Journal of Undergraduate Chemistry Research, **2020**,19(1),1

DETECTION OF NITROAROMATICS BY QUENCHING OF FLUORESCENCE FROM CHLOROPHYLL IN DETERGENT MICELLES

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Abstract

Nitroaromatic compounds are present in an array of products like pesticides and explosives. The environmental and safety hazards of nitroaromatics make the ability to detect their presence extremely important. This experiment describes a simple, economical method for detecting nitroaromatics using chlorophyll. Chlorophyll was inserted into various detergent micelles in order to minimize photooxidation. The critical micelle concentration values for each detergent with respect to chlorophyll were measured and the chlorophyll-containing micelles were then exposed to 2,4-dinitrotoluene, a nitroaromatic explosive simulant and a fluorescence quenching agent. The emission spectra of the chlorophyll-containing micelles were obtained to determine which detergent provided the greatest sensitivity to quenching. Based upon the K_{sv} values, the detergent most sensitive to nitroaromatic quenching using both chlorophyll a and chlorophyll b was determined to be octyl-β-D-glucopyranoside. Here we have shown that environmentally friendly reagents can be used to stabilize chlorophyll from photooxidation for use in detecting nitroaromatic compounds.

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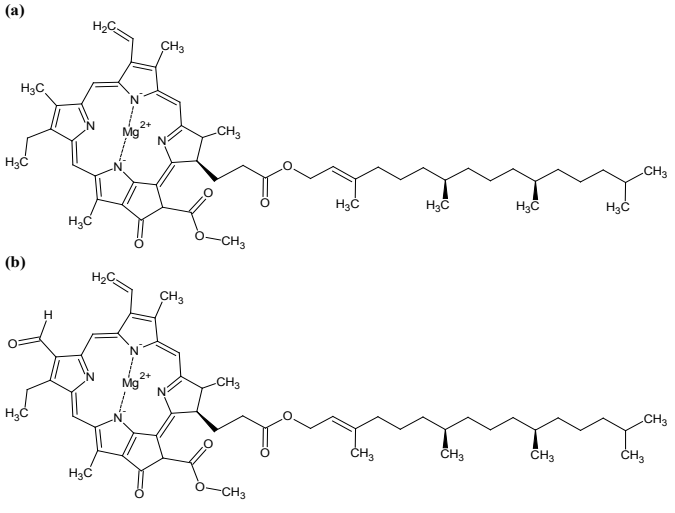
Keywords: chlorophyll, CMC, detergent, fluorescence, micelles, nitroaromatic, quenching

Introduction

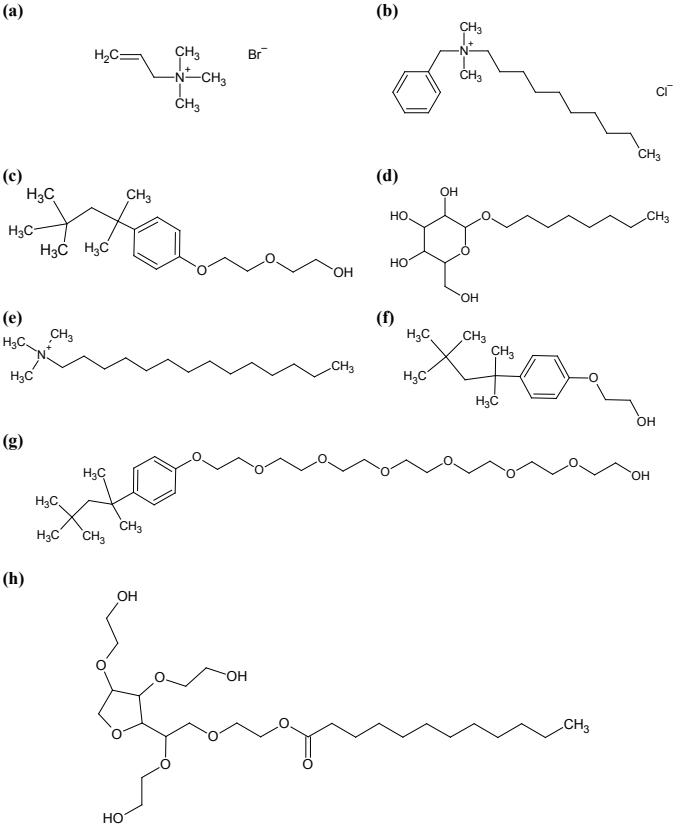
There are two major analogs of chlorophyll (Chl) found in plants: chlorophyll a (Chla) and chlorophyll b (Chlb) (Scheme 1) (1-2). Chl fluorescence can be quenched by numerous agents including oxygen, quinone, and nitrobenzene (3-5). Nitroaromatics like nitrobenzene are used in explosives and fertilizers, making detection of the molecule critical for environmental health and for society's safety (6). Several methods already exist for detecting nitroaromatics, including detection by quenching of Chl fluorescence (7-8). These methods, however, do not account for the photooxidation of Chl. Photooxidation reduces Chl's fluorescence; so failure to account for the photooxidation of Chl results in inaccurate nitroaromatic detection, specifically regarding concentration (9). The research described herein explores quenching of Chl incorporated into detergent micelles by 2,4-dinitrotoluene (DNT) as an inexpensive, reliable method for nitroaromatic detection.

Chl was incorporated into 8 different detergent micelles in order to protect Chl from photooxidation (Scheme 2). Chl's hydrophobic phytol chain is embedded within the micelle, preventing it from being photooxidized. This orientation also protects the carbonyl group on Chl's porphyrin ring from photooxidation. The portion of the porphyrin ring that interacts with DNT remains exposed at the hydrophilic membrane of the micelle. The critical micelle concentrations (CMC) for the Chl-detergent micelles were measured in order to determine which detergent best incorporates Chl into micelles. The micelles were then exposed to DNT, and the fluorescence spectra of the micelle-DNT complexes were collected

Scheme 1. Structures of **(a)** Chla and **(b)** Chlb.



Scheme 2. Structures of detergents. **(a)** Alkyl trimethyl ammonium bromide **(b)** Benzyldimethylhexadecylammonium chloride **(c)** Nonidet P-40 **(d)** Octyl β-D-glucopyranoside **(e)** Trimethyl-tetradecylammonium chloride **(f)** Triton X-100 **(g)** Triton X-114 **(h)** TWEEN 20.



in order to determine K_{sv} values. The K_{sv} values indicate which detergent promotes the greatest interactions between Chl and DNT, allowing the most accurate nitroaromatic detection method.

Experimental Section

Unless otherwise specified, all reagents used were purchased from Sigma-Aldrich, St. Louis, MO. Absorbance and fluorescence data were collected using both a Synergy H4 spectrofluorimeter (BioTek, Winoosky, VT) and a Cary Eclipse fluorescence spectrophotometer (Varian, Mulgrave, Victoria Australia).

Chlorophyll Fluorescence

Chla in hexane (100 µg/mL, Alfa Aesar, Ward Hill, MA) and Chlb in hexane (100 µg/mL, Alfa Aesar, Ward Hill, MA) were diluted 20-fold with methanol in order to prepare standard Chl solutions (5 µg/mL). A blank was prepared using the same volumes of hexane and methanol. Fluorescence of Chla, Chlb, and the blank were measured in a black UV transparent 96-well plate using a Synergy H4 spectrofluorimeter. The emission spectra were collected from 600 nm to 800 nm with a step of 2 nm, an emission slit width of 9 nm, a fixed λ_{ex} of 435 nm, an excitation slit width of 9 nm, and gain of 90. The excitation spectra were collected from 350 nm to 690 nm with a step of 2 nm, an emission slit width of 9 nm, a fixed λ_{ex} of 720 nm, an excitation slit width of 9 nm, and gain of 110.

Chlorophyll Fluorescence in Detergent Micelles

1% (m/v) stock solutions of each detergent were prepared by dissolving 0.5 g of detergent in 50 mL of 50 mM phosphate buffer (pH 7.3). 0.8% detergent-Chl micelle solutions were prepared using 960 µL of 1% detergent stock solution, 12 µL of Chla standard, and 228 µL of the phosphate buffer. Fluorescence emission was measured in a black UV transparent 96-well plate using a Synergy H4 spectrofluorimeter. The emission spectra for each detergent were collected from 600 nm to 800 nm with a step of 2 nm, an emission slit width of 9 nm, a fixed λ_{ex} of 430 nm, an excitation slit width of 9 nm, and gain of 100. The process was repeated using the Chlb standard.

Detergent-Chlorophyll Micelle CMC Determination

The 1% (m/v) stock solutions of each detergent were diluted 100-fold to 0.01% (m/v) using the 50 mM phosphate buffer. Ten detergent-Chl micelle solutions ranging from detergent concentrations of 0.005% (m/v) to 0.800% (m/v) were prepared with 12 µL of Chla (20 µg/mL in hexane) and enough phosphate buffer to prepare volumes of 1200 µL. The process was repeated for Chlb. A blank was made from 1% detergent stock and phosphate buffer.

Fluorescence was measured in a black UV transparent 96-well plate using a Synergy H4 spectrofluorimeter. Fluorescence of Chla was collected at an λ_{ex} of 430 nm, an excitation slit width of 9 nm, an λ_{em} of 676 nm, an emission slit with of 9 nm, and gain of 95. Fluorescence of Chlb was collected at an λ_{ex} of 480 nm, an excitation slit width of 9 nm, an λ_{em} of 660 nm, an emission slit width of 9 nm, and gain of 115.

As is standard when determining CMC based on fluorescence, the log of detergent concentration was plotted against fluorescence emission (10). The CMC was then mathematically derived based on the detergent concentration corresponding to the inflection point of the curve.

Chlorophyll Fluorescence in 2,4-dinitrotoluene

Twelve DNT assays ranging from concentrations of 0 µM to 2000 µM were prepared using 12 µL of Chla (20 µg/mL in hexane), 960 µL of 1% (m/v) Nonidet P-40 (Bohringer, Mannheim, Germany) in sodium acetate buffer, and 108 µL of sodium acetate buffer. Methanol was added to bring the volumes to 1200 µL. Twelve additional assays were prepared using Chlb. Fluorescence spectra were collected in a black UV transparent 96-well plate using a Synergy H4 spectrofluorimeter. The emission spectra of Chla were collected from 600 nm to 800 nm with a step of 2 nm, an emission slit width of 9 nm, a fixed λ_{ex} of 430 nm, an excitation slit width of 9 nm, and gain of 90. The emission spectra of Chlb were collected from 600 nm to 800 nm with a step of 2 nm, an emission slit width of 9 nm, a fixed λ_{ex} of 480 nm, an excitation slit width of 9 nm, and gain of 110.

Photostability of Chlorophyll in Organic Solvents

The relative photostabilities of Chla and Chlb in the organic solvent methanol and in Triton X-100 were determined. The organic samples were prepared with 12 µL Chl (20 µg/mL in hexane) and 1188 µL of methanol. A 1% (m/v) solution of Triton X-100 was prepared with 50 mM phosphate buffer (pH 7.3).The detergent samples were prepared with 12 µL of Chl (20 µg/mL in hexane), 960 µL of Triton X-100 solution, and 228 µL of phosphate buffer pH. The emission spectra for the samples were collected in a black UV transparent 96-well plate with a Cary Eclipse fluorescence spectrophotometer.

Chla emission was collected from 600 to 800 nm with a fixed λ_{ex} of 430 nm, a scan rate of 360 nm/min, 30 scans/min, an averaging time of 0.025 s, a data interval of 0.15 nm, and a detector voltage of 725 V. Chlb emission was collected from 600 to 800 nm with a fixed λ_{ex} of 470 nm, a scan rate of 360 nm/min, 30 scans/min, an averaging time of 0.025 s, a data interval of 0.15 nm, and a detector voltage of 625 V.

Results and Discussion

Chlorophyll Fluorescence

Figure 1 shows the fluorescence spectra of Chla and Chlb. For Chla, the maximum of the Soret band occurs at 430 nm, the maximum of the Q-band occurs at 670 nm, and the maximum emission for Chla occurs at 676 nm. For Chlb, the maximum of the Soret band occurs at 475 nm, the maximum of the Q-band occurs at 654 nm, and the maximum emission for Chlb occurs at 670 nm. These parameters were used to collect the fluorescence spectra for Chla and Chlb throughout the experiment.

Figure 2 shows the fluorescence emission spectra of Chla and Chlb in micelles of each detergent analyzed. A majority of the spectra resemble those of Chl that has not been incorporated into detergent micelles. The micelles comprised of Triton X-114 reduced the Stokes shift of both Chla and Chlb. The micelles comprised of Nonidet P-40 reduced the Stokes shift of Chla. This can be attributed to the incorporation of a portion of the pi-bonded groups of Chl into the detergent micelles.

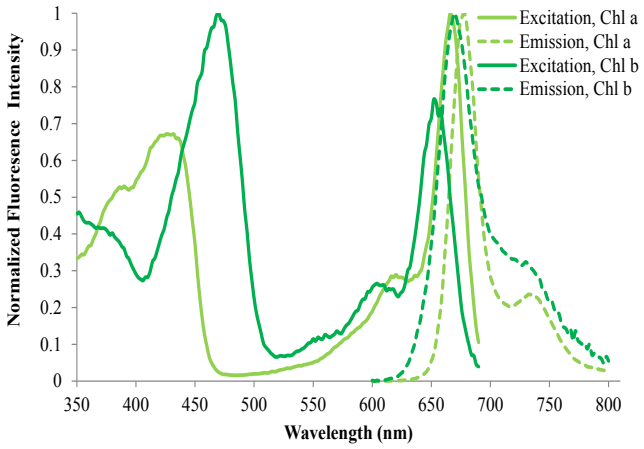


Figure 1. Fluorescence of Chla and Chlb. Chla reaches maximum excitation at 430 nm. Chlb reaches maximum excitation at 475 nm. Both molecules undergo a red shift in their emission spectra. Both Chla and Chlb exhibit emission from 600 nm to 800 nm.

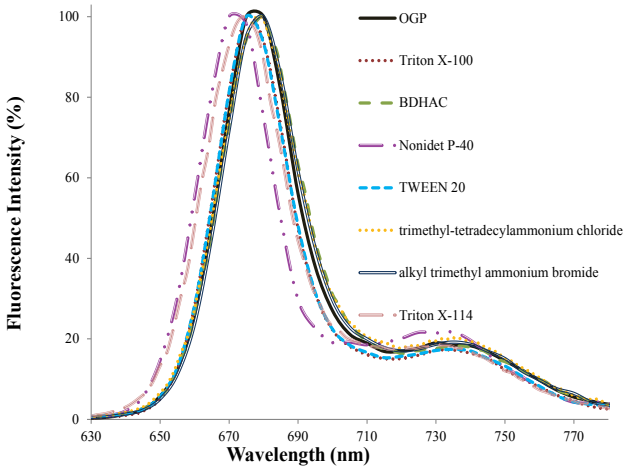


Figure 2a. Plot of fluorescence intensity versus wavelength for Chla in each detergent analyzed. Nonidet-P-40 and Triton X-114 micelles reduce redshift, indicating protection of some of Chla's pi-bonded groups.

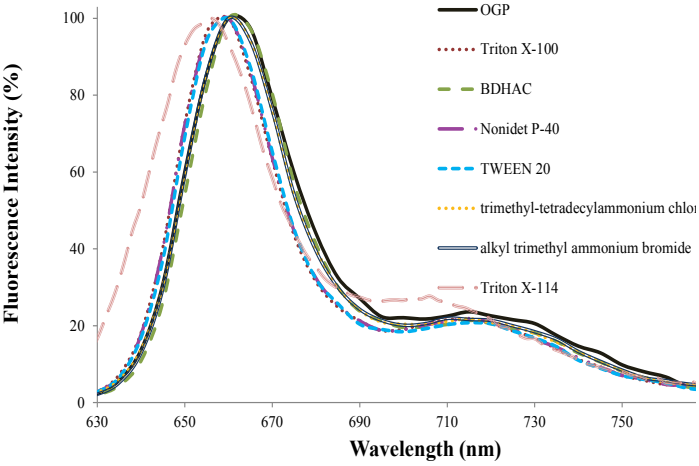


Figure 2b. Plot of fluorescence intensity versus wavelength for Chlb in each detergent analyzed. Triton X-114 micelles reduce redshift, indicating protection of some of Chla's pi-bonded groups.

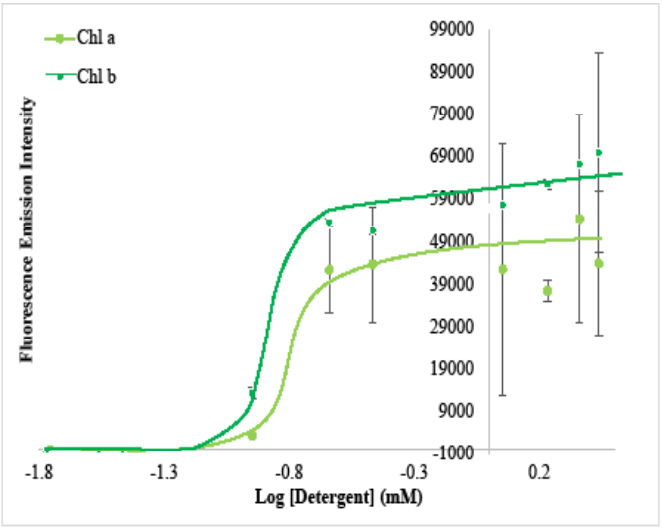


Figure 3. CMC determination of trimethyl-tetradecyl ammonium chloride-Chl micelles. CMC for the detergent with Chla is 0.106 mM. CMC for the detergent with Chlb is 0.090 mM.

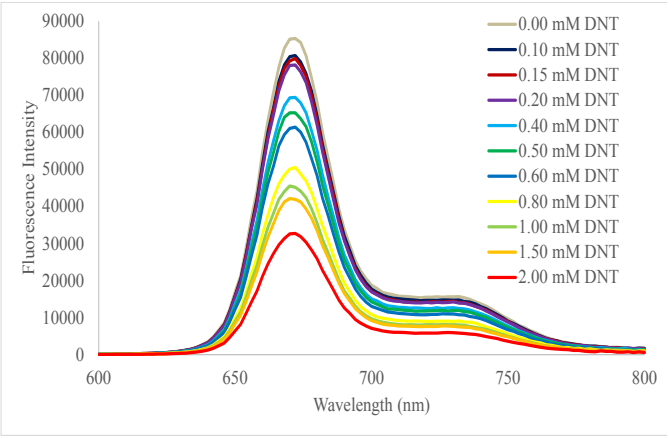


Figure 4a. Fluorescence intensity of Chla in the presence of various concentrations of DNT. As DNT concentration increases, quenching of Chla's fluorescence intensity increases.

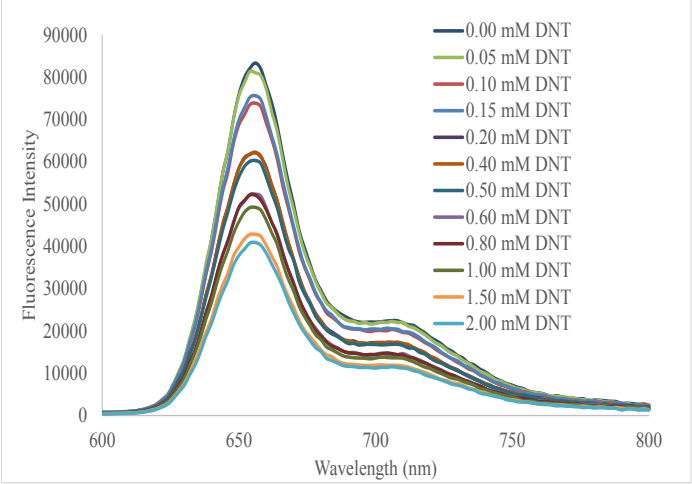


Figure 4b. Fluorescence intensity of Chlb in the presence of various concentrations of DNT. As DNT concentration increases, quenching of Chlb's fluorescence intensity increases.

Detergent-Chlorophyll Micelle CMC Determination

The critical micelle concentration is the concentration of detergent required for all molecules to be incorporated into micelles. Figure 3 shows the trimethyl-tetradecylammonium chloride and Chl micelle fluorescence emission intensity for Chla and Chlb versus the log of detergent concentration. The CMC value occurs at the most vertical portion of the graph. The CMC for the detergent with Chla was determined to be 0.106 mM; and the CMC for the detergent with Chlb was determined to be 0.090 mM.

The CMC values were determined for each detergent-Chl micelle combination. Table I depicts the experimentally-determined values along with the published CMCs for each detergent micelle. In general, the experimental values tended to be lower than the published values. This can likely be explained by the incorporation of Chl's relatively large phytol chain into the micelles. The phytol chain occupies more space, which would require a lower concentration of detergent molecules to reach the CMC.

Chlorophyll Fluorescence in 2,4-dinitrotoluene

Figure 4 shows the fluorescence emission intensities of Chl incorporated into detergent micelles in the presence of varying concentrations of DNT. The emission spectra indicate a steady increase of fluorescence quenching as DNT concentration increases. The spectra can be used as a standard curve for both detecting and quantifying the presence of DNT. Similar standard spectra can be created for use in indicating the presence of other Chl-quenching, nitroaromatic species.

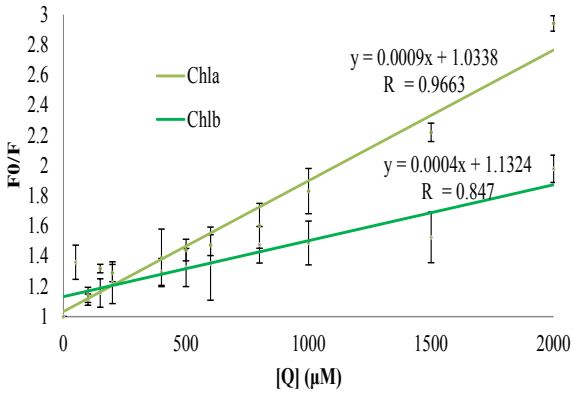


Figure 5. Stern-Volmer relationship between Triton® X-114-Chl micelles and DNT as the quencher. The K_{sv} values for micelles containing Chla and Chlb are 900 μM^{-1} and 400 μM^{-1}, respectively.

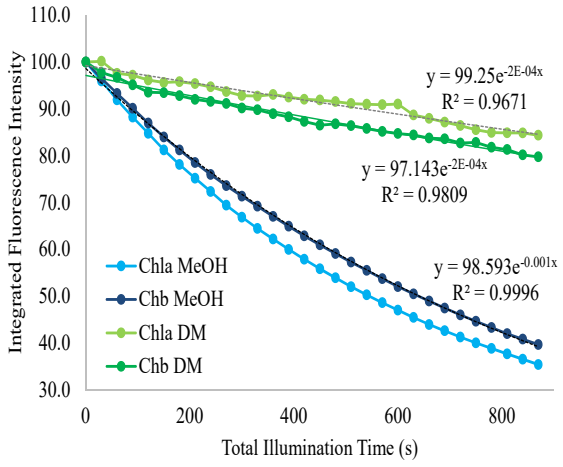


Figure 6. Photostability of Chl in MeOH versus detergent micelles. The gradual decrease in fluorescence intensity of Chl in detergent micelles compared to Chl in MeOH indicates the protective properties of micelles in regard to preventing photooxidation of Chl.

The Stern-Volmer plot in Figure 5 depicts DNT quenching for the Triton® X-114-Chl micelles. The slope of the plot corresponds to the Stern-Volmer constant (K_{sv}) of the micelles quenched with DNT. The K_{sv} values for each detergent-Chl micelle are summarized in Table II. The precision of the data was not affected by the detergent; so a higher K_{sv} indicates a greater quenching interaction. Therefore, lower concentrations of DNT can be detected as K_{sv} for the detergent-Chl micelles increases. With respect to DNT, the most effectively quenched micelles are those comprised of n-octyl-β-d-glucopyranoside and Chl.

In most instances the K_{sv} values for Chlb are lower than those for Chla. This can likely be attributed to the conjugated aldehyde on Chlb. This could be due to differing degrees of exposure of the two porphyrin analogs when incorporated into the micelles. Alternatively, the higher degree of conjugation in Chlb results in greater fluorescence of the analog as compared to Chla. This would result in a lesser degree of quenching by DNT, and, therefore, lower K_{sv} values.

Table I. Experimental and Published CMC Values for Detergent Micelles

Detergent	Experimental CMC: Chla (mM)	Experimental CMC: Chlb (mM)	Published CMC 1 (mM)	Published CMC 2 (mM)
Alkyltrimethylammonium bromide	1.8	1.6	15[11]	16[12]
Benzyldimethylhexadecylammonium chloride	0.0050	0.00079	0.0042[13]	0.59[14]
n-octyl-β-D-glucopyranoside	2.1	2.1	25[15]	26[16]
Nonidet P-40	0.0010	0.00014	0.25[17]	0.059[18]
Trimethyl-tetradecylammonium chloride	0.11	0.090	0.0038[19]	0.039[20]
Triton® X-100	0.016	0.016	0.55[21]	0.23[22]
Triton® X-114	0.0063	0.20	0.20[23]	0.009[24]
TWEEN® 20	0.19	0.16	0.050[25]	0.042[26]

Table II. Experimentally-Determined Stern-Volmer constants (K_{sv}) of Detergent-Chl Micelles. *Estimated error in K_{sv} not determined

Detergent	K_{sv} Chla (μM^{-1})	K_{sv} Chlb (μM^{-1})
Alkyl trimethyl ammonium bromide	500 ± 38.8	200 ± 17.7
Benzyldimethylhexacylammonium chloride	867 ± 104	367 ± 116
n-octyl-β-d-glucopyranoside	2400 ± 161	1500 ± 117
Nonidet P40	800 ± 75.2	500± 53.6
Trimethyl-tetradecylammonium chloride	600 ± 22.5	600± 18.9
Triton® X-100	40 ± 4.8	30 ± 3.5
Triton® X-114	900 ± 44.9	400 ± 53.0
TWEEN® 20	3*	70*

Photostability of Chlorophyll in Organic Solvents

Figure 6 depicts the photostability of Chla and Chlb in an organic solvent compared to a detergent. As seen in the figure, Chla and Chlb in a detergent are much more photostable than Chla and Chlb in methanol. The incorporation of Chl into detergent micelles protects Chl photooxidation over time.

Conclusion

Previous studies using chlorophyll to quantify fluorescence quenching have been done in organic solvents (7,8). The solubility of oxygen in organic solvents is orders of magnitude higher than in aqueous solution, and in such studies, simultaneous loss of fluorescence due to photooxidation and quenching make accurate quenching measurements problematic. Both Chla and Chlb, with long aliphatic phytol chains, incorporate into all detergents tested, and can in fact, be used to estimate detergent CMC values. Exposure of the mixed detergent-Chl micelles to nitroaromatic compounds results in the quenching of Chl fluorescence without significant photooxidation of the porphyrin, and is thus an improvement over previously published studies. Based upon the K_{sv} values, the most sensitive detergent-Chl combination for nitroaromatic quenching is Chla in micelles of octyl-β-D-glucopyranoside.

Acknowledgement

USAFA Department of Chemistry Research Center.

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