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14. ABSTRACT

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3,3′-Dioctadecyloxacarbocyanine perchlorate (DiO) as a fluorogenic probe for measurement of critical micelle concentration†

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Critical micelle concentration (CMC) is a key factor in applications of amphiphilic molecules, as it dictates the assembly state of the molecules. Nile Red (NR) is often used as a fluorogenic dye for the measurement of CMC values, but we have observed that NR is prone to aggregation, which leads to reduced reliability in CMC measurements. Here we evaluate 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO) as an alternative fluorogenic dye for the measurement of CMC values. Both NR and DiO provide values comparable to those reported in the literature. However, we demonstrate that DiO provides improved consistency and is more user friendly than NR for measurement of CMC values.

Introduction

Amphiphilic molecules, generally referred to as surfactants, can undergo phase-driven assembly to form higher order structures such as vesicles, bilayers, and micelles. Of these structures, micelles are the most common.[1] In aqueous environments, the micelle structure is solvated by the hydrophilic portion of the amphiphile to minimize unfavorable interactions between the hydrophobic region and the polar solvent. Because of their ability to sequester hydrophobic guest molecules, micelles have shown significant utility in applications including drug delivery, separations, and reaction catalysis.[2–6] In order to utilize micelles in these applications, their assembly must be well-characterized. Micelle assembly is a concentration-dependent process that is characterized by a sharp transition at the critical micelle concentration (CMC). Below this concentration, the surfactant molecules can be free in solution or form a monolayer at the air–solvent interface. However, as the surfactant concentration increases above the CMC, the molecules assemble to form micelles. While the CMC is largely dictated by the chemical properties of the surfactant, it is also dependent on environmental conditions such as pH, temperature, or ionic strength.[7]

CMC values have been measured using a variety of methods including tensiometry,[8] conductivity,[9] dynamic light scattering (DLS),[10] fluorescence polarization,[11] and capillary electrophoresis.[12] However, these procedures require specialized equipment and are not well suited for all surfactants. For example, conductivity requires a charged state, and thus is not capable of measuring the CMC values of non-ionic surfactants. In contrast, a number of methods utilize fluorogenic probe molecules that undergo a change in fluorescence intensity or emission wavelength upon sequestration in the hydrophobic core of a micelle. The key benefit of this approach is that analysis is carried out using a fluorimeter or fluorescence plate reader, which is more commonly available in research labs. A number of fluorogenic probes have been reported including coumarin, curcumin, 1,6-diphenyl-1,3,5-hexatriene (DPH), pyrene, and Nile Red (NR).[13–15] Of these probes, pyrene and NR are used most commonly. In the presence of micelles, pyrene undergoes a change in the relative intensity of emission at 373 and 384 nm.[16,17] Though useful, resolving these two wavelengths requires a more sophisticated fluorimeter, and we have found that a standard fluorescence plate reader does not provide sufficient resolution to enable CMC measurement using pyrene. In contrast, with NR (Fig. 1a), the fluorescence intensity increases in the presence of micelles and often undergoes a blue shift in emission wavelength. These changes are of sufficient magnitude to enable CMC measurement using a standard fluorescence plate reader.[18,19] However, instances have been reported in which the emission wavelength of NR instead undergoes a red shift as surfactant concentration increases.[20,21] In these cases, the authors hypothesize that this anomalous behavior may be the result of dye aggregation. This is consistent with a recent report by Mohr and coworkers describing aggregation of NR to form non-emissive dimers *via* π–π stacking interactions.[22] This behavior creates a significant challenge for CMC measurement, as aggregation results in a hydrophobic environment similar to the core of a micelle, producing misleading results.

After witnessing challenges with NR aggregation in our own lab, we sought to find an alternative fluorogenic dye that could offer greater reliability while remaining suitable for measuring CMC values using a standard fluorescence plate reader. We

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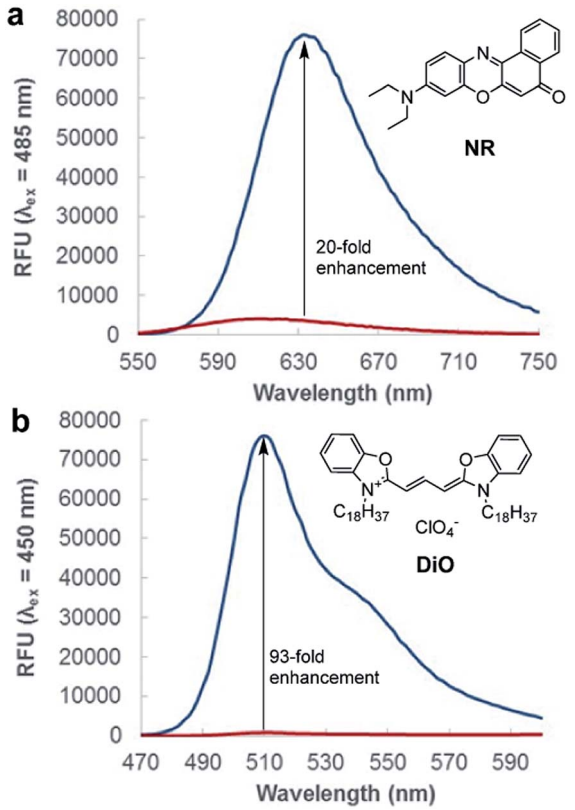

Fig. 1 Chemical structure and emission spectra for (a) NR and (b) DiO in DMSO (blue) and water (red). RFU = relative fluorescence units.

found that 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO, Fig. 1b) shows similar fluorogenic properties to NR, and has been previously used for lipophilic staining[23] and monitoring guest exchange dynamics in micelles and nanogels.[24] Upon transitioning from water to DMSO, DiO undergoes a dramatic increase in fluorescence intensity, with an emission maximum at 510 nm. Impressively, DiO shows a 93-fold fluorescence enhancement upon transition from water to DMSO, compared to only 20-fold enhancement observed for NR (Fig. 1).

Herein, we evaluate the utility of DiO for fluorescence-based CMC measurement, and directly compare its performance to that of NR. We find that DiO is compatible with a variety of surfactant types, and while NR and DiO both provide CMC measurements that agree with literature values, DiO did not suffer from failed measurements, as NR often did. Additionally, DiO was easier to handle than NR, as solubility and aggregation problems were not observed with DiO, but were frequent with NR. Therefore, DiO provides an accurate and reliable method for measuring CMC values without the need for specialized equipment.

Experimental section

All chemicals were purchased from commercial sources and used without further purification. NR and DiO stock solutions were prepared by dissolving the dye in DMSO to a concentration of 900 μM. Concentrated stock solutions of each surfactant were prepared in water (MilliQ), then combined with dye solution and additional water (MilliQ) to provide the appropriate final concentrations. The solutions were sonicated for 30 min at 35 °C then incubated at 25 °C for 5 hours. Following incubation, the solutions were transferred to a 96- or 384-well microplate, centrifuged, and allowed to equilibrate at 25 °C for 10 minutes.

All fluorescence measurements were carried out using a Biotek Synergy MX plate reader at 25 ± 0.5 °C. Excitation/emission wavelengths of 450/510 nm (DiO) and 485/636 nm (NR) were used, with a bandwidth of ±9 nm. Fluorescence intensity was plotted as a function of surfactant concentration, and each CMC value was calculated by one of two methods: (1) if the data formed two distinct lines, the concentration at which these lines intersect was calculated and determined to be the CMC. (2) If the transition was too sharp to provide a second intersecting line, the data were fit to a sigmoid function using Origin Pro 9.0. The second derivative was then used to determine the surfactant concentration at the lower inflection point, which is analogous to the intersection of the two lines, and thus represents the CMC.[1,25]

Results and discussion

To test the feasibility of using DiO as a fluorogenic dye for CMC measurement, we first employed the widely-used non-ionic surfactant Triton X-100. As shown in Fig. 2, increasing the surfactant concentration produces an increase in fluorescence intensity with a λ_{max} of 510 nm. This increase mirrors the change in fluorescence intensity observed when DiO is transferred from aqueous to organic solvent, strongly suggesting that DiO is being sequestered to the hydrophobic core of the Triton X-100 micelles.

Having established that DiO displays a dramatic fluorescence enhancement in the presence of Triton X-100, we set out to evaluate the accuracy and versatility of DiO for CMC measurement. We selected a set of 12 commonly used surfactants that included examples from each of the four ionic states and spanned a wide range of CMC values. For each surfactant, we carried out parallel experiments using DiO and NR to

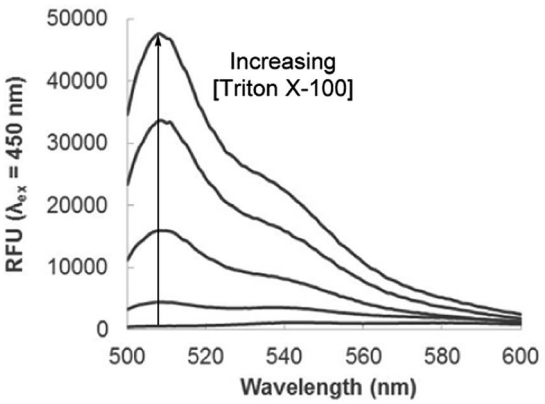

Fig. 2 DiO shows increasing fluorescence intensity with increasing concentrations of Triton X-100 surfactant.

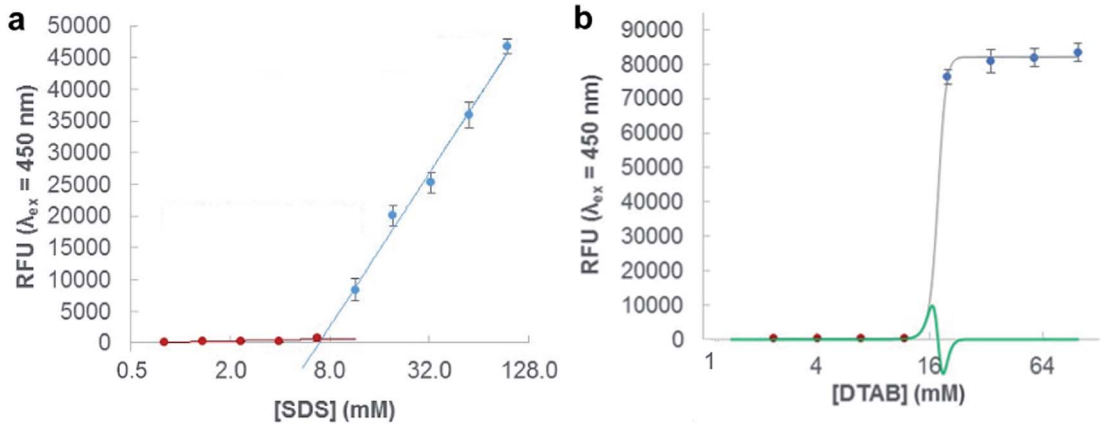

Fig. 3 Representative fluorescence data for CMC calculation. (a) If three or more data points can be obtained for the transition region, the CMC value can be calculated using the two-line method. (b) If the slope of the transition region is too steep to enable a linear fit, the second derivative method is used.

compare the accuracy and consistency of each probe. We initially used a dye concentration of 2 μM, as this is typical for NR studies.[19] While this dye concentration provided accurate CMC values for most surfactants, we observed atypical results in some cases, and found that increasing the dye concentration to 10 μM produced consistent results for all surfactants. Thus, unless otherwise noted, all reported data were collected using DiO or NR concentrations of 10 μM. In CMC measurements using fluorogenic dyes, an incubation time is required to allow equilibration of the dyes binding in the micelles. We found that for all surfactants, accurate CMC values could be measured after 5 hours of incubation, and for some surfactants, DiO provided accurate results with only 2 hours of incubation. This allows for fast screening of CMC values compared to some NR protocols that suggest overnight incubation.[26]

The λ_{max} values for DiO and NR show small variations based on surfactant structure, but at concentrations near or above the CMC of the surfactants, the emission maxima for DiO and NR were found to be centered upon 510 and 636 nm, respectively. Thus, these wavelengths were used in all CMC calculations. For each dye, plotting the fluorescence intensity as a function of surfactant concentration yields a sigmoidal curve, if a wide enough concentration range is used. In the region far below the CMC, fluorescence intensity is constant or increases slightly. Upon approaching the CMC, fluorescence intensity increases sharply as micelles form and sequester the dye molecules. Then, this trend levels off as all of the dye molecules become bound in micelles. Typically, the transition is sufficiently gradual to allow linear fits of the lower and middle regions of the sigmoid, and the intersection point of these two lines provides the CMC value (Fig. 3a).[1,11] In some cases, the transition is too sharp to provide a linear fit for the transition region. In these cases, the entire curve can be fit to a sigmoid, and the CMC value calculated by finding the maximum of the second derivative. This value represents the lower transition point, which is analogous to the intersection of the two lines in the former approach (Fig. 3b).

Table 1 CMC values obtained using DiO and Nile Red compared to those reported in the literature. Most CMC values were calculated using the two-line method. CMC values calculated using a sigmoidal fit are noted with an asterisk. The error represents the average of at least three trials

Charge state	Surfactant	CMC (lit)	CMC (DiO)	CMC (NR)	References
Non-ionic	Triton X-100	240 μM	195 ± 2 μM	271 ± 19 μM	7 and 25
	Tween 20	60–80 μM	66.5 ± 0.5 μM	79.9 ± 1.5[a] μM	7, 8 and 25
	Tween 80	12 μM	13.0 ± 0.2 μM	11.4 ± 1.6 μM	7, 8 and 25
	Brij 58	24–77 μM	32.4 ± 2.4 μM	36.7 ± 7.3 μM	8 and 25
Anionic	SDS	8.2 mM	7.11 ± 0.77 mM	8.37 ± 0.45* mM	7
	NaGC	4–14 mM	14.2 ± 0.1 mM	9.12 ± 0.34 mM	24
	NaTC	6–11 mM	14.3 ± 0.5 mM	6.10 ± 0.18 mM	7 and 23
Cationic	DTAB	14–16 mM	12.7 ± 0.8* mM	14.1 ± 1.0* mM	7
	CTAB	0.9–1.0 mM	2.65 ± 0.14 mM	0.780 ± 0.135* mM	7 and 25
Zwitterionic	CHAPS	6–10 mM	8.25 ± 0.20 mM	8.46 ± 2.13 mM	7 and 25
	EMPIGEN BB	1.6–2.1 mM	1.95 ± 0.04 mM	1.38 ± 0.07 mM	7
	Zwittergent 3-14	100–400 μM	268 ± 14 μM	259 ± 26 μM	25

[a] The CMC value for Tween20 was obtained using 1.25 μM NR.

The data in Table 1 show the CMC values obtained using DiO and NR with each of the twelve surfactants tested. Both dyes show good accuracy, giving CMC values comparable to those reported in the literature.[7,8,27–29] However, DiO provided overall greater precision, as some measurements using NR gave inconsistent data, leading to higher standard deviations. This is especially pronounced in the cases of Triton X-100, Brij 58, and Zwittergent 3-14. Fig. 4 shows data collected for Triton X-100 using both DiO and NR. The first NR trial gave two distinct lines, while the subsequent two trials resulted in noisy data that were more difficult to fit. On the other hand, each of the three DiO trials provided consistent data. It is also important to note that in the case of DiO, the change in slope between the two lines is much greater than that observed for NR. This made the assignment of data points to their respective regions easier when working with DiO, further demonstrating its superior accuracy and precision.

We also found that DiO consistently provided usable data, whereas many trials using NR provided data that could not be used to calculate a CMC value (see ESI† for examples). These failures on the part of NR are not directly reflected in the data, as we repeated these experiments numerous times to obtain at least three usable data sets with NR that were then utilized to generate the CMC values reported in Table 1. This highlights the fact that CMC measurement using NR generally required greater time and resources compared to DiO, further convincing us of the superiority of DiO.

In addition to lower precision and success rate, we found that NR often showed a problematic shift in λ_{max} as a function of surfactant concentration, while the λ_{max} values for DiO remained consistent across surfactant concentrations. For example, in the case of NaGC, DiO maintains a λ_{max} value of 508–510 nm across all surfactant concentrations. In contrast, the λ_{max} for NR undergoes a gradual increase, followed by a sharp decrease, as surfactant concentration decreases (Table 2). While working with both of the dyes in our lab, we found that DiO consistently showed excellent solubility at 10 μM concentration, while NR often left a ring of dye adhered to the side of the microcentrifuge tube (Fig. 5). This observation is worrisome, as it indicates that the actual concentration of NR in the solutions is not necessarily reproducible, which may be the

Table 2 λ_{max} for DiO and NR emission in the presence of NaGC

[NaGC] (mM)	DiO (nm)	NR (nm)
51.0	508	638
26.0	508	638
18.0	508	642
6.47	510	650
2.24	508	654
0.77	510	620

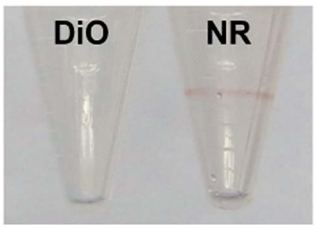

Fig. 5 NR often left a visible ring of aggregated dye (right) while DiO did not (left).

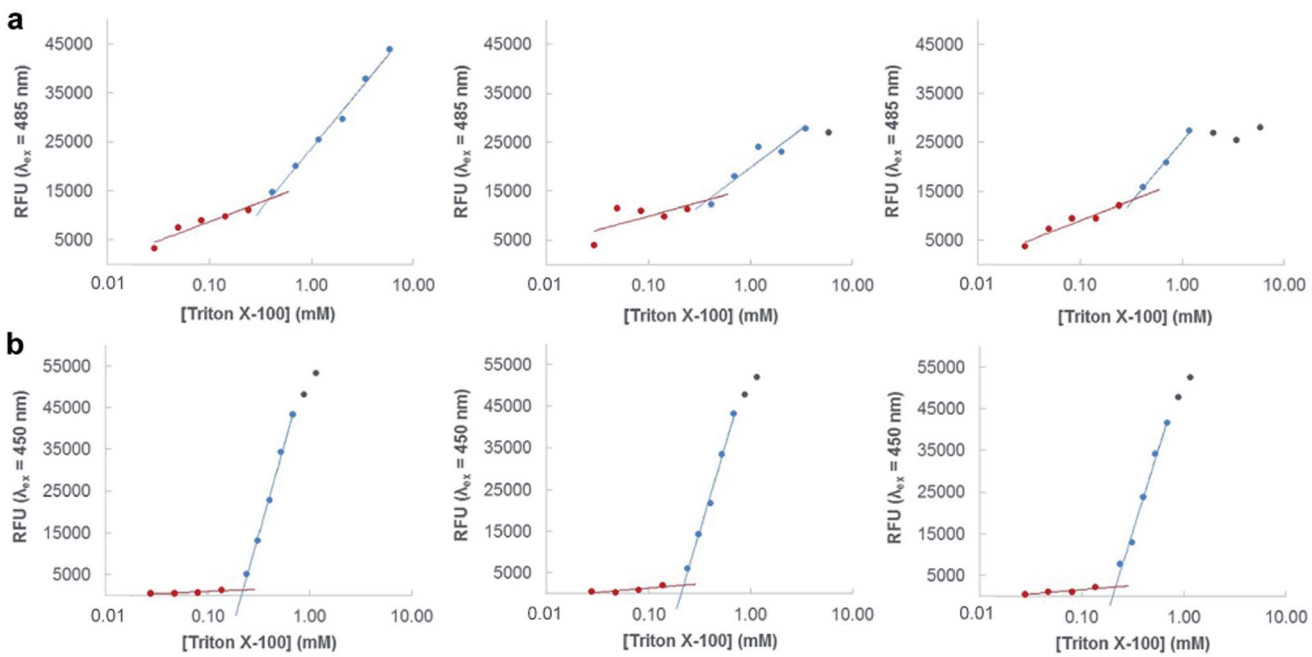

Fig. 4 CMC curves collected for Triton X-100 using (a) NR and (b) DiO. DiO shows superior consistency as well as greater change in slope upon micelle formation.

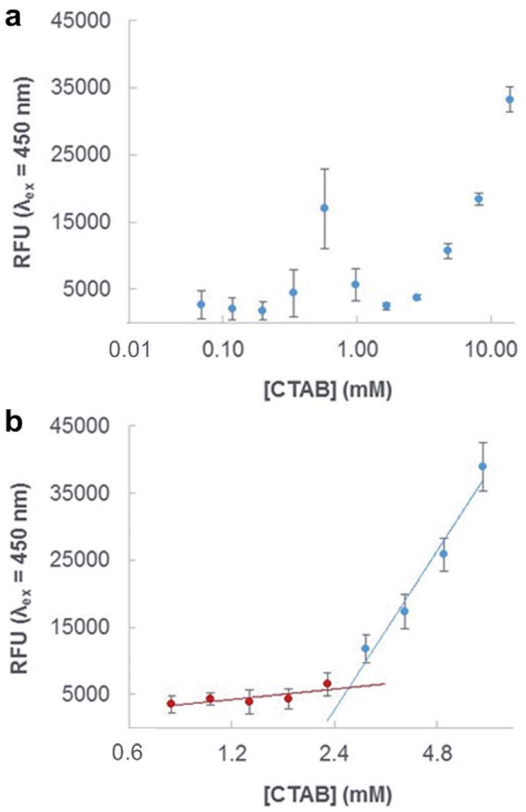

Fig. 6 (a) The emission intensity of DiO temporarily spikes at approximately 0.6 mM CTAB. (b) However, the CMC can be calculated for CTAB using a smaller concentration interval range.

source of many of the issues discussed above. It also accounts for the inconsistencies observed in λ_{max} value, as the NR is likely aggregating in solution, leading to a change in local environment and thus emission wavelength.

Among the four classes of surfactants, we found that the cationic surfactants CTAB and DTAB proved to be the most challenging for measuring CMC values using either DiO or NR. Despite surveying very narrow intervals of surfactant concentration, we were typically unable to acquire a sufficient number of data points in the transition region to calculate CMC using the two-line method. Thus, in the analysis of DTAB using either DiO or NR and the analysis of CTAB using NR, we instead employed the second derivative method. Despite using a different analysis method, CMC values in line with reported values were still obtained (Table 1). In the case of CTAB analysis using DiO, we observed an interesting and reproducible peak in fluorescence intensity at approximately 0.6 mM surfactant (Fig. 6a). While the source of this peak is unclear, we did observe the expected transition as surfactant concentration was increased, and were able to measure the CMC of CTAB by using data points at concentrations above this anomalous signal (Fig. 6b). Our calculated CMC value using this method is slightly higher than the previously reported values, demonstrating that for this surfactant, NR does provide greater accuracy than DiO. However, it is important to note that cationic surfactants represent only about 5% percent of all commercially available surfactants,[30] and thus this limitation associated with DiO is relatively minor.

Conclusions

The data presented here demonstrate that DiO is a promising alternative to NR for the measurement of surfactant CMC values. We find that both DiO and NR provide CMC values that are consistent with those previously reported in the literature. However, DiO provides superior precision and reproducibility. We hypothesize that the inconsistency of results obtained using NR largely stems from its propensity to aggregate in aqueous solution, especially in the presence of low surfactant concentrations. In our hands, this led to difficulties in sample handling as well as multiple instances of failed experiments. Thus, we find that DiO is generally a more user-friendly and reliable fluorogenic dye for the measurement of surfactant CMC values.

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