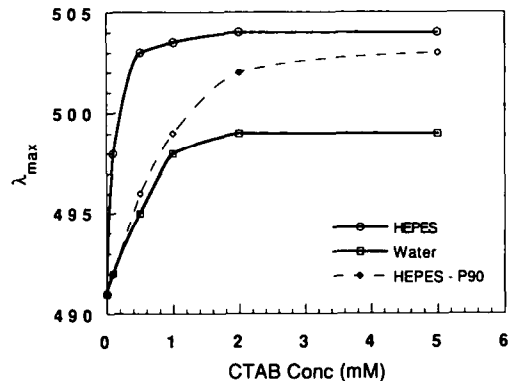

Figure 4
Effect of increasing CTAB concentration on the absorbance maxima (λ_{max}) of 10^{-5} M calcein in water, in the pH 7.5 HEPES (10^{-3} M) buffer alone and in the presence of a sonicated dispersion of P90 (0.3 mg ml^{-1}).

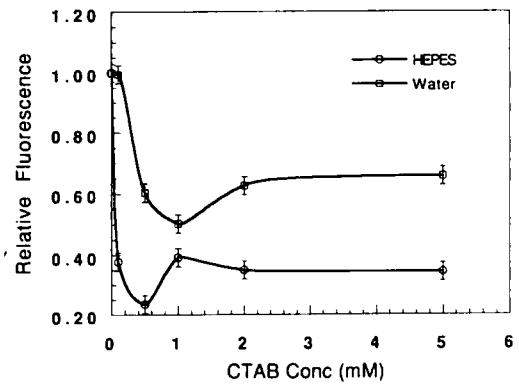

Figure 5
Effect of increasing CTAB concentration on the fluorescence intensity of 10^{-5} M calcein in water and in the pH 7.5 HEPES (10^{-3} M) buffer. Fluorescence changes are expressed as the ratio of the value observed in the presence of surfactant to that of the reference. The absorbance λ_{max} was used as the excitation wavelength and each time the emission wavelength was appropriately checked. The values refer to the corresponding maximum value obtained.

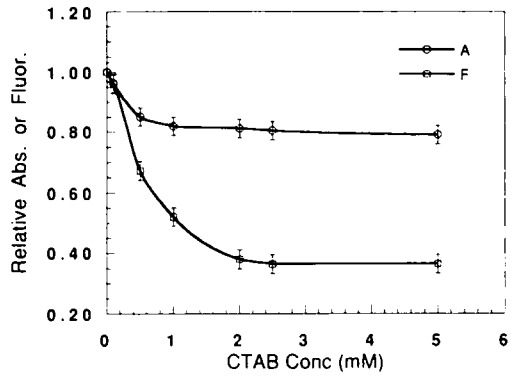

Figure 6
Effect of increasing CTAB concentration on the absorbance (*A*) and fluorescence (*F*) values of 10^{-5} M calcein in the pH 7.5 HEPES (10^{-3} M) buffer in the presence of a sonicated dispersion of P90 (0.3 mg ml^{-1}). Absorbance and fluorescence changes are expressed as the ratio of the value observed in the presence of surfactant to that of the reference. The absorbance was determined at the corresponding λ_{max} value and this was also used as the excitation wavelength in fluorescence measurements. Each time the emission wavelength was appropriately checked and the values refer to the corresponding maximum value obtained.

It must be pointed out that effects on λ_{max}, absorption and fluorescence similar to those induced by CTAB were obtained also in the presence of other cationic surfactants like benzalkonium chloride; in contrast, the presence of other substances having the same charge but not behaving as surfactants (e.g. trimethylammonium and triethylammonium chloride/bromide) did not affect the spectral behaviour of calcein. It may consequently be assumed that the observed effects cannot be simply related to electrostatic interactions between the dye and the surfactant molecules.

The same measurements on the spectral behaviour of calcein were repeated in the presence of P90, after sonication. Results obtained in the HEPES buffer are reported in Fig. 4 where it is shown how the λ_{max} value again increased with increasing CTAB concentration. At the same time, fluorescence and absorbance decreased accordingly (Fig. 6). All the observed variations were this time less sharp than those detected without phospholipids.

A comparison between turbidity and spectral behaviour indicates that the main variations in the photophysical properties of calcein took place before the complete transformation of liposomes into mixed micelles.

Anionic surfactant

In Figs 7 and 8 the absorbance and fluorescence of calcein determined in the presence of an anionic surfactant (SDS) are reported, respectively. The experimental conditions were the same as those reported above, but a larger range of surfactant concentrations (1–40 mM) was investigated in order to include its c.m.c. value. Absorbance measurements were performed at 491 nm because SDS, below and above its c.m.c., did not affect the λ_{max} value of calcein; 491 nm was also used as the excitation wavelength for fluorescence measurements. It was observed that in the HEPES solution the presence of this anionic surfactant did not induce appreciable variations of absorbance and fluorescence, but in water both absorbance and fluorescence intensity appreciably decreased with increasing SDS concentration.

The difference in spectral behaviour between the buffered and the non-buffered calcein solution can be simply interpreted in terms of SDS hydrolysis [18] which affects pH and consequently the spectrum of the dye [10]. The same experiments carried out without this surfactant but in calcein solutions, buffered at the same value as a 2×10^{-3} M SDS solution in water, gave results that were similar to those obtained with SDS in water.

As expected, in sonicated dispersions of P90 in HEPES, no appreciable changes in fluorescence or absorbance were detected.

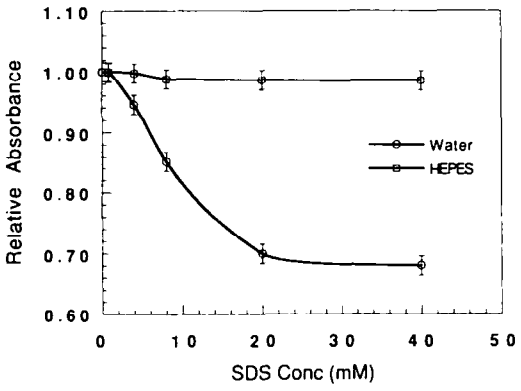

Figure 7
Effect of increasing SDS concentration on the absorbance of 10^{-5} M calcein in water and in the pH 7.5 HEPES (10^{-3} M) buffer. Absorbance changes are expressed as the ratio of the value observed in the presence of surfactant to that of the reference. The absorbance was determined at the wavelength of 491 nm.

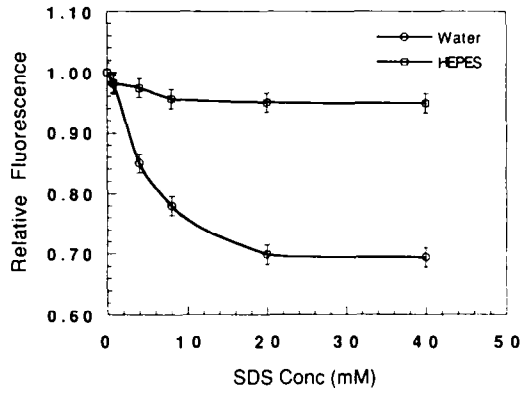

Figure 8
Effect of increasing SDS concentration on the fluorescence intensity of 10^{-5} M calcein in water and in the pH 7.5 HEPES (10^{-3} M) buffer. Fluorescence changes are expressed as the ratio of the value observed in the presence of surfactant to that of the reference. Excitation and emission wavelengths were 491 and 516 nm, respectively.

Non-ionic surfactant

Within a Triton X-100 concentration of 0.05–1 mM, no variations of calcein absorption maximum and absorbance values were detected. On the other hand, a sharp decrease of fluorescence intensity ($\lambda_{ex} = \lambda_{max}$; λ_{em} = 516 nm) was correspondingly observed in the presence of this non-ionic surfactant (Fig. 9). This decrease, that was less in HEPES than in water, starts well below the Triton c.m.c. and then remains almost constant.

When the same measurements were performed after sonication in the presence of P90, no variations of the results reported in Fig. 9 were observed.

Results so far reported refer to experiments performed a few minutes after the preparation

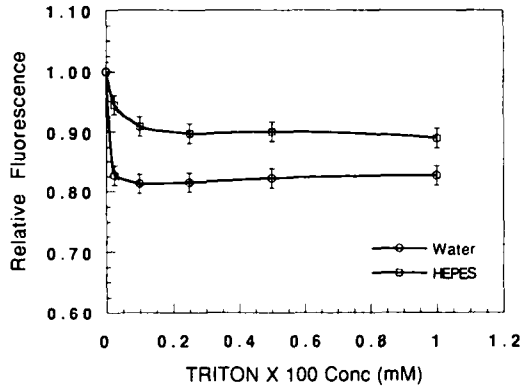

Figure 9
Effect of increasing Triton X-100 concentration on the fluorescence intensity of 10^{-5} M calcein in water and in the pH 7.5 HEPES (10^{-3} M) buffer. Fluorescence changes are expressed as the ratio of the value observed in the presence of surfactant to that of the reference. Excitation and emission wavelengths were 491 and 516 nm, respectively.

of the samples; however, it must be considered that micelles and, even more, liposomes may undergo considerable structural variations with time and that spectral properties of the dye interacting with such aggregated forms could be correspondingly affected. Consequently, λ_{max}, absorbance and fluorescence were again determined, on each sample, 24 and 72 h after its preparation. In no case were appreciable variations of these spectral properties detected.

Conclusions

The observed changes in the spectral behaviour of calcein in surfactant solutions can be explained mainly in terms of micellar effects [8]. However, the reported results showed that the photophysical properties of calcein were influenced even below the c.m.c. This effect, in the case of hydrophobic fluorescent probes, was explained by the formation of premicellar aggregates [8]. For calcein, i.e. a hydrophilic molecule, the different shape of some curves (CTAB in water, in HEPES or in the presence of P90) can be related to the slight but significant reduction of c.m.c. induced by the buffer and/or by the phospholipids [7, 18]. This suggested hypothesis is considered to be in accordance with the observation that the presence of substances with the same charge of CTAB but not leading to the formation of micelles showed no effect on calcein spectral behaviour. Besides a complete explanation of the mechanisms involved, these results clearly indicate how the presence of surfactants may

remarkably affect the absorbance and fluorescence behaviour of a dye that often must be quantitatively determined for the evaluation of the properties of phospholipid vesicles. Thus, for these systems, absorbance and fluorescence should be carefully checked each time under the appropriate experimental conditions in order to calculate the actual dye concentrations.

References

[1] J.M.R. Eftink, in *Biophysical and Biochemical Aspects of Fluorescence Spectroscopy* (T.G. Dewey, Ed.), pp. 1–41. Plenum Press, New York (1991).

[2] G. Strauss, F. Alhaique, A. Memoli, E. Santucci and F.M. Riccieri, *Biophys. J.* **53**, 130 (1988).

[3] A. Baillie, A.T. Florence, L.R. Hume, G.T. Muirhead and A. Rogerson, *J. Pharm. Pharmacol.* **37**, 863–868 (1985).

[4] Y. Nagawa and S.L. Regen, *J. Am. Chem. Soc.* **114**, 1668–1672 (1992).

[5] M.T. Paternostre, M. Roux and J.L. Rigaud, *Biochemistry* **27**, 2668–2677 (1988).

[6] J. Ruiz, F.M. Goñi and A. Alonso, *Biochim. Biophys. Acta* **937**, 127–134 (1988).

[7] A.K. Mathur, C. Agarwal, B.S. Pangtey, A. Sing and B.N. Gupta, *Int. J. Cosmet Sci.* **10**, 213–218 (1988).

[8] Y.H. Paik and S.C. Shim, *J. Photochem. Photobiol. A, Chem.* **56**, 349–358 (1991).

[9] N.J. Turro, M. Grätzel and A.M. Braun, *Angew. Chem. Int. Ed. Engl.* **19**, 675–698 (1980).

[10] T.M. Allen, in *Liposome Technology*, Vol. III (G. Gregoriadis, Ed.), pp. 177–182. CRC Press, Boca Raton, FL (1984).

[11] N. Weinstein, F. Ralston, L.D. Leserman, R.D. Klausner, P. Dragsten, P. Henkart and R. Blumenthal, in *Liposome Technology*, Vol. III (G. Gregoriadis, Ed.), pp. 183–204. CRC Press, Boca Raton, FL (1984).

[12] S.A. Weston and C.R. Parish, *Cytometry* **13**, 739–749 (1992).

[13] A. Memoli, L.G. Palermiti, V. Travagli and F. Alhaique, *J. Soc. Cosmet. Chem.* **44**, 123–128 (1993).

[14] H. Diehl and N. Horchak-Morris, *Talanta* **34**, 739–741 (1987).

[15] H. Ringsdorf, B. Schlarb and J. Venamer, *Angew. Chem. Int. Ed. Engl.* **27**, 113–158 (1988).

[16] D. Lichtenberg, R.J. Robson and E.A. Dennis, *Biochim. Biophys. Acta*, **737**, 285–304 (1983).

[17] K. Anzai, H. Utsumi, K. Inoue, S. Nojima and T. Kwan, *Chem. Pharm. Bull.* **28**, 1762–1767 (1980).

[18] J.L. Kurz, *J. Phys. Chem.* **66**, 2239–2245 (1962).

[19] J.P. Andersen, M. Le Maire, T. Gulik-Krzywicki, U. Kragh-Hansen, P. Champeil and J.V. Moller, *Eur. J. Biochem.* **134**, 205–214 (1983).

[Received for review 21 May 1993; revised manuscript received 2 August 1993]