The determination of the critical micelle concentration (CMC) value of surfactants under different environmental conditions is important for a number of different biological and chemical processes. Because the CMC is not a constant value, shifting with different environmental conditions, it is important that a rapid, reliable and easy methodology be available to facilitate testing. Here we describe the rapid semi-automated determination of CMC values for surfactants in 384-well microplates using fluorescence polarization.

**Introduction**

Surfactants are amphiphilic compounds that have a molecular structure containing both a hydrophilic (water loving) and a hydrophobic (water hating) region. The hydrophobic region is usually a long chain aliphatic hydrocarbon, whereas the hydrophilic portion can be composed of an ionic or non-ionic polar group. The physical nature of these molecules bestows the ability to reduce surface tension of solutions and to self-aggregate into colloids known as micelles.

A micelle is an aggregation of surfactant molecules in a colloidal suspension. A typical micelle in aqueous solution forms with the hydrophilic head regions in contact with the water and the hydrophobic aliphatic tail regions buried in the inner portion of the micelle. Useful surfactants are soluble to some degree in aqueous solution and only aggregate into micelles when they reach a sufficient concentration. This concentration is referred to as the critical micelle concentration (CMC) (Figure 1). Below the CMC micelles are not present and the surface tension of the solution decreases and osmotic pressure increases with an increase in surfactant. Above the CMC, the concentration of unaggregated surfactant will stay constant and the number of micelles will increase as the total surfactant concentration increases. This results in increases in solution turbidity and solubilization with increased surfactant concentration. Once the CMC is reached the change in surface tension with surfactant concentration is significantly reduced or eliminated with further increase in surfactant.

Amphiphilic surfactants have numerous properties other than lowering of surface tension and are often labeled as to the use (e.g. soap, detergent, wetting agent, dispersant, emulsifier, foaming agent, bactericide, corrosion inhibitor, antistatic agent etc.). While commercially classified by their use, scientifically they are classified based on their dissociation in water. Anionic surfactants dissociate in water into an amphiphilic anion (neg. charge) and a simple cation (e.g. Na+, K+). Anionic surfactants are the most commonly used surfactants, accounting for about 50% of the world’s production [1].

Nonionic surfactants account for approximately 45% of all surfactants. These agents do not ionize in solution and typically have a hydrophilic group composed of an alcohol, phenol, ether, ester or amide. Many nonionic surfactants contain polyethylene glycol chains. Cationic surfactants form an amphiphilic cation and an anion in aqueous solution. Often this class contains nitrogen compounds such as fatty amine salts of quaternary ammoniums linked to one or more long chain alkyl hydrophobic moieties.
This group is less popular as a result of the cost of their manufacture and is only used when a less expensive substitute cannot be found. Typically they are used as a bactericide, antistatic, and for corrosion inhibition. When a single surfactant molecule contains both anionic and cationic dissociations it is referred to as amphoter or Zwitterionic. These compounds include synthetic betaines or sulfobetaines and natural substances such as amino acids and phospholipids.

Considerable effort has been made on predicting CMC values for surfactants. Accurate prediction of CMC prior to synthesis of new compounds would enable the customization of surfactants to meet specific needs [14]. For some surfactant types the predicted CMC agrees quite well with the observed values under defined conditions, primarily in simple aqueous solution. Empirical relationships have been utilized to generate mathematical relationships between surfactant structure and CMC. Most notable is the linear relationship between the log of CMC and the number of alkane carbon atoms in linear alkyl hexaethoxylates [14]. Thermodynamic models have also been used to predict CMC for various surfactants [17]. Most recently a systematic quantitative structure-property relationship (QSPR) approach has allowed for predictive modeling equations to be generated for more classes of surfactants [18]. Despite the effort made in predicting surfactant behavior under many conditions, the only way to determine the CMC is to do so empirically.

Critical micelle concentrations have been experimentally determined using a number of different methodologies. UV-spectroscopy of benzoylecetone (BZA) has been used to determine CMC [13]. BZA exists in an equilibrium mixture of keto and enol tautomers when dissolved in water. The amount of enol tautomer increases dramatically in the presence of surfactant above the CMC, as the enol form partitions to the inner portion of the micelle [13]. Lipid soluble dyes such as Hoechst 33342 [6] or Nile red [7] demonstrate enhanced fluorescence in a hydrophobic environment such as when micelles begin to form and the dye partitions in its hydrophobic core. Nile red's spectral shift in different solvents has also been exploited to monitor micelle formation [8]. The fluorescent compound pyrene exhibits five major vibrational fluorescent peaks, which vary depending on the solvent. The ratio of the fluorescence intensity of peak 1 to that of peak 3 is indicative of the local environment. The pyrene 1:3 ratio plots of surfactant titrations generate decreasing sigmoidal shaped curves. Surfactant concentrations below the micelle concentration result in a polar environment indicative of a high peak 1 to peak 3 ratio. As surfactant concentrations increase and approach the CMC, the pyrene 1:3 ratio begins to decrease rapidly to reaching a new lower constant value that reflect the 1:3 ratio at surfactant concentrations above the CMC [9].

In addition to fluorescent methods, changes in conductivity [10], increase in light scattering [11] and even solid state electrodes [12] have been used to determine critical micelle concentrations of surfactant molecules in solution.

The fluorescence polarization of fluorescent molecules that have been modified to interact with micelles can be exploited to determine CMC of surfactants [3]. 5-dodecanoylaminofluorescein (DAF), is essentially a fluorescent probe connected to an aliphatic tail, which can be inserted into the micellar inner region, but not become completely immersed in the interior of the micelle. By doing so the effective molecular volume of the fluorescent compound DAF is that of the micelle, which is significantly larger than the lipophilic probe alone. As such the rotational speed differential can be exploited through fluorescence polarization measurements.

Fluorescence polarization (FP) is a fluorescence detection technique first described in 1926 by Perrin [2]. It is based on the observation that fluorescent molecules, when excited by polarized light, will emit polarized light. In solution, the polarization of the emitted light is inversely proportional to the molecule’s rotational speed, which is influenced by molecular volume or by approximation, molecular weight. Fluorescence polarization is measured using the ratio of the fluorescence emission returned through two polarizing filters, one parallel (‖) to and one perpendicular (┴) to the plane of polarized excitation light. Fluorescence polarization (P) is calculated using the following formula, where G is an instrument and assay dependant correction factor.

\[
\text{P} = \frac{(\text{exp} - \text{blank}) - G \ast (\text{┴ exp} - \text{┴ blank})}{(\text{exp} - \text{blank}) - G \ast (\text{‖ exp} - \text{‖ blank})}
\]

Eq 1.  

Data is often multiplied by 1000 and expressed and expressed as millipolarization (mP).

Calculation of CMC:
The fluorescence polarization data generated in these experiments produces a sigmoidal shaped curve that can be described using a 4-parameter logistic curve fit [5] which is given by

\[
\gamma = A - \frac{D}{1 + \left(\frac{x}{C}\right)^b} + 1
\]

Eq 2.  

Where variable y corresponds to the polarization value of a given surfactant concentration at a concentration x and A is the theoretical response at the lower concentration, B is the relative slope of the curve at its inflection point, C is the concentration value at the inflection point, and D is the response at the highest concentration.
While there are a number of different methods to calculate the CMC value from experimental plots. One method that was originally described for use with the pyrene 1:3 method [4], utilizes the interception of the rapidly changing portion of the curve and the nearly horizontal lower concentration portion of the curve [3]. This method can be modified for use with an increasing sigmoidal curve that is observed with fluorescence polarization (Figure 2).

![Figure 2. Schematic of CMC determination from Sigmoidal shaped plot.](image)

In a 4-parameter logistic fit the slope of the tangent line can be described using the equation

Eq 3. \( \gamma = LS \times \log(x) + LSb \)

Where LS is the LogSlope and LSb is the y intercept of the line. The LogSlope (LS) can be calculated from the individual parameters of the 4-parameter logistic equation used to describe the original data using the equation

Eq 4. \( LS = B \times (D - A) \times \ln(10)/4 \)

LSb is calculated using the information provided by the inflection point according to the equation.

Eq 5. \( LSb = (A + D)/2 - LS \times \log(C) \)

As previously stated the CMC value is the intersection (Ix) between the lower horizontal portion of the curve and the tangent line. Thus

Eq 6. \( A = LS \times \log(Ix) + LSb \)

or

\( \log(Ix) = A - LSb/LS \)

The antilog of the resultant value is the calculated CMC value.

**Materials and Methods**

The surfactants domiphen bromide (P/N 247480), sodium lauryl sulfate (P/N L4509), N-Benzyl-N,N-dimethyl-1-dodecanaminium chloride (P/N 13380), N-Benzyl-N,N-dimethyl-1-tetradecanaminium chloride (P/N 13401), N-Benzyl-N,N-dimethyl-1-hexadecan-aminium chloride (P/N B4136), Ipegal 630 (P/N 18896), Triton X-100 (T8787), polysorbate 20 (P/N P1379), polysorbate 40 (P/N P1504), polysorbate 60 (P/N 95754), polysorbate 80 (P/N 59924), zwittergent (P/N T7763) were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions (200 mM) of these surfactant compounds were made in Milli-Q water. Solid black 384-well microplates (3573) were obtained from Corning (Corning, NY) and 5-dodecanoylamino-fluorescein (P/N D109) was purchased from Life Technologies. A 5X stock solution of Hepes buffer (125 mM, pH 8.0) and 2.5X solutions of sodium chloride (0.25%, 2.5%, and 25% w/v) were prepared, filter sterilized and stored at room temperature.

Assays were run in 384-well plates such that different compound dilutions, fluorescent stains, buffer constituents and salt concentrations could be used interchangeably. Reagents were added as 5X or 2.5X solutions to achieve the intended final concentrations. Compound dilutions were made fresh daily from 200 mM stock solutions and pipetted (15 µL) into microplates manually. After compound titration 5X Hepes reaction buffer (pH 8.0) solution (15 µL) was added using a syringe pump on a MultiFlo™ automated dispenser (BioTek Instruments, Winooski VT). Sodium chloride solutions (30 µL) were then immediately added using the MultiFlo peripump dispenser. For experiments where multiple salt concentrations were required, different tubes from the 8-tube peristaltic pump pulled from different reagent reservoirs. After 3-minute incubation, 15 µL of DAF fluorescent dye (5 µM) was added using a MultiFlo syringe pump dispenser. The fluorescent polarization was measured after 20 minute incubation (Figure 3) using a Synergy™ Neo (BioTek Instruments, Winooski, VT). Parallel and perpendicular readings were made simultaneously using filter cubes 4 and 61 with 485/20 excitation and 528/25 emission filters.
The reader was controlled and the data captured using Gen5 Data Analysis software (BioTek Instruments). Experimental differences in fluorescence polarization data were adjusted using the G-factor to return a value of 29 mP for free unbound DAF tracer. The data was automatically plotted as a 4-parameter logistic fit and the CMC calculated by the Gen5 software.

Results

When different surfactant molecules are compared, markedly different curve shapes and CMC values are evident. Of the molecules tested, the non-ionic polysorbate 20 (Tween 20) had the lowest CMC, while the quat C12 compound formed micelles at much higher molar concentrations.

In addition to the differential concentration at which micelles begin to form it is important to note that the polarization value in the presence of micelles of each compound is quite different, despite correcting the 100% unbound tracer polarization wells to the same value. This suggests that each surfactant forms micelles of different sizes.

The concentration at which many surfactants form micelles is affected by sodium chloride concentration. As demonstrated in Figure 5, the fluorescence polarization curves and by inference the CMC for domiphen bromide is affected by the amount of sodium chloride present. The marked increase in polarization, which denotes the formation of micelles occurs at lower concentrations in the presence of sodium and chloride ions. The CMC for each concentration can be determined mathematically (Table 1) and the fold change from the no salt value plotted as a function of sodium chloride concentration (Figure 6). With increasing sodium chloride concentration, the CMC of Quat C12 and domiphen bromide decrease nearly 50-fold. However, this large effect is not uniform with all surfactants as polysorbate 20 is minimally affected by changes in sodium chloride. This is no doubt due to it being a non-ionic detergent and so the salt will not affect micelle formation to the same degree.

<table>
<thead>
<tr>
<th>NaCl Conc (% w/v)</th>
<th>CMC (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.351</td>
</tr>
<tr>
<td>0.05</td>
<td>0.389</td>
</tr>
<tr>
<td>0.1</td>
<td>0.192</td>
</tr>
<tr>
<td>0.5</td>
<td>0.0817</td>
</tr>
<tr>
<td>1.0</td>
<td>0.0657</td>
</tr>
<tr>
<td>5.0</td>
<td>0.0153</td>
</tr>
<tr>
<td>7.5</td>
<td>0.0104</td>
</tr>
<tr>
<td>10.0</td>
<td>0.00791</td>
</tr>
</tbody>
</table>

Table 1. Determined CMC values for domiphen bromide at different sodium chloride concentrations.
Hydrogen ion concentration can play a significant role in micelle formation also. As demonstrated in Figure 7, Quat C12 and domiphen form micelles at an approximately 50-fold lower concentrations in an acidic environment, as compared to a neutral pH. SDS and Ipegal 630 are much less affected by hydrogen ion concentration, showing little to no change from pH 5.5 to 9.0. Because the hydrophilic polar region of the molecule is dependent on the ionization state of the molecule, positively charged molecules such as Quat C12 or domiphen bromide would certainly be expected to be influenced by hydrogen ion concentration and their effectiveness at forming micelles at low pH is not surprising. Non-ionic detergents (e.g. Ipegal 630) are more resistant to changes in hydrogen ion concentration. Somewhat surprising is the resistance of the CMC for the negatively charged SDS to change with the pH levels tested. Although the slight decrease in the fold change at a pH of 9.0 for SDS suggests that micelles may form at significantly lower surfactant concentrations at higher pH. This 10-fold decrease in CMC with increasing tail length is not an absolute phenomenon. As demonstrated in Figure 9, where three different polysorbate surfactants are compared, only small differences in the CMC is observed with molecules having different length aliphatic tails. Polysorbate 20 and 60, which have tail lengths of 12 and 18 respectively has a decrease in CMC of approximately 2-fold, despite an increase in tail length of 6 carbons.
In addition, the presence of a double bond does not significantly influence the formation of micelles with polysorbate compounds (Figure 10.) Polysorbate 60 and polysorbate 80 differ by the presence of a double bond linkage present in the aliphatic tail (see appendix). When the CMC value is calculated only a very small difference is observed (Table 2).

<table>
<thead>
<tr>
<th>Compound</th>
<th>CMC (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quat C12</td>
<td>3.375</td>
</tr>
<tr>
<td>Quat C14</td>
<td>0.194</td>
</tr>
<tr>
<td>Quat C16</td>
<td>0.0421</td>
</tr>
<tr>
<td>Polysorbate 20</td>
<td>0.00694</td>
</tr>
<tr>
<td>Polysorbate 40</td>
<td>0.00381</td>
</tr>
<tr>
<td>Polysorbate 60</td>
<td>0.00367</td>
</tr>
<tr>
<td>Polysorbate 80</td>
<td>0.00300</td>
</tr>
</tbody>
</table>

Table 2. Determined CMC values for Quat and polysorbate surfactants with different aliphatic tails.

While increasing aliphatic tail length reduces the CMC value for surfactants, compounds with small polar heads are influenced by the length of the aliphatic tail to a much greater extent than surfactants with large non-ionic polar-regions. The ionic quaternary compounds tested show an approximately 10 fold decrease in CMC with each additional increase of a 2 carbon length. Non ionic detergents such as simple polyoxyethylenes (e.g. Triton X-100) or the polysorbitans, such as polysorbate 20, are polyoxyethylene derivatives which show more modest decreases.

Preparations of these many non-ionic surfactants are often mixtures of slightly different compounds with the number of oxyethylene moieties represented as an average. All of the polysorbitans have a total of 20 oxyethylene moieties linked to a sorbitol sugar (see Appendix), with differences being in the fatty acid tail, which are designated by the number present in the name. Ipegal 630 and Triton X-100, while having different names and described uses in the literature, are essentially the same molecule and have approximately 9 oxyethylene groups (see Appendix). A number of studies have shown that head size of alcohol ethoxylates directly influences the CMC. Head size is proportional to the number of ethylene oxides present [16]. In either molecular class the polar head structure is relatively large as compared to the ionic polar regions of the positively charged Quat compounds or the negatively charged SDS. The CMC for these compounds appear to be much less influenced by length of the aliphatic tail.

The effect of sodium chloride on micelle formation is two-fold. Sodium chloride significantly decreases the CMC of ionic surfactants such as domiphen bromide or the quat compounds. In addition polarization values of surfactants above the CMC in the presence of salt are higher than those without, which suggest that the size of the micelle aggregates is larger. A slight increase in micelle size is also observed with non-ionic surfactants. This increase in size could be manifested by either more molecules on average in each micelle or a swelling of the micelles as a result of ionic forces.

These data demonstrate that the use of DAF fluorescence polarization as a means to determine the CMC values for surfactants is not only easy and accurate, but that the method can also be easily scaled for large sample numbers. Unlike fluorescence intensity, fluorescence polarization uses a ratio of two measurements on each well, correcting for differences in intensity brought about by experimental conditions, such as pH, temperature, and surfactant concentration. The Synergy Neo reader is a high throughput reader specifically designed for the measurement of large numbers of samples.
The reader uses modular optic cubes to measure numerous read modalities, which include UV-Vis absorbance, luminescence, fluorescence intensity, time resolved fluorescence, HTRF®, AlphaScreen®, and fluorescence polarization. In regards to fluorescence polarization, the reader is capable of simultaneously determining parallel and perpendicular measurements. Gen5™ software (BioTek instruments) not only controls reader function, but also is capable of automatically performing the 4-parameter logistic fit and calculating CMC values.

References

15. Kubota, Y. M. Kodama, and M. Miura (1979) Bull

Acknowledgements

I would like to acknowledge Eddie Delpierre (BioTek Instruments) and Sara Held (Community College of Vermont) for their assistance with the mathematical determination of CMC values from the 4-parameter logistic fit equitation.