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# Effects of surfactants on the spectral behaviour of calcein (II): a method of evaluation

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**Abstract**

The spectral behavior of calcein, a water-soluble self quenching fluorescent marker often used in biomedical analysis, can be considerably affected by the prescence of surfactants. With this study we intend to obtain further information on the photophysical properties of calcein, in the presence of surfactants and in the concentration range commonly used to investigate the release of such marker from vesicle dispersions. The experiments were carried out both in water and in a physiological buffer (HEPES, pH 7.5), in the presence of Triton X-100, sodium dodecyl sulphate and centyltrimethylammonium bromide, both below and above their critical micelle concentration (c.m.c.). The obtained results confirm that calcein flourescence can be affected by the presence of surfactants. Thus, environmental conditions must always be carefully checked for the actual quantitative evaluation of this dye. Furthermore, this study sheds some light on the nature and mechanism of calcein quenching. 

*Keywords:* Calcein; Fluorescence changes; Surfactants; Quenching

## 1. Introduction

In the wide range of the available spectroscopic techniques, fluorescence quenching has become more and more frequently used when probing biological macromolecular assemblies [1–9]. In particular, the self-quenching property shown by some fluorescent molecules [10] can provide interesting information about vesicle structure, stability and behaviour [11]. With respect to similarly behaving compounds (e.g. fluorescein), calcein (or Fluorexon, CAS 1461-15-0) is characterised by features (i.e. stability at physiological pH values, water solubility and chelating properties [12]) that make this substance a suitable water-soluble self-quenching fluorescent marker in vesicle methodology [13–17]. It was therefore selected for the present investigation.

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In a previous work, according to the specific aim of that study, we evaluated the changes in the spectral behaviour of a fixed calcein concentration ($10^{-5}$ M) in aqueous solutions in the presence of different surfactants (i.e. Triton X-100, sodium dodecylsulphate, cetyltrimethylammonium bromide), commonly used as membrane-like solubilizers [18]. In this study, in order to acquire further information, we investigated the photophysical properties of calcein, alone as well as in the presence of the same surfactants, broadening the concentration range of the fluorescent probe between $10^{-6}$ and $10^{-4}$ M. Such a concentration range was chosen because it is related to the actual values obtained during calcein release experiments from vesicle dispersions. The experiments were performed both in water and in a physiological buffer (HEPES, pH 7.5), in the presence of surfactant concentrations below and above their critical micelle concentration (c.m.c.) values.

The present investigation emphasises the importance and the difficulties of the quantitative evaluation of calcein concentration in the presence of surfactants, often used in pharmaceutical studies. It also contributes to the knowledge of the nature and the mechanism of calcein self-quenching. At the same time, it is meant to provide a tool for the evaluation of the influence that surfactants may exert on the spectral behaviour of a fluorescent tracer.

## 2. Experimentals

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

An adequate amount of crystalline calcein (SIGMA) was dissolved into the minimum effective amount of NaOH (1.0 M) and then diluted with HEPES to 0.01 M.

Triton X-100 (TX-100), sodium dodecyl sulphate (SDS), cetyltrimethylammonium bromide (CTAB) and all other products used were of analytical grade or purer.

The absorbance was measured by a Perkin Elmer Lambda 3A spectrophotometer, appropriately equipped with 10 or 1 mm quartz cuvettes.

Fluorescence measurements were made on a Perkin Elmer LS-5 spectrofluorimeter. An excitation wavelength of 492 nm and slit widths 2.5/2.5 of the monochromators, unless otherwise specified, were used. Furthermore, in order to detect both absorbance and fluorescence in the same samples, it was necessary to attenuate, by means of an optical filter, the signal at the excitation window.

Both spectrometers were coupled with a Perkin Elmer 3600 Data Station. All the photophysical measurements were performed at room temperature. The reported results represent the mean of at least three separate determinations $\pm$ RSD (Relative Standard Deviation). An iterative non-linear least-squares fitting method (NLLSQ) was applied to the data.

## 3. Results and discussion

The fluorescence of increasing calcein concentrations ($10^{-6}$–$10^{-4}$) was determined in aqueous solutions containing different surfactants levels above and below their c.m.c., as indicated in Table 1.

The presence of surfactants induced appreciable fluorescence variations with respect to the reference solutions (i.e. those without surfactants), but such variations did not follow a regular trend. For each surfactant, in fact, decreases or increases in the relative fluorescence values (i.e. ratio between the values obtained in the presence of surfactant and the reference ones) were observed. For this reason, it seemed more reasonable to consider the overall trend of the calcein fluorescence plot as a function of its concentration; furthermore, for an appropriate comparison among the fluorescence data obtainable in the different sets of experiments (e.g. in the presence or in absence of surfactants), all the values–within each set–were normalised with respect to the fixed concentration of $10^{-5}$ M ($10^{-5}$ M = 100 in arbitrary units).

Table 1
Test concentrations of TRITON X-100, sodium dodecyl sulphate (SDS) and cetyltrimethylammonium bromide (CTAB) and critical micelle concentration (c.m.c.) values determined in water and HEPES solutions

| Surfactant | Test concentrations (M) | c.m.c. water (M) | c.m.c. HEPES (M) |
|---|---|---|---|
| TRITON X-100 | $3.0\times10^{-5}$ | | |
| | $1.5\times10^{-3}$ | $2.5\times10^{-4}$ | $2.5\times10^{-4}$ |
| SDS | $8.0\times10^{-4}$ | | |
| | $4.0\times10^{-2}$ | $6.0\times10^{-3}$ | $1.0\times10^{-3}$ |
| CTAB | $1.0\times10^{-4}$ | | |
| | $5.0\times10^{-3}$ | $2.0\times10^{-3}$ | $2.0\times10^{-3}$ |

In order to evaluate the effect of surfactants, the fluorescence of solutions containing calcein alone was preliminarily determined. In Fig. 1 the fluorescence values are reported as a function of the dye concentration. The trend of the obtained data can be fitted by numerous equations: in this sense, the Stern–Volmer Eq. (1), that is usually adopted for these type of studies and that consequently might seem to be the most appropriate one, even if modified in such a way as to take into account the self-quenching related to calcein dimerization, is not applicable for the present investigation. Such an approach, that should give important information on the dynamic and static quenching processes (e.g. dimerization process, quenching rate constant and collisional self-quenching), provides good results only as far as the mathematical description of the curve (NLLSQ analysis; $r^2 > 0.990$) is concerned; in fact at the tracer concentrations we employed, the calculated constants of the Stern–Volmer equation have no physical meaning and could therefore lead to formally incorrect conclusions [19–21]. For these reasons, a different mathematical interpretation of the observed phenomena is proposed, in analogy to the approach indicated by Georges [22] in order to justify variations of fluorescence versus concentrations that can originate from instrumental and/or chemical effects.

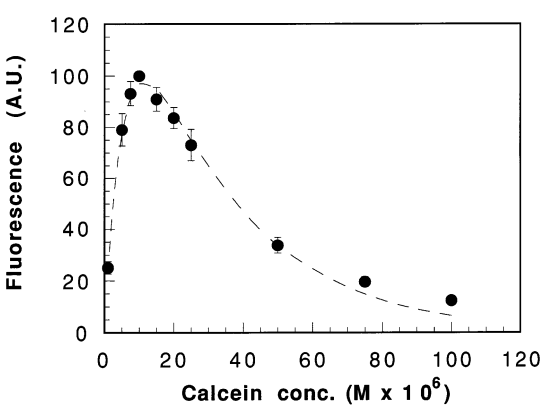


Fig. 1. Fluorescence values ($\pm$ RSD) of calcein as a function of its concentration, in HEPES. $10^{-5}$ M = 100 (AU). The dashed curve ($r^2 = 0.990$) represents the curve obtained from Eq. (2) where $\varepsilon_m = 63{,}700$; $\beta_f$ (proportionality constant) = 175.2; $k_{ife}$ (fluorescence decrease constant due to the inner filter effect) = 32 875 (see also Table 2).

Considering that the fluorescence intensity is proportional to the amount of energy absorbed, by neglecting the secondary absorption effects as well as the factors related to the instrumental prefilter and to the cuvette transmission, the following equation can be written:

$$I_0 = \beta_f(1 - 10^{-\varepsilon_m[C_t]}) \quad (1)$$

where $I_0$ is the fluorescence intensity in the absence of fluorescence decrease, $\beta_f$ is a proportionality constant that takes into account, among other factors, the fluorescence quantum yield, the frequency of the excitation radiation, the average emission fluorescence frequency, as well as the instrumental characteristics.

The adopted experimental conditions give rise to a 'trivial' self-quenching of calcein fluorescence due to the inner filter effect on the spectral region investigated [23]. In fact, the absorbance at the path length used for fluorescence measurements (i.e. 10 mm standard cuvette, that must be used for lower calcein concentrations) causes a considerable inner filter absorption. Consequently, the

Table 2
Molar extinction coefficients ($\varepsilon_m$), statistical parameters (confidence interval, C.I.; linear regression coefficient, $r^2$) and constant values (proportionality constant, $\beta_f$; fluorescence decrease constant, $k_{ife}$) of the equations describing fluorescence trends in HEPES and in water, in the presence of TRITON X-100 (TX100), sodium dodecyl sulphate (SDS) and cetyltrimethylammonium bromide (CTAB) below and above their critical micelle concentration (c.m.c.)

| | $\varepsilon_m$ | $\beta_f$ | 95% C.I. | $k_{ife}$ | 95% C.I. | $r^2$ |
|---|---|---|---|---|---|---|
| *(a) Medium: HEPES* | | | | | | |
| Calcein alone | 63 700 | 175.2 | 163.4 ÷ 187.0 | 32 875 | 29 021 ÷ 36 728 | 0.990 |
| Calcein-[TX100] < c.m.c. | 63 700 | 187.9 | 169.8 ÷ 206.1 | 37 574 | 31 629 ÷ 43 518 | 0.983 |
| Calcein-[TX100] > c.m.c. | 63 700 | 201.1 | 184.3 ÷ 217.9 | 41 974 | 36 513 ÷ 47 436 | 0.988 |
| Calcein-[SDS] < c.m.c. | 63 700 | 198.8 | 177.8 ÷ 219.8 | 42 181 | 35 250 ÷ 49 113 | 0.981 |
| Calcein-[SDS] > c.m.c. | 63 700 | 179.5 | 167.3 ÷ 191.7 | 31 777 | 27 976 ÷ 35 777 | 0.989 |
| Calcein-[CTAB] < c.m.c. | 63 700 | 175.8 | 157.8 ÷ 193.8 | 33 153 | 27 631 ÷ 38 675 | 0.985 |
| Calcein-[CTAB] > c.m.c. | 63 700 | 160.0 | 150.8 ÷ 169.2 | 25 500 | 22 678 ÷ 28 321 | 0.991 |
| | 51 000 | 183.0 | 162.9 ÷ 193.0 | 28 640 | 25 895 ÷ 31 385 | 0.993 |
| *(b) Medium: water* | | | | | | |
| Calcein alone | 42 500 | 207.3 | 197.7 ÷ 216.9 | 27 764 | 25 599 ÷ 29 928 | 0.994 |
| Calcein-[TX100] < c.m.c. | 42 500 | 205.9 | 190.7 ÷ 221.2 | 26 252 | 22 905 ÷ 29 598 | 0.983 |
| Calcein-[TX100] > c.m.c. | 42 500 | 203.6 | 193.8 ÷ 213.4 | 25 581 | 23 443 ÷ 27 719 | 0.993 |
| Calcein-[SDS] < c.m.c. | 42 500 | 210.9 | 182.9 ÷ 238.9 | 26 266 | 20 280 ÷ 32 253 | 0.939 |
| Calcein-[SDS] > c.m.c. | 42 500 | 235.7 | 204.7 ÷ 266.6 | 39 487 | 31 862 ÷ 47 112 | 0.966 |
| | 35 200 | 276.4 | 233.4 ÷ 319.4 | 43 721 | 34 537 ÷ 52 904 | 0.955 |
| Calcein-[CTAB] < c.m.c. | 42 500 | 195.1 | 150.9 ÷ 239.2 | 24 088 | 14 455 ÷ 33 721 | 0.790 |
| Calcein-[CTAB] > c.m.c. | 42 500 | 201.2 | 193.9 ÷ 208.5 | 25 449 | 23 856 ÷ 27 043 | 0.996 |

concentration above which the fluorescence values start to decrease (ca $10^{-5}$ M) becomes much lower than that corresponding to the 'true' self-quenching due to dimerization processes [19,22]; thus explaining why the Stern–Volmer equation is inapplicable, as previously discussed. On the basis of the above-reported considerations, the experimental data of Fig. 1 can be interpreted as the result of an overall phenomenon consisting of two phases occurring at the same time and competing with each other; i.e. a behaviour somehow similar to the usual trend of the kinetic describing a drug absorption/elimination process [24].

The adopted equation describing the calcein fluorescence behaviour (Fig. 1) becomes therefore:

$$I = \beta_f \cdot (1 - 10^{-\varepsilon_m [C_t]}) \cdot e^{k_{ife} \cdot [C_t]} \quad (2)$$

where $I$ is the experimental value of the relative fluorescence intensity; the term $\beta_f(1 - 10^{-\varepsilon_m[C_t]})$ represents $I_0$ of Eq. (1) (i.e. the fluorescence intensity in the absence of fluorescence decrease), $[C_t]$ is the total concentration of calcein, $\beta_f$ is the proportionality constant and $k_{ife}$ is a constant representing the fluorescence decrease-due almost totally to the inner filter effect-under the various conditions examined.

According to Eq. (2), fluorescence data of calcein obtained in the individual experiments were fitted by a non-linear least square analysis procedure in order to calculate a significant value for the $\beta_f$ and $k_{ife}$ parameters. The approximation of the fitting was evaluated by the 95% C.I. and by $r^2$. Results are summarised in Table 2. The curve obtained from Eq. (2) is shown in Fig. 1 and shows the fitting of the experimental data.

Calcein $\varepsilon_m$ values, representing the molar extinction coefficients (Table 2), were calculated from the slope of the corresponding absorbance/concentration plots, both in water and in the HEPES buffer. Since $\varepsilon_m$ values were, in most cases, almost constant (at least within small differences having an order of magnitude that can be negligible for our purposes, i.e. always below 1%), the same value (63 700 or 42 500, HEPES or water, respectively) was considered for the calculation, using Eq. (2), of the $\beta_f$ and $k_{ife}$ parameters. On the other hand, when the calculated $\varepsilon_m$ differed more than 1%, the corresponding values are

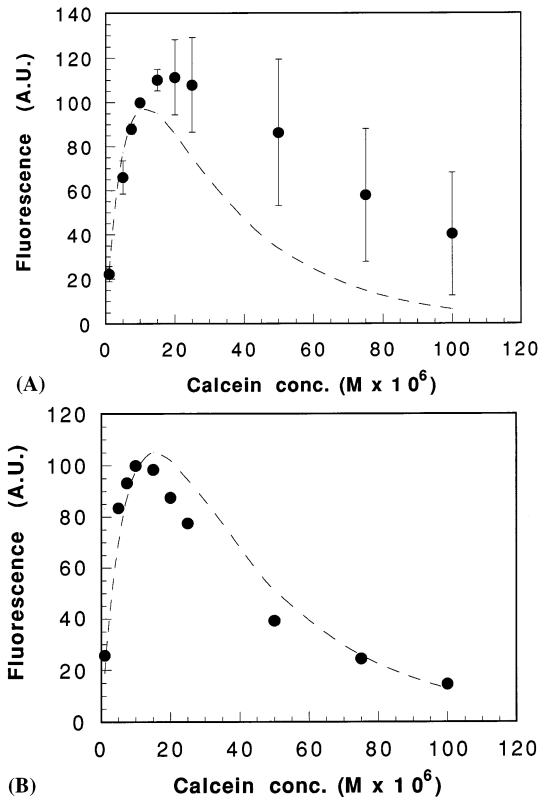


Fig. 2. (A) Fluorescence values ($\pm$RSD) of calcein as a function of its concentration, in CTAB above c.m.c., in HEPES. $10^{-5}$ M = 100 (AU). The dashed curve is that of Fig. 1. (B) Fluorescence values (C.V.% < 3) of calcein as a function of its concentration, in SDS above c.m.c. in water. $10^{-5}$ M = 100 (AU). The dashed curve represents the curve of calcein in water obtained from Eq. (2) where $\varepsilon_m = 42{,}500$; $\beta_f$ (proportionality constant) = 207.3; $k_{ife}$ (fluorescence decrease constant due to the inner filter effect) = 27 764 (see also Table 2).

reported in the same Table 2 and are used for our calculations. As shown, $\varepsilon_m$ value variations were obtained in the solutions of CTAB > c.m.c. (HEPES and water) and of SDS > c.m.c. (water). In such cases differences of the $\beta_f$ and $k_{ife}$ values, and consequently of $r^2$, were found. It must also be pointed out that, owing to the presence of the surfactants affecting calcein fluorescence, appreciable variations of $\beta_f$, $k_{ife}$ and $r^2$ were detectable when a constant $\varepsilon_m$ value was used (Table 2; in particular CTAB < c.m.c. in water).

As an example, in Fig. 2 the remarkable differences between the theoretical curve calculated from Eq. (2) for a solution containing calcein alone and the actual experimental values obtained in the presence of CTAB > c.m.c. in HEPES (Fig. 2A) and of SDS > c.m.c. in water (Fig. 2B) are reported. An interpretation of the observed effect can be derived by the following considerations: according to previously reported results [16], cationic CTAB is capable of interactions with the dye molecules as indicated by the relevant height of the error bars at high CTAB concentrations (Fig. 2A). On the other hand, the anionic SDS undergoes hydrolysis in water, leading, at the higher tested concentrations, to a significant pH variation (that of course does not occur when the buffer is used): such a variation affects calcein spectral behaviour as reported in Fig. 2B where in particular is possible to observe how fluorescence starts to decrease at lower calcein concentrations.

In Table 3 the increase (+) or decrease (−) in the relative fluorescence values, obtained in the

Table 3
Calcein fluorescence values in the presence of TRITON X-100 (TX100), sodium dodecyl sulphate (SDS) and cetyltrimethylammonium bromide (CTAB) below and above critical micelle concentration (c.m.c.), expressed as percentage of increase (+) or decrease (−) with respect to the reference determined in the absence of surfactants

| Calcein M × $10^6$ | [TX100] < c.m.c. | [TX100] > c.m.c. | [SDS] < c.m.c. | [SDS] > c.m.c. | [CTAB] < c.m.c. | [CTAB] > c.m.c. |
|---|---|---|---|---|---|---|
| (a) Medium HEPES | | | | | | |
| 5 | +5.3 | +11 | +15 | +7.2 | +16.9 | −16.6 |
| 25 | +5.5 | +7.8 | +4.1 | +19.2 | +20.8 | +33.1 |
| 75 | −30.1 | −26 | −22.4 | −2 | −14.8 | +4.6 |
| (b) Medium water | | | | | | |
| 5 | +6.1 | +2.6 | +21.4 | +18.9 | +30.6 | −1 |
| 25 | +3.2 | +1.3 | +4.2 | −14.4 | −5 | +6.6 |
| 75 | +20.8 | +9.9 | +23.7 | −10.2 | +60.9 | +15 |

presence of surfactants, with respect to the reference are reported. As can be observed, the values appear to be rather irregular and unpredictable and therefore could lead to unreliable evaluations of the actual calcein concentrations if calculated on the basis of each single experimental point. In such a situation, the type of approach given here, that takes into account the overall trend of the curve obtained from Eq. (2) as well as the calculated $\beta_f$ and $k_{ife}$ parameters, could be suitable for an appropriate calculation of calcein concentration in the presence of surfactants.

## 4. Conclusions

The reported results confirmed that the presence of surfactants affects calcein fluorescence and show how the surfactant influence on calcein spectral behaviour is a function of the relative concentration of both substances. Furthermore, it must be underlined that even negligible differences in the operating conditions (e.g. sampling, dilution, different instrumentation, etc.), that could be disregarded in other experimental contexts, may lead to appreciable differences of the final results in this type of quantitative evaluation. It becomes therefore difficult to evaluate, by means of single fluorescence determinations, the actual calcein concentration under the various experimental conditions. The use of the proposed equation, capable of describing the overall phenomenon within a sufficiently wide range of dye concentration, provides a tool that could be useful for the overcoming of this type of problems.

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