Determination of the aggregation number of detergent micelles using steady-state fluorescence quenching

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ABSTRACT The development of a simple, reliable method for determination of detergent micelle aggregation number that relies solely on measurement of steady-state fluorescence quenching is presented. The degree of steady-state fluorescence quenching of a micelle-solubilized fluorophore (pyrene) by a quencher that partitions greatly into the micelles (coumarin 153) is dependent on the micelle concentration, which can therefore be determined. The aggregation number is calculated as the micelle concentration/detergent monomer concentration (the total detergent concentration above the critical micelle concentration). For the determination to be accurate, the partition coefficient of the quencher into the micelle phase is determined and used to calculate the micellar concentration of quencher. Also, the quenching of pyrene by a coumarin 153 molecule within the same micelle must be complete, and this was confirmed by time-resolved fluorescence measurements. Aggregation numbers were determined for one cationic and several nonionic detergents and were found to be consistent with literature values. The approach presented is an improvement on a previous luminescence quenching technique (Turro, N. J., and A. Yekta. 1978. *J. Am. Chem. Soc.* 100:5951–5952) and can be used on cationic, anionic, and nonionic detergents with micelles ranging greatly in size and under varying conditions, such as detergent concentration, ionic strength, or temperature.

INTRODUCTION

Studies of membrane proteins are frequently conducted with the protein dissolved in detergent micelles and knowledge of the physical characteristics of the detergent is of great importance (1). The aggregation number (n_{agg}) of the micelle, i.e., the number of detergent monomers per micelle, is an important characteristic that has not been reported for many detergents (2) and is often dependent on experimental conditions (3). Conventional methods used to determine micelle size include light scattering, sedimentation, membrane osmometry, and small-angle neutron scattering (for review see reference 4). Although reliable, these methods are not well suited for routine use by most researchers. A method for n_{agg} determination of a detergent under a variety of experimental conditions that is easily performed is therefore of interest.

A method for determination of aggregation number on the basis of luminescence quenching was developed by Turro and Yekta (5) and was used to calculate the micelle size of sodium dodecyl sulfate. The method, which measures the luminescence intensity of a micelle-bound probe as a function of quencher concentration, is simple and fast but has significant limitations. As described, it can only be used on anionic surfactants with $n_{agg} < 120$ (6) and assumes complete partitioning of the quencher into the micelle phase. Several groups have reported improvements on this method (6–8), but these require time-resolved fluorescence measurements, a technique not available to most researchers.

The purpose of the current work was to develop a method for aggregation number determination on the basis of steady-state fluorescence quenching that makes few assumptions, can be generally used on the majority of detergents, and is simple to perform. The experimental system measures the quenching of the steady state fluorescence of pyrene in detergent micelles by coumarin 153 (C153), which partitions greatly into the micelles. This method is shown to work for a variety of micelles of different size (n_{agg} = 53–143) and is used to accurately determine n_{agg} of several nonionic and one cationic detergent. Quencher partitioning is determined and the method can be applied under varying conditions, such as detergent concentration, ionic strength, or temperature, unlike some of the methods mentioned previously. It may also provide useful information about the micelle structure when the results are compared with those obtained from other techniques, such as light scattering or sedimentation, which actually determine micelle size (not n_{agg}).

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Abbreviations used in this paper: C153, coumarin 153, {2,3,6,7-tetrahydro-9-(trifluoromethyl)-1*H*,5*H*,11*H*-[1]benzopyrano[6,7,8*ij*]quinolizin-11-one}; cmc, critical micelle concentration; DDM, *n*-dodecyl-β-D-maltoside; HTAB, hexadecyltrimethylammonium bromide; Mops, 3-[*N*-morpholino]-propanesulfonic acid; n_{agg}, aggregation number.

MATERIALS AND METHODS

Reduced (hydrogenated) Triton X-100, Triton X-100, Brij 35, Tween 80, DDM, HTAB, and pyrene were purchased from Sigma Chemical Co (St. Louis, MO). C153 {2,3,6,7-tetrahydro-9-(trifluoromethyl)-1*H*,5*H*,11*H*-[1]benzopyrano[6,7,8*ij*]quinolizin-11-one} was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). DCM was purchased from Exciton, Inc. (Dayton, OH).

Micelle solution preparation

For reduced Triton X-100, Triton X-100 and Brij 35, pyrene was dissolved completely in slightly heated (<60°C) pure detergent and di-

0006-3495/93/05/1580/08 $2.00

luted with water to a stock solution of 10 μM pyrene, 1.0% (wt/vol) detergent, 10 mM 3-[*N*-morpholino]propanesulfonic acid (Mops), pH 7.2. For Tween 80, HTAB, and DDM, pyrene was not soluble in high concentrations of these detergents even with mild heating. Therefore, 10 mM pyrene in pure ethanol was added to detergent solutions to obtain the following stock solutions: 4 μM pyrene in 2.0% (wt/vol) Tween 80, 20 μM pyrene in 1.5% HTAB, and 10 μM pyrene in 1.0% DDM. Using the literature values for aggregation number, these relative concentrations were chosen so that the micelle/pyrene ratio was ≥10, to reduce the fraction of micelles with more than one pyrene molecule. The ratio was later verified using the aggregation numbers determined. Since there was no n_{agg} reported in the literature for Tween 80, several pyrene/detergent concentrations were evaluated (as described in Results) until the above stock solution was chosen, which has a micelle/pyrene ratio of 36.

Calculation of R_0 for pyrene/coumarin 153 in reduced Triton X-100 as a donor/acceptor pair for energy transfer

The Förster distance, R_0 (in ångströms), is the distance at which the energy transfer efficiency between a donor and an acceptor is 50% (for review see reference 9) and is shown in Eq. 1.

$$R_0 = (J\kappa^2 Q_0 n^{-4})^{1/6} \times (9.7 \times 10^3)\ \text{Å}. \qquad (1)$$

J is the spectral overlap integral (see Fig. 1); κ^2, the orientation factor between donor and acceptor dipoles; Q_0, the fluorescence quantum yield of the donor in the absence of acceptor; and n is the refractive index of the medium. The value of 2/3 was used for κ^2, n is approximated as 1.42 for the reduced Triton, and Q_0 = 0.58 for pyrene in cyclohexane (10), which is a similar solvent to the micelle octylcyclohexyl core of reduced Triton X-100.

Fluorescence measurements

Steady-state fluorescence was measured with a fluorometer (model Fluorolog II; Spex Industries Inc., Edison, NJ). All fluorescence experiments were performed at ambient temperature, except those using the detergent HTAB. Since the Krafft point of HTAB is 22°C, determination of its P_m and its n_{agg} were performed at 30°C.

For P_m determination of C153 in each detergent, the excitation and emission spectra of this dye were measured in 10 mM Mops buffer, pH 7.2, and in detergent solution (5–10%) in the same buffer. The presence of any of the detergents used caused a great increase in fluorescence, with no change in the excitation peaks but with a shift in the emission peak from 540 to ~528 nm. For all P_m determinations, the sample was excited at 427 nm and emission was measured at 490 nm since this emission wavelength had the highest $C153_{(detergent)}$ fluorescence/$C153_{(buffer)}$ fluorescence of ~40, on average. The fluorescence of 5.0 μM C153 was measured in concentrations of detergent from 0 to 4.0%. Background fluorescence for each detergent concentration was subtracted from each fluorescence value.

Pyrene in reduced Triton X-100 has excitation peaks at 274, 321, and 337 nm and emission peaks at 372 and 395 nm. To minimize inner filter effects from C153 in all steady state fluorescence quenching experiments, pyrene was excited at 337 nm and emission was measured at 372 nm. 20 μM C153 (the highest final concentration used) in 1.0% reduced Triton X-100 has an Abs (337 nm) = 0.022 and Abs (372 nm) = 0.088 with a 10-mm path length. The fluorescence was measured at a right angle to the excitation light through a 10 × 2.0 mm cuvette. The C153 absorbance in the fluorescence experiments is estimated to be fivefold lower than that with a 10-mm path length and, therefore, inner filter effects were expected to be insignificant in all quenching experiments. This was verified by performing an identical pyrene quenching experiment with C153 in reduced Triton X-100 by addition of C153 and measuring front-face fluorescence emission. The n_{agg} calculated from the front-face fluorescence experiment is within the range of values from several experiments performed measuring fluorescence at a right angle, confirming the inner filter effects in the fluorescence quenching experiments to be insignificant.

TABLE 1 **Critical micelle concentration and density values used in P_m and aggregation number calculations**

Detergent	CMC	Reference	Density	Reference
	mM		*cm³/g*	
Reduced Triton X-100	0.185	1	0.908*	13
Triton X-100	0.22	14	0.908	13
Tween 80	0.012	15	0.896	13
Brij 35	0.092	16	0.925‡	17
HTAB	0.92	18	0.995	13
DDM	0.15	19	0.95§	

* Estimated to be the same as nonreduced form.
‡ Estimated to be the same as the similar detergent Lubrol WX.
§ Estimate.

In each experiment, the fluorescence of a pyrene solution (1.0–4.0 μM) in detergent was first measured; all measurements with a 10-s integration time. Aliquots of C153 (1.0 or 0.5 mM stock in 100% ethanol) were then added to the sample, remeasuring fluorescence after each addition. The maximal final concentration of ethanol was 2%, which is not expected to affect the aggregation number because of its high water solubility. There was no measurable time dependence to the quenching. Background fluorescence, always <1.0% of initial pyrene fluorescence, was subtracted from each value. All fluorescence values, along with all fluorophore, quencher, and detergent concentrations, were adjusted for dilution upon quencher addition.

Time-resolved fluorescence was measured by the correlated single-photon counting method using an instrument previously described (11). DCM was used in the dye laser to excite pyrene at its 321-nm absorption peak. The fluorescence decay of pyrene in each of the six detergents was measured at 380 nm. The samples used were the stock solutions previously described plus 10% ethanol and had fivefold higher pyrene and detergent concentrations than the samples used in the steady-state experiments. This was done to increase the fluorescence decay signal from the samples. Note, though, that detergent micelle/pyrene ratios used were identical to those in the steady state fluorescence experiments. The fluorescence decays of identical detergent samples (10% ethanol) with 100 μM C153 were also measured. All decay kinetics were analyzed in an identical manner by a Marquardt nonlinear least-squares algorithm (12) over the same number of channels, with none of the parameters being fixed.

Determination of partition coefficient of C153 into detergent micelles

As described previously C153 fluorescence was measured versus detergent concentration, which can be expressed as α_m, the micelle volume/total volume of the solution. α_m was calculated by subtracting the cmc from the total detergent monomer concentration (both in mol/liter) and multiplying this by the molecular weight and specific volume (in liters/g) of the detergent. Values for cmc and density (for calculation of specific volume) of the detergent were obtained from the literature and are shown in Table 1. The partition coefficient of C153 into a micellar phase is given by:

$$P_{m(C153)} = \frac{[C153]_m}{[C153]_w} \qquad (2)$$

Since the fluorescence of C153 greatly increases upon partitioning into detergent micelles, this fluorescence was used to monitor the relative amount of the fluorophore in micelles at different α_m values. Assuming that $F_m/F_w \gg 1$, where F is C153 fluorescence, one obtains:

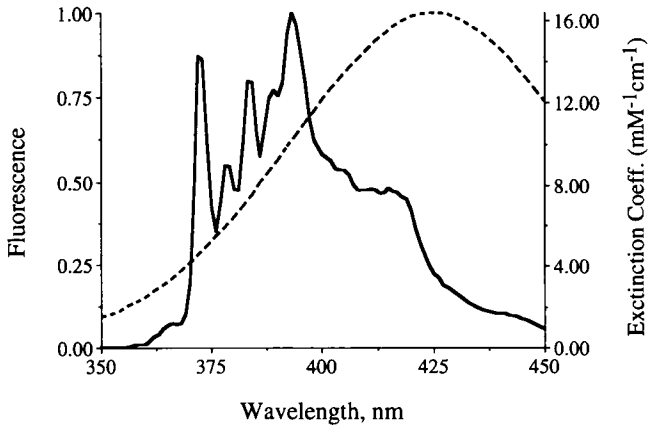

FIGURE 1 Overlap of pyrene fluorescence spectrum (*solid line*) and coumarin 153 absorption (*dotted line*), both in 1.0% (wt/vol) reduced Triton X-100, 10 mM Mops, pH 7.2. Pyrene was excited at 337 nm and the fluorescence peak normalized to 1.0. The spectra were used to calculate a spectral overlap integral, $J = 2.93 \times 10^{-14}$ cm^3/M.

$$\frac{F}{F_\infty} = \frac{\alpha_m[C153]_m}{\alpha_m[C153]_m + (1 - \alpha_m)\dfrac{[C153]_m}{P_m}}, \quad (3)$$

where F_∞ is the fluorescence when all of the C153 is in the micelle phase. Eq. 3 can be rewritten:

$$\frac{1}{F} = \frac{1}{F_\infty} \times \frac{\alpha_m[C153]_m + (1 - \alpha_m)\dfrac{[C153]_m}{P_m}}{\alpha_m[C153]_m}, \quad (4)$$

and thus:

$$\frac{1}{F} = \frac{1}{F_\infty} - \frac{1}{P_m F_\infty} + \left(\frac{1}{P_m F_\infty} \times \frac{1}{\alpha_m}\right). \quad (5)$$

A plot of inverse F versus inverse α_m has a value equal to inverse F_∞ at $\alpha_m = 1.0$, and a slope equal to inverse $P_m F_\infty$. Therefore, intercept/slope gives P_m. This value was then adjusted through a series of iterations for the small contribution to the total fluorescence (<1%) due to aqueous C153.

Determination of micelle aggregation number from fluorescence quenching data

The following equations were developed assuming an ensemble of N identical micelles with m quencher molecules, randomly distributed among the micelles, with no limit on the number of quencher molecules per micelle (no interaction between the quencher molecules). Under these conditions, the probability of a micelle being unoccupied by quencher is:

$$P_{empty} = \left(1 - \frac{1}{N}\right)^m, \quad (6)$$

which can be written as:

$$P_{empty} = \left[\left(1 - \frac{1}{N}\right)^N\right]^{m/N}. \quad (7)$$

When m, N increase indefinitely while their ratio is kept constant, then:

$$P_{empty} = \lim_{N,m\to\infty}\left[\left(1 - \frac{1}{N}\right)^N\right]^{m/N} = e^{-m/N}. \quad (8)$$

If the partition of one quencher molecule into a micelle completely quenches the fluorescence of pyrene in that micelle, then:

$$\frac{F}{F_0} = P_{empty} = e^{-m/N} \quad (9)$$

$$\ln\frac{F}{F_0} = -\frac{m}{N}, \quad (10)$$

where F_0 and F represent the fluorescence intensity in the absence and in the presence of quencher, respectively. A plot of $\ln F/F_0$ versus the concentration of m has a slope of $-1/N$, where N is the concentration of micelles. Knowledge of N and of the detergent concentration above the cmc allows for the calculation of n_{agg}, the number of detergent monomers per micelle. The values of m used in this calculation were adjusted to yield the amount of C153 in the micelles, based on the P_m determined and the α_m value.

RESULTS

The overlap of pyrene fluorescence (excitation wavelength 337 nm) with the absorbance of C153 is shown in Fig. 1. The spectral overlap integral (J) was calculated from these spectra to be 2.93×10^{-14} cm^3/M for the donor–acceptor pair. Using this value, R_0 was evaluated to be ~34 Å.

The P_m for C153 partitioning into reduced Triton X-100 was determined from the data shown in Fig. 2 using Eq. 5. Similar plots were obtained for C153 partitioning into five of the six detergents studied, and the fitted slopes had an average R^2 value of 0.97. HTAB differed in that, although C153 fluorescence greatly increased in its presence, there was no change in fluorescence versus the α_m of HTAB. This indicates that essentially all C153 is in the HTAB micelle phase, and the P_m is above the measurable limits of the method (ability to distinguish a 1% change in fluorescence). The P_m values, calculated using the cmc and density values given in Table 1, are presented in Table 2. Since the density of DDM has not been reported in the literature, the value used here is a median value for nonionic detergents. It is important to note that, although the value used for the density affects the P_m, it has no effect on the n_{agg} determined since the density terms in P_m and α_m used in the calculation cancel out. Note that C153 partitions greatly into all the detergents studied, therefore, ≥78% of C153 was in the micelle phase under the experimental conditions used for fluorescence quenching.

The quenching of pyrene by C153 in reduced Triton X-100 micelles occurs in the manner predicted by Eq. 10, and is shown in Fig. 3. The slope of the curves increases with decreasing detergent concentration, which is expected since the slope is dependent on micelle concentration. Note that the concentration of C153 used in each quenching experiment is the amount in the micelle phase (calculated using the P_m and α_m values)/total solution volume. The aggregation numbers calculated from each of the slopes in Fig. 3 are summarized in Table 2 and are similar, indicating that the method is not sensitive to micelle concentration. Similar experiments were

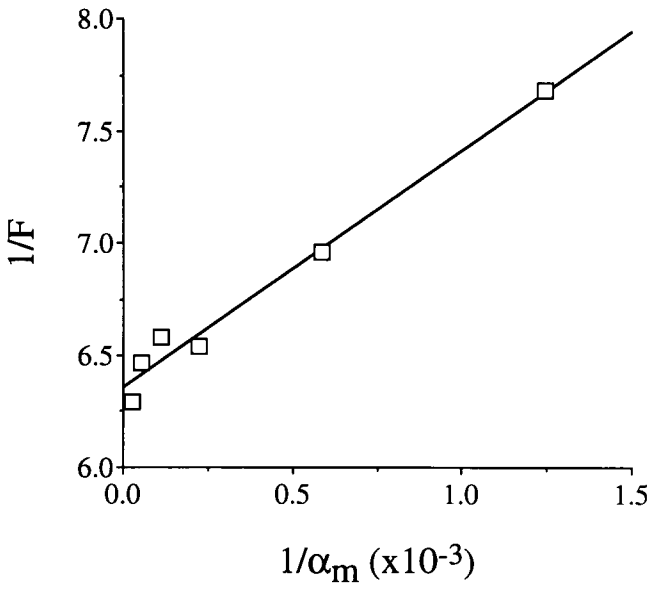

FIGURE 2 Reciprocal intensity of coumarin 153 fluorescence vs. reciprocal α_m of reduced Triton X-100 micelles in 10 mM Mops, pH 7.2. Excitation wavelength 427 nm, emission monitored at 490 nm. The inverse of the value of 1/F at $1/\alpha_m = 1$ is equal to the coumarin fluorescence when it is completely in micelles.

used to calculate the n_{agg} values of five other detergents, and the results are also presented in Table 2. All slopes from plots similar to those in Fig. 3 had excellent fits, with an R^2 average of 0.991. The determination for Triton X-100 was conducted in concentrations of 0.1, 0.2, and 0.3% detergent whereas the determination for the remaining four detergents were conducted three times at one concentration.

To verify that the presence of a quencher molecule in a pyrene-containing micelle completely quenches pyrene fluorescence, time-resolved fluorescence of the pyrene was measured without and with C153. Fig. 4 shows fluorescence decays for pyrene in reduced Triton X-100 in the absence (*top*) and in the presence (*bottom*) of coumarin 153. In the absence of C153, 98.6% of the fluorescence from the sample is due to the long-lived component, T_3, indicative of pyrene monomers. Fluorescence from reduced Triton X-100, with a lifetime much shorter than pyrene, probably contributes to the two shorter components, T_1 and T_2, whereas a very small amount of pyrene excimers may also contribute to T_2. When coumarin 153 was added to the sample in an amount equal to the highest quencher/micelle ratio used in the n_{agg} experiments (in which 60% of the pyrene steady-state fluorescence is quenched), the relative amplitude of pyrene was reduced as the contribution of background fluorescence increased, but no significant effect on its decay constant was found. This clearly indicates that C153 in a reduced Triton micelle completely quenches the fluorescence of the pyrene in that micelle. The remaining unaltered long-lived component in the bottom panel of Fig. 5 is due to pyrene in micelles with no C153 present whereas pyrene/C153-containing micelles do not fluoresce at all. Time-resolved fluorescence was measured in a similar manner for each detergent in the absence and presence of C153. The results showed that, as with reduced Triton X-100, there is complete quenching of pyrene fluorescence by C153 in the micelles of four of the five other detergents at concentrations of pyrene and detergent as used in the experiments.

Pyrene in Tween 80 was not completely quenched by C153, probably because of exciplex formation between pyrene and an impurity in the detergent. The time-resolved fluorescence of this sample, initially measured with 2 μM pyrene and 0.2% detergent, had a short lifetime (55 ns), making up 12% of the total fluorescence that could not be accounted for by background emission from the detergent. The sample used had a relatively low micelle/pyrene ratio of 7.2, therefore, the ratio was increased to determine whether the 55-ns component was due to pyrene excimers, in which case the contribution from this component would decrease with an increasing micelle/pyrene ratio because of the reduced probability of having two pyrenes per micelle. Increasing the detergent concentration with constant pyrene concentration resulted in increased fluorescence intensity in the range of 370–430 nm shown in Fig. 5. The small fluorescence peak at 470 nm, indicative of pyrene excimers, did decline with dilution of pyrene (see Fig. 5, *inset*) but its

TABLE 2 Aggregation numbers determined by fluorescence quenching for six detergents and corresponding literature values

Detergent	$P_m \times 10^{-3}$	n_{agg}, determined*	n	n_{agg}, value	Reference
Reduced Triton X-100	5.56	143 (±10)	4	111	20
				125	21
				140	22
				156	23
Triton X-100	8.10	121 (±1)	3	111	20
				125	21
				140	22
				156	23
Tween 80	4.16	133 (±5)	3	—	
Brij 35	2.02	53 (±3)	3	40	16
HTAB	>50	63 (±3)	3	59	24
				61	18
				63	25
				80	26
DDM	4.10	138 (±3)	3	98‡	27
				111	28

* ±SD.

‡ From size-exclusion chromatography.

The values of n_{agg} were determined at ambient temperature in 10 mM Mops, pH 7.2, with the exception of HTAB determined at 30°C. The determination by fluorescence quenching requires knowledge of the P_m for coumarin 153 between the aqueous solution and the detergent (values shown); the calculation is described in Methods. There are published aggregation numbers for five of the six detergents studied, with no literature values for Tween 80.

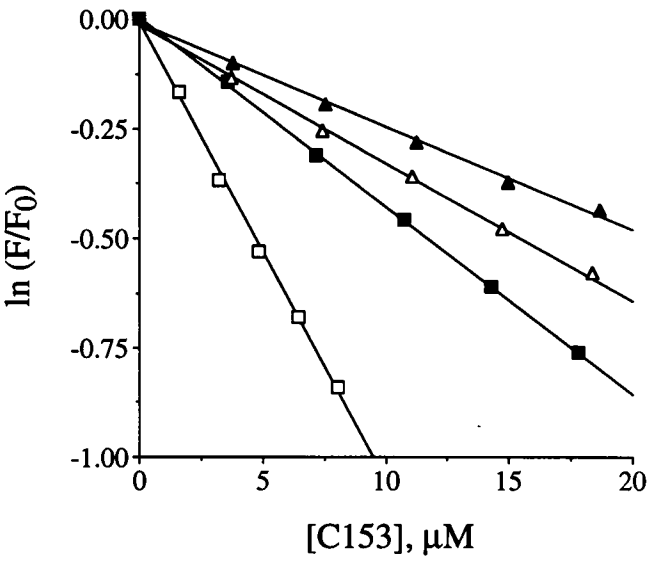

FIGURE 3 Quenching of pyrene fluorescence by coumarin 153 at four different reduced Triton X-100 concentrations. Excitation wavelength 337 nm, emission monitored at 372 nm, 2.0 μM pyrene in reduced Triton X-100, 10 mM Mops, pH 7.2. (□) 0.1% (wt/vol) reduced Triton X-100, (■) 0.2%, (△) 0.3%, (▲) 0.4%. Coumarin 153 in pure ethanol was added to the sample from a 1.0 mM stock ($[\text{ethanol}]_{max}$ = 2%). Quencher concentrations, adjusted for dilution, represent the amount expected in micelles considering the partition coefficient (P_m). Fluorescence values were adjusted for dilution and background fluorescence.

intensity is far too low to account for the 12% of total fluorescence at 380 nm. The time-resolved fluorescence of pyrene in Tween 80 was also measured with a high micelle/pyrene ratio of 36, where the amount of pyrene excimers should be insignificant; the results are shown in Table 3. The contribution of the pyrene short lifetime to total fluorescence increased to 0.20 with the increased micelle/pyrene ratio, clearly ruling out pyrene excimers as the short-lifetime fluorophore. The detergent is also not responsible for the 52-ns lifetime since its decay is much shorter than this (results not shown). The short pyrene lifetime may be due to exciplex formation with an impurity in the detergent, consistent with the increasing fluorescence from this component with increasing detergent. More important for determination of n_{agg}, the degree of quenching by C153 was determined to be 21% of the 52-ns component and 83% of the total fluorescence; the latter value was used in the n_{agg} calculation.

DISCUSSION

The quenching of pyrene fluorescence in detergent micelles by coumarin 153 presents a simple technique based on steady state fluorescence for the determination of n_{agg} that can be applied to most detergents and that relies on few assumptions.

Pyrene was chosen as a fluorophore for the current study because its micelle solubility/water solubility > 10^5 (18) and because its long fluorescence decay (~200 ns) may allow for greater quenching due to diffusion. The concentrations of pyrene and the micelle/pyrene ratios used were chosen so that the pyrene would not affect the aggregation number of the detergent (29). C153 was chosen as a fluorescence quencher because of the good overlap of its absorbance with the fluorescence emission of pyrene shown in Fig. 1 and because of its low absorbance at the pyrene short emission peak of 372 nm. Thus, pyrene quenching can be monitored at 372 nm with insignificant inner filter effects below 20 μM C153 under the conditions used (see Methods). This was verified by performing one quenching experiment while monitoring front-face fluorescence, which practically eliminates inner filter effects, and obtaining an n_{agg} within the range of values determined by right-angle fluorescence quenching experiments. Determination of n_{agg} using the pyrene–C153 pair with quencher concentrations $\leq$ 20 μM and measuring right-angle fluorescence can be applied to many detergents with a low cmc, which includes most of those that are nonionic. Detergents with a higher cmc will require a higher detergent concentration, hence a higher C153 concentration, and may require the use of front-face fluorescence. C153 was a useful quencher also because its high partition coefficient into detergent micelles ensures that the majority of quencher is within the micelle phase even with low α_m values. Despite the high partitioning, there is still a significant fraction of quencher in the aqueous phase, therefore, the C153 concentration was corrected (using the P_m) to reflect quencher within the micelle phase only in the determination of aggregation numbers. C153 is also a useful quencher because its fluorescence greatly increases in the micelle phase, making it simple to determine P_m by Eq. 5.

The results from the time-resolved fluorescence measurements show that a C153 molecule will completely quench the fluorescence of pyrene when both occupy the same micelle, even in large micelles (n_{agg} = 143). Complete quenching was found using five of the six detergents despite the variation in their monomer structure, which is a good indication that complete quenching is likely in other micelles of comparable size. Complete quenching in these detergents is interesting in view of the fact that R_0 for the pyrene–C153 pair (34 Å) is comparable to the size of the micelle core of Triton (and possibly other detergents). This indicates that C153 may quench completely by diffusing greatly within the micelle during the 200-ns lifetime of pyrene.

Complete quenching was not observed with Tween 80 probably because of exciplex formation between pyrene and a detergent impurity. Steady-state fluorescence shows that the contribution of pyrene excimer fluorescence to total fluorescence is insignificant under the conditions used and could not account for the short-lived component. Also, an increased degree of incomplete quenching consistent with the possibility of an interac-

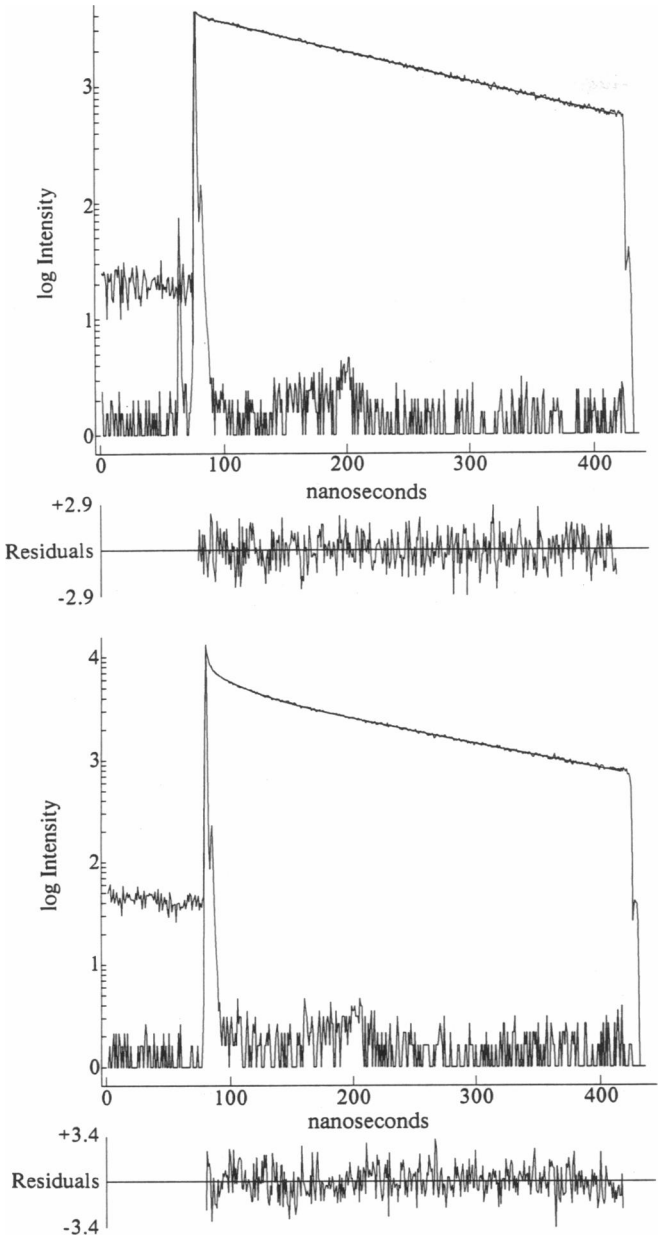

FIGURE 4 The fluorescence decay of 10 μM pyrene in 1.0% (wt/vol) reduced Triton X-100, 10% (vol/vol) ethanol, 10 mM Mops, pH 7.2, in the absence and presence of coumarin 153. Excitation wavelength 321 nm, emission wavelength 380 nm. (*Top*) Without C153. A three-exponential decay fits the data; $A_1 = 0.207$, $T_1 = 2.45$ ns, $F_1 = 0.004$, $A_2 = 0.026$, $T_2 = 55.5$ ns, $F_2 = 0.011$, $A_3 = 0.767$, $T_3 = 176$ ns, $F_3 = 0.986$, and $\chi^2 = 1.03$. (*Bottom*) With 100 μM coumarin 153. A three-exponential decay fits the data; $A_1 = 0.661$, $T_1 = 2.08$ ns, $F_1 = 0.033$, $A_2 = 0.137$, $T_2 = 26.0$ ns, $F_2 = 0.085$, $A_3 = 0.202$, $T_3 = 181$ ns, $F_3 = 0.882$, and $\chi^2 = 1.18$. The residuals are the difference between the theoretical fit and the data, divided by the square root of the data.

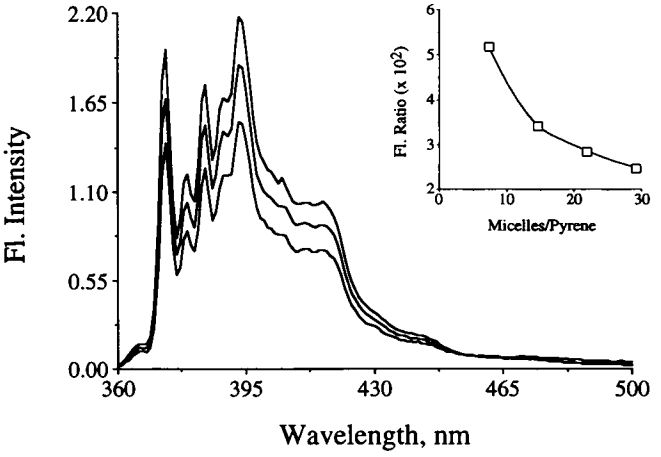

FIGURE 5 The fluorescence spectrum of pyrene in Tween 80 as a function of the micelle/pyrene ratio. 5.0 μM pyrene in Tween 80, 10 mM Mops, pH 7.2, excitation wavelength 337 nm. Lower spectrum obtained with 0.2% (wt/vol) detergent, middle spectrum obtained with 0.4%, upper spectrum obtained with 0.6%. The spectrum with 0.8% Tween 80 (not shown) was identical to that containing 0.6%. (*Inset*) The ratio of pyrene fluorescence intensity at 470 nm to that at 393 nm vs. the ratio of Tween 80 micelles/pyrene. The actual micelle/pyrene ratio used in the n_{agg} determination for this detergent was 36.

tion between pyrene and a detergent impurity was found with increased detergent/pyrene ratio. It is noteworthy, though, that the mechanism for incomplete pyrene quenching can not be fully elucidated from the current results. For the purpose of this study, however, the incomplete quenching was easily detected and corrected for by time-resolved fluorescence. In order for the n_{agg} determination using solely steady-state fluorescence to be a reliable technique, one of two approaches should be taken. A second donor–acceptor pair could be used where the donor is significantly different in structure from pyrene and n_{agg} determinations would be performed with both pairs on the same detergent. Consistent results would give a very strong indication that there was complete quenching and that the n_{agg} values determined were accurate since it is highly unlikely that a detergent impurity would interact with two different do-

TABLE 3 Fluorescence decay parameters of pyrene in Tween 80 micelles in the absence and presence of coumarin 153

Parameters	Pyrene in Tween	Pyrene in Tween + C153
A_1	0.520	0.499
T_1	4.25	6.02
F_1	0.044	0.066
A_2	0.193	0.255
T_2	51.5	40.6
F_2	0.196	0.228
A_3	0.287	0.246
T_3	135	130
F_3	0.761	0.705
χ^2	1.30	1.16

The fluorescence decay of pyrene, 2.0 μM, in 1.0% (wt/vol) Tween 80, 4.0% ethanol, 10 mM Mops, pH 7.2, was fit to three components and the results shown in column 2. Column 3 shows the results of the three-component fit of the fluorescence decay of an identical sample that also contains 40 μM coumarin 153. Details of the measurement and analysis are given in Materials and Methods.

nors to decrease quenching. A second, more conclusive approach would be to demonstrate complete quenching with the pyrene–C153 pair on many of the commonly used detergents for biological research. Once complete quenching is demonstrated in a detergent, the n_{agg} determination could then be performed using steady state fluorescence alone. It is believed that the incomplete quenching found using this donor–acceptor pair in Tween 80 is an exception and complete quenching will occur in the majority of detergents.

The aggregation numbers determined here correspond well to those reported in the literature and determined by other methods. The n_{agg} of reduced Triton X-100 has not been reported, but this detergent has been shown to have the same micelle size as its nonreduced counterpart (1), reflecting the similarities in their monomer structure and in their physical characteristics (30). Our values suggest that the reduced form may have a slightly larger micelle size than the nonreduced one, but further study is required to clarify this point. There is also good agreement between the aggregation number determined for HTAB and the literature values whereas in the cases of Brij 35 and DDM our values are somewhat higher than literature values. The determination of n_{agg} for DDM by Rosevear et al. (27) may be inaccurate because the method used involved analytic size exclusion chromatography using globular proteins as standards. This assumes that the nonionic micelles and globular proteins of the same size migrate through the column matrix at the same rate. Tanford and Reynolds (31) found that this may not be true, since micelle particle asymmetry and detergent/matrix interaction may alter migration rates. It is important to note that there is a range of literature values for any detergent under the same conditions, often using the same technique. The literature values chosen for comparison were all determined in water at 20–25°C. However, since the n_{agg} of some detergents is clearly sensitive to detergent concentration (8), ionic strength (32), and/or temperature (33), there may be some experimental differences that account for the small difference between the values reported here and the literature values.

A luminescence quenching method for n_{agg} determination was first presented by Turro and Yekta (5) and was modified in the current work with the purpose of maintaining its simplicity while increasing its general usefulness and reliability. The method of Turro and Yekta (5), which measures the quenching of ruthenium(bipyridine)$_3^{2+}$ bound to sodium dodecyl sulfate by 9-methylanthracene, can only be used with anionic detergents. Also, it has been shown by time-resolved fluorescence that there is only complete quenching in micelles with an n_{agg} of $\leq$120 (6) and thus the technique is not effective on larger micelles. The method also assumes complete partitioning of the quencher into the micelle phase. Although a high partition coefficient is expected for methylanthracene, as is the case with coumarin 153, a significant amount of quencher may still be in the aqueous phase, yielding inaccurately low n_{agg} values. The technique presented here relies on quenching of pyrene by coumarin 153 mostly by energy transfer, enhanced by diffusion of quencher in the micelle during the pyrene excited state lifetime. The results presented show complete quenching in large micelles (reduced Triton X-100, n_{agg} = 143, $mw = 9.0 \times 10^4$), and the fluorophore/quencher pair could be tested in even larger micelles to determine its limits. Complete quenching will need to be demonstrated under conditions known to alter the micelle shape of specific detergents, since micelle shape may affect the degree of quenching. Also, the technique may be less accurate for small micelles ($n_{agg} <$ 30) where the pyrene molecule may change the n_{agg}. The technique was used successfully with nonionic and cationic detergents and should also be applicable to anionic detergents since the affinity of the fluorophore and quencher for the micelle phase is due to hydrophobicity, a characteristic of all micelle interiors. Finally, the partition coefficient of C153 is determined in each detergent to accurately calculate quencher concentration in the micelle phase.

The importance of having a simple method for n_{agg} determination is great since there is no value reported in the literature for many detergents commonly used in biological research (2). Moreover, the n_{agg} of many detergents varies with temperature, ionic strength, and/or detergent concentration, as previously described. In studies in which the n_{agg} under the specific experimental conditions must be known, the fluorescence quenching method would be most useful. Another advantage of this method is that the determination is unaffected by intermicellar interactions, where the commonly used techniques of light scattering and sedimentation may be affected. A study of micelle size using both fluorescence quenching and one of the commonly used techniques would yield the most convincing aggregation numbers and may provide information about micelle shape and hydration, which do not affect quenching but affect the other two techniques.

We are grateful to Dr. Joseph A. Schauerte for his assistance in the time-resolved fluorescence experiments.

This work was supported by a grant from the U.S. Office of Naval Research. P. J. Tummino was supported by a training grant from the National Institute on Aging (contract T32 AG 00114).

Received for publication 22 September 1992 and in final form 28 December 1992.

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