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# Fluorescence Quenching of Xanthene Dyes during Amide Bond Formation Using DMTMM

Kristen N. Patterson, Misael A. Romero-Reyes, and Jennifer M. Heemstra*

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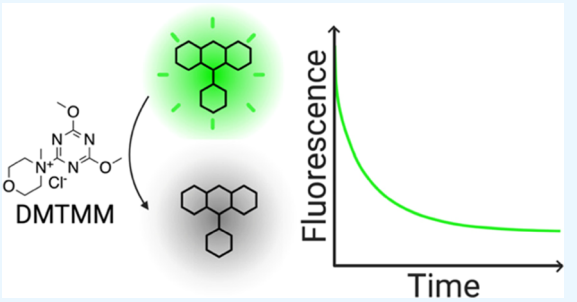
**ABSTRACT:** Fluorophore bioconjugation to proteins, nucleic acids, and other important molecules can provide a powerful approach to sensing, imaging, and quantifying chemical and biological processes. One of the most prevalent methods for fluorophore attachment is through the formation of amide bonds, which are often facilitated by coupling agents to activate carboxylic acid moieties for subsequent nucleophilic attack by amines. 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride (DMTMM) is among the most popular of these coupling agents for bioconjugation due to its ability to facilitate amide bond formation in water. After observing quenching of 5-fluoresceinamine (5-FAM)-conjugated oligonucleotides in the presence of DMTMM, we sought to evaluate the magnitude and scope of this challenge by surveying the effect of DMTMM on a range of fluorescent dyes. A higher quenching effect was consistently observed for xanthene dyes compared to that for cyanine dyes. Further analysis of the impact of DMTMM on FAM shows that quenching occurs independently of whether the dye is free in solution or attached to an oligonucleotide or antibody. Furthermore, we found that FAM-conjugated DNA was unable to recover its fluorescence after the removal of DMTMM, and UV−vis and NMR analyses suggest the formation of new products, such as an adduct formed between FAM and the dimethoxytriazine of DMTMM. As such, DMTMM at high concentrations is not recommended for coupling reactions where targets are fluorescently labeled. This research serves as a word of caution to those utilizing xanthene-containing fluorophores in bioconjugation reactions involving DMTMM.




## INTRODUCTION

Amines are ubiquitous in nature and thus offer a convenient handle for bioconjugation. Consequently, amide bond formation is an essential reaction used to functionalize a wide range of molecules including drugs, proteins, antibodies, and aptamers. Generating amide bonds typically involves the conjugation of an amine and a carboxylic acid and necessitates the release of water, making it an inherently difficult reaction to conduct under aqueous conditions.[1,2] There are few coupling agents compatible with these conditions, and of these, the organic triazine derivative 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride, or DMTMM, has emerged as a highly favorable option (Figure 1, Scheme S1).[1] DMTMM shows comparable performance to coupling agents such as *N*-hydroxysulfosuccinimide and hexafluorophosphate azabenzotriazole tetramethyl uronium in organic solvents and has become highly desirable for its ability to facilitate coupling reactions in aqueous environments under atmospheric conditions.[2,3] While bioconjugation can be used for attachment of a wide range of molecules, coupling with fluorophores is highly common in chemical biology as this supports numerous applications including biomolecule quantification, real-time imaging and tracking, and sensing of conformation changes or the presence of analytes.[4−7] This makes the selection of a well-tuned fluorophore and coupling strategy crucial to experimental design.

While exploring amide bond formation to immobilize fluorophore-labeled aptamers on a solid support, we observed what appeared to be quenching of our fluorophore in the presence of DMTMM.[8] This was a significant challenge as we had aimed to use fluorescence intensity to quantify aptamer attachment, and we recognized that this effect warranted further investigation. We subsequently exposed FAM to various concentrations of DMTMM over a period of 24 h to determine the degree to which fluorescence intensity was affected by the presence of the coupling agent. After observing a consistent trend of fluorescence, we sought to evaluate a series of commonly used fluorescent dyes to determine how widespread this challenge was and which fluorophore structures were most susceptible to quenching in the presence of DMTMM. We also evaluated the impact of buffer and pH

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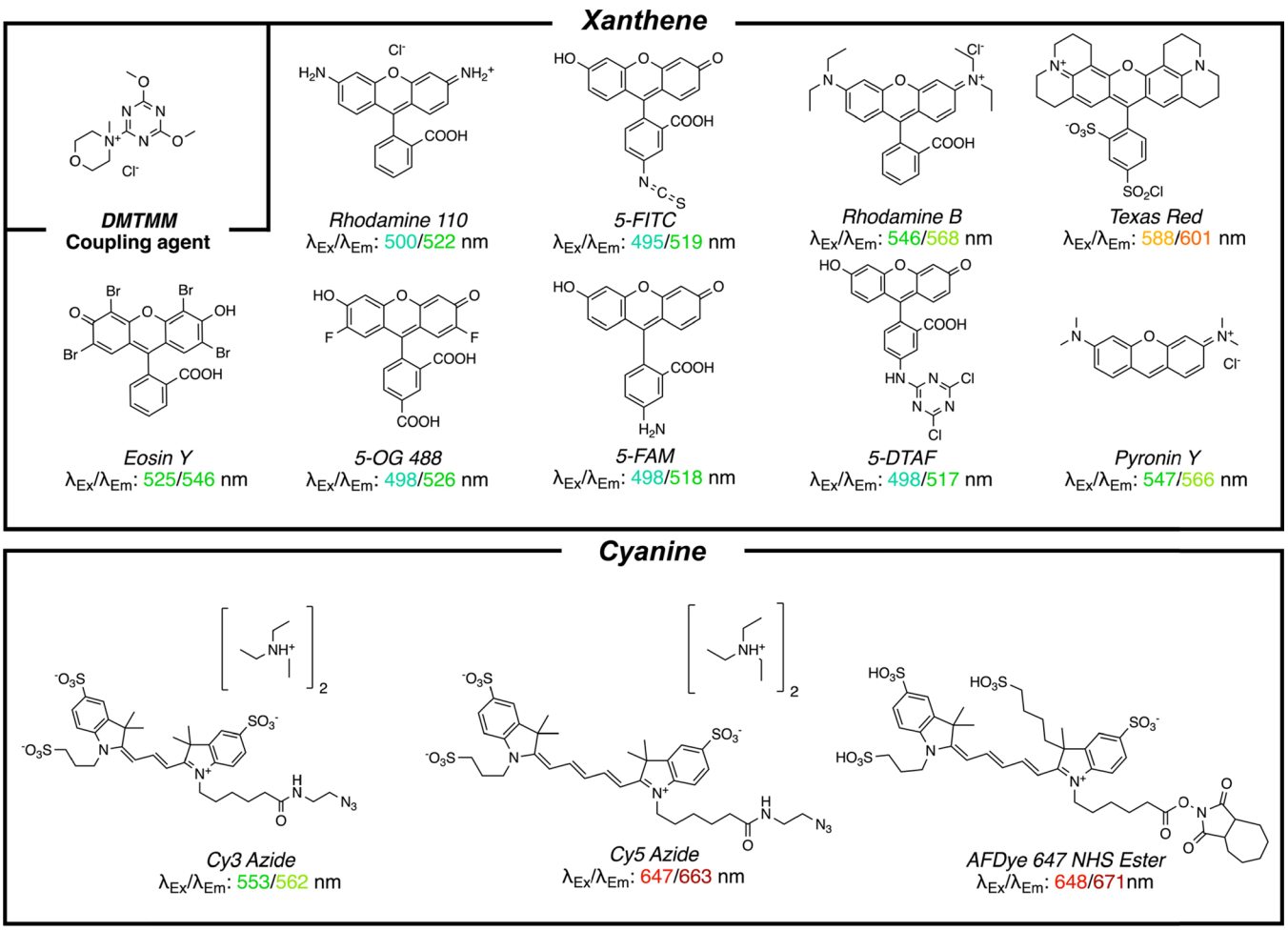


**Figure 1.** Chemical structures and optical properties of DMTMM and the xanthene and cyanine dyes investigated.

conditions on FAM quenching and determined whether the reaction between DMTMM and fluorophores was reversible. We then compared quenching for xanthene and cyanine dyes, either free in solution or attached to an aptamer or antibody, to determine whether the bioconjugation context impacted quenching. Finally, we determined whether this pattern of FAM fluorescence quenching was specific to FAM or observed with other common coupling agents. Throughout these experiments, we observe a consistent and irreversible fluorescence depletion for xanthene-based fluorophores, whereas cyanine fluorophores are only mildly impacted. These results suggest that researchers using DMTMM for fluorophore bioconjugation should be judicious in their choice of fluorophore structure and reaction conditions. More specifically, in generating amide bonds in bioconjugation conditions utilizing DMTMM, cyanine-based dyes such as Cy3 may be a considerable alternative to FAM and other xanthene-labeled fluorophores.

## RESULTS AND DISCUSSION

The first step of our investigation was to determine how widespread the effect of fluorescence quenching in the presence of DMTMM was. Most fluorescent dyes are based on xanthene or cyanine scaffolds, and we probed multiple examples from each structural class to elucidate relationships between dye properties and fluorescence depletion in the presence of DMTMM (Figure 1).[9] We decided to scan a variety of xanthene-core dyes that have different substituents on the pendant phenyl ring to observe the breadth of the impact of DMTMM across dyes that are routinely used by researchers. To also determine the dose-dependent nature of the quenching, each dye was incubated at 1 $\mu$M in the presence of varying concentrations of DMTMM ranging from 10 $\mu$M to 1 M. A calibration curve was generated for each of the dyes, and fluorescence measurements were taken over 24 h to observe the kinetics of depletion. To assure no photobleaching or evaporation was taking place, the 96-well plate holding the samples was covered using an opaque lid and stored in the dark. Furthermore, to minimize error, the calibration curve and samples were read on the same plate (Figures 2 and S1). Raw fluorescence values were compared to the calibration curve and normalized to initial fluorescence intensity. Full fluorescence emission spectral scans were taken of 5-FAM and Cy3 azide over the 24 h period (see page S4 for protocol).

As can be observed in Figure 2a, quenching occurred in a dose-dependent fashion as higher concentrations of DMTMM resulted in faster reactions as well as greater overall quenching at 24 h. However, the majority of FAM quenching was observed within the first hour of reaction. A similar general pattern of fluorescence depletion was observed across the other xanthene dyes tested, but we did observe some significant variations as a function of dye structure (Figure S2). In comparison, dyes having a cyanine core were universally more resistant to quenching after being exposed to DMTMM,


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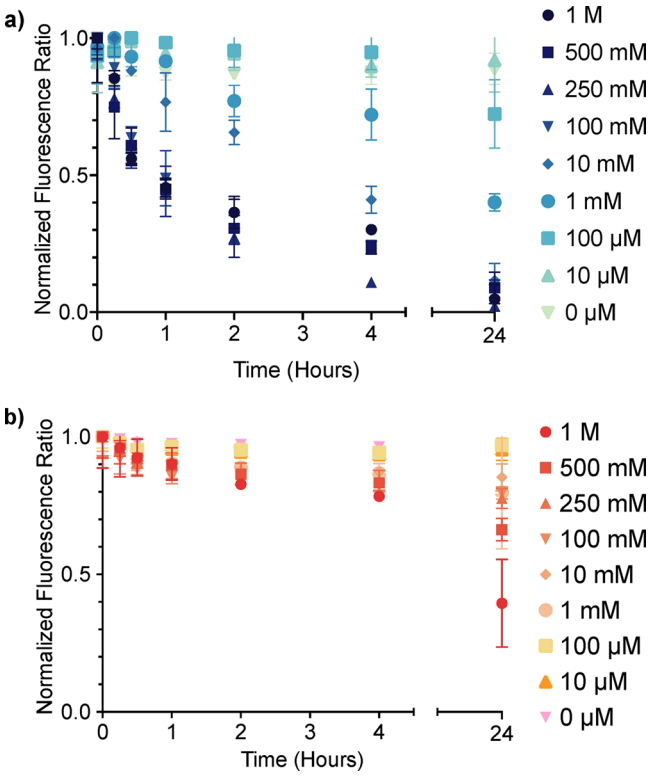


**Figure 2.** Fluorescence depletion of representative examples of (a) xanthene dye (5-FAM) and (b) cyanine dye (Cy5 azide) upon exposure to varying concentrations of DMTMM. All fluorescence experiments were performed at $N = 6$, and error bars represent standard deviation.

especially within the first hour of reaction (Figures 2b and S2). In full spectral scans of 5-FAM and Cy3 azide, the quenching effect is similar, in that while quenching was observed almost immediately with FAM and more significantly with higher concentrations of DMTMM (Figure 3a,b), Cy3 was comparably stable, with a slight drop in fluorescence but the majority of signal retained over the 24 h period (Figure 3c,d).

As a point of comparison, we plotted percent fluorescence depletion at 24 h with 100 mM DMTMM (Figure 4) Consistent with the results described above, the dyes that suffer from the greatest quenching are xanthene-based such as fluorescein isothiocyanate (FITC), oregon green/5-OG 488, 5-(4,6-dichlorotriazinyl)aminofluorescein, and 5-FAM. In addition, cyanine dyes such as Cy3 and Cy5 are among the most stable. It is of note that while the xanthene dyes examined in this study generally experience quenching within the first few hours of reaction and level off after a 4 h period, the cyanine dyes remained resistant to depletion within the first few hours, experiencing a drop in fluorescence after 24 h, which is more likely caused by dye stability than reactivity with DMTMM. We also observe some dyes that defy this trend, namely, pyronin Y and rhodamine 110.

Many of the xanthene dyes in this study exist in an equilibrium between a closed ring, non-fluorescent form and an opened ring, fluorescent form. The interconversion between these two states is pH-sensitive as acid shifts the equilibrium toward the closed form and base shifts the equilibrium toward the open form.[10] Importantly, the open form features a carboxylic acid functionality, and without this group, dyes are

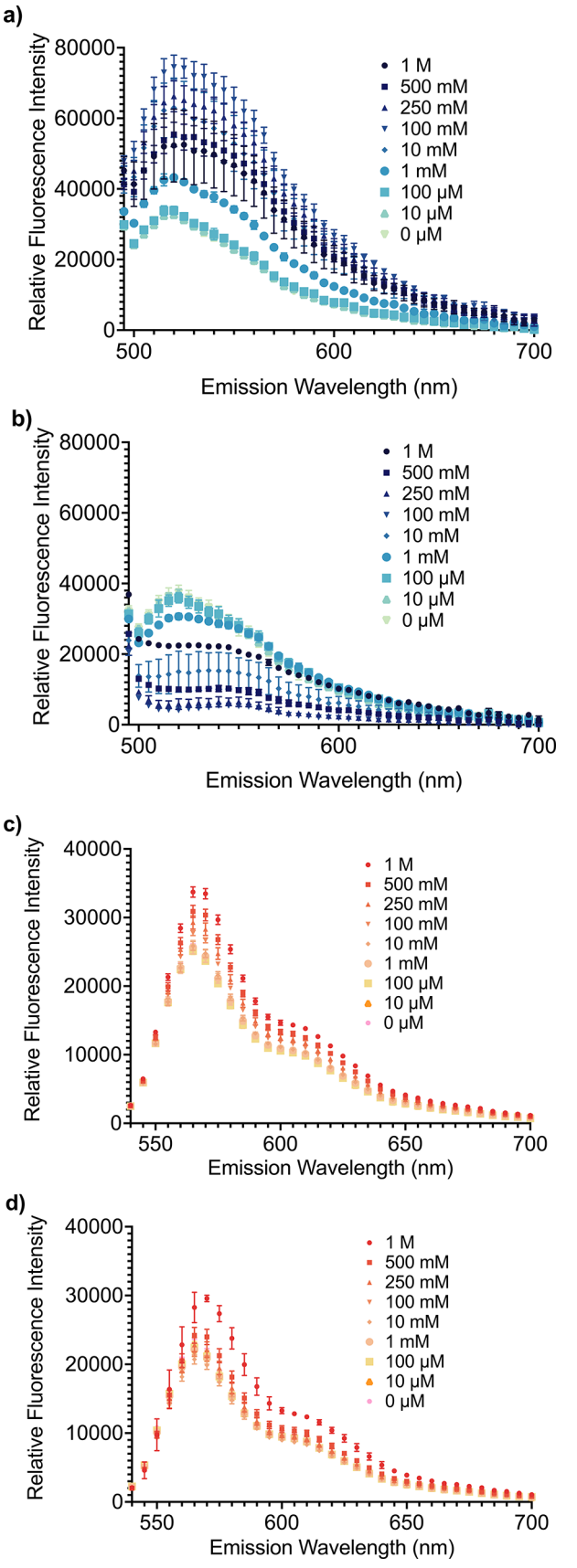


**Figure 3.** Fluorescence excitation spectral scan of 5-FAM exposed to DMTMM at (a) 0 h and (b) 24 h as well as fluorescence excitation spectral scan of Cy3 azide exposed to varying concentrations of DMTMM at (c) 0 h and (d) 24 hperformed at $N = 6$, and error bars



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**Figure** 3. continued

represent standard deviation. For instance, without a carboxylic acid, pyronin Y is unable to interconvert between open and closed forms and consequently retains its fluorescence over the course of 24 h, regardless of its environmental conditions (Figures 4 and S2).

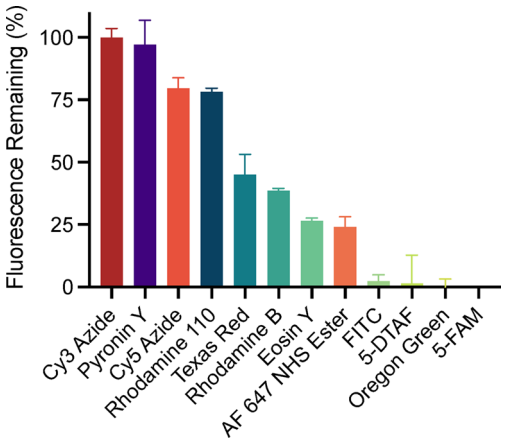


**Figure 4.** Percent fluorescence remaining after 24 h of exposure to 100 mM DMTMM. All fluorescence experiments were performed at $N = 6$, and error bars represent standard deviation.

unable to interconvert and maintain fluorescence independent of their environment.

In contrast, dyes such as rhodamine have been utilized as fluorescent probes for environmental conditions, given the sensitivity of their fluorescence.[11] Thus, it is logical that reagents that can impact the carboxylic acid functionality could have profound impacts on dye fluorescence. In contrast, the fluorescence functionality of cyanine dyes included in this study was either unaffected or affected to a lesser extent compared to the effects observed across xanthene dyes, reinforcing that structure plays a key role in the quenching observed.[12−14]

In addition to evaluating the reactivity of several dyes, we were also curious to explore the effect of varying reaction conditions, including pH and buffer.[15] Similar to the dye structure scan, solutions of DMTMM were prepared in a 96-well plate such that the dye concentration was fixed at 1 $\mu$M in each well. This was exposed to DMTMM at concentrations ranging from 10 $\mu$M to 1 M. Fluorescence intensity was observed over the course of 24 h. Across all buffer and pH conditions, the fluorescence intensity of FAM decreased with increasing concentrations of DMTMM in a similar fashion to that observed in the general dye scan (Figure S3). While significant quenching was observed under all conditions when using higher than 10 mM DMTMM, we do observe that quenching is also faster and more pronounced at higher pH. More importantly, however, these data show that the challenge of DMTMM quenching occurs not only in water but also in buffer systems typically used for amide bond formation.

We note that we first observed the effect of DMTMM on fluorescence when conjugating a dye-labeled aptamer to a solid support. Using the free dyes for our initial investigation of this phenomenon provided a convenient approach to rapidly test for the effect of dye structure on quenching and assess kinetics as a function of reaction conditions. However, we recognize that bioconjugation reactions are often carried out using fluorophore-labeled biomolecules such as in our example. Thus, we wanted to determine whether the impact of DMTMM on fluorescent dyes would be different when they are conjugated to biomolecules such as oligonucleotides or antibodies. We purchased the kanamycin A binding aptamer (Ky2) and bisphenol A binding aptamer modified with either 5'-FAM or 5'-Cy3 (Table S1) and monitored fluorescence quenching of the 10 $\mu$M aptamer in 1X MOPS buffer, pH 8, in the presence of 3 mM DMTMM for 24 h.[16] This concentration of coupling agent was selected as it was the maximum volume we observed retained in our initial experiments' system prior to exposure to oligonucleotides. It is of note that in principle, the free carboxylic acid and amine groups on the protein can cross-react. However, this is the case in all bioconjugation reactions performed using amide bond formation, and considering the structure of most proteins and conditions of the experiments, it would be expected that the intermolecular reactions would be slow at the low concentrations used, making this of minimal concern. A significant quenching effect was observed for both fluorophores but was much greater in magnitude for FAM, where fluorescence was depleted by nearly 98%, while fluorescence was retained after a 24 h period in the Cy3-labeled aptamer (Figure 5a). In a

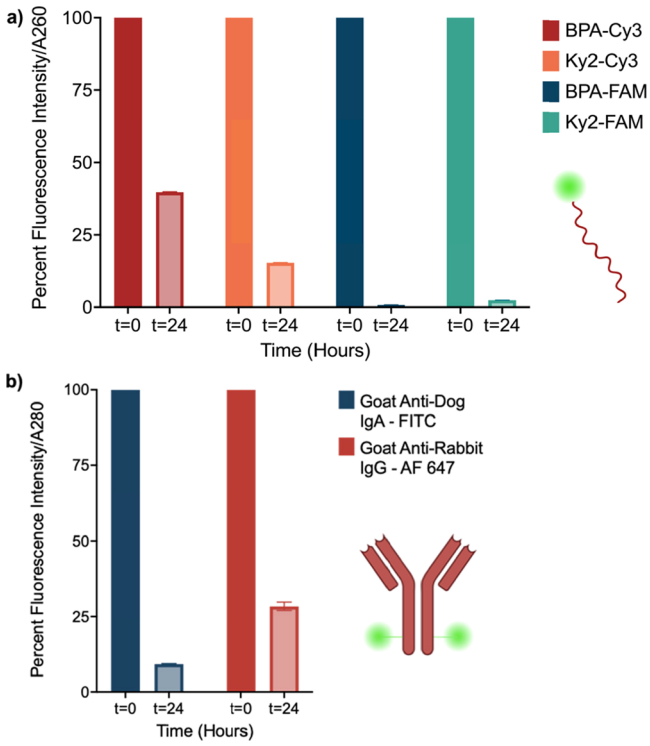


**Figure 5.** Fluorescence depletion of fluorophore-labeled (a) aptamers and (b) antibodies. Fluorescence was monitored as a ratio to biomolecule concentration using absorbance at 260 nm for the aptamer and absorbance at 280 nm for the antibody. All fluorescence experiments were performed at $N = 2$, and error bars represent standard deviation.

similar experimental design, 0.1 ug/mL goat anti-dog IgA antibody labeled with FITC and a goat anti-rabbit IgG (H + L) highly cross-adsorbed secondary antibody labeled with Alexa Fluor Plus 647 were incubated in 1X PBS buffer with 30 $\mu$L of 300 mM DMTMM (3 mM DMTMM) for 24 h, and a similar fluorescence depletion trend was observed (Figure 5b).



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We were especially curious to determine whether this quenching effect with DMTMM was reversible. If so, then the removal of DMTMM should result in a restoration of fluorescence, and the effect of the coupling reagent on bioconjugation reactions would be a somewhat less significant issue. To probe this question, a solution of 10 $\mu$M FAM-labeled Ky2 aptamer was exposed to 3 mL of 300 mM DMTMM (3 mM DMTMM) in 1X MOPS buffer at pH 8 for 24 h.[16] The reaction mixture was purified using a Monarch PCR and DNA Cleanup kit to remove excess DMTMM that would otherwise interfere with DNA quantification. The DNA was then resuspended in buffer, and the DNA concentration was then measured by absorbance at 260 nm. To observe whether recovery would be possible, we then monitored fluorescence for up to 24 h after the removal of DMTMM. As shown in Figure 6, the FAM-labeled aptamer was unable to recover a significant degree of fluorescence even 24 h after DMTMM removal. This suggests that the reaction of DMTMM with FAM is irreversible and that use of this coupling reagent permanently damages fluorophore activity.

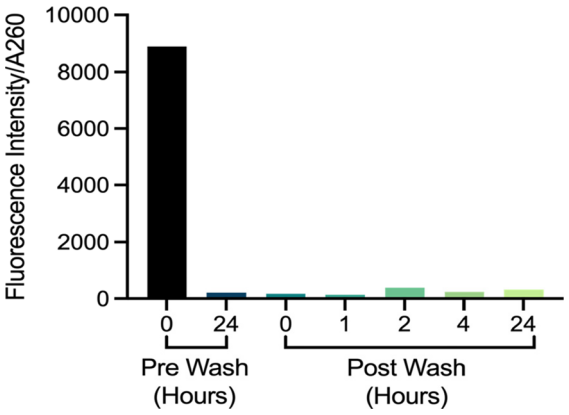


**Figure 6.** Probing the reversibility of the effect of DMTMM on FAM fluorescence intensity. Fluorescence decreases as expected after 24 h after exposure to DMTMM and is not appreciably restored even 24 h after DMTMM removal.

As the reaction between FAM and DMTMM was identified as being non-reversible, at least on a timescale of hours, our next step was to evaluate whether DMTMM might be covalently modifying the FAM structure. To study this reaction, we utilized UV−vis and $^1$H NMR analyses. For UV−vis analysis, a solution of 50 $\mu$M FAM in 1% DMSO was incubated with 50 $\mu$M DMTMM, and absorbance was monitored from 200 to 800 nm over a 24 h period. This spectrum was compared to solutions of 50 $\mu$M FAM and 50 $\mu$M DMTMM to identify which changes in spectra were the result of interactions between the two analytes. Initially, we observe a characteristic spectrum of the neutral species of unsubstituted 5-FAM ($H_2R$), wherein absorbance increased followed by a decrease and change in the ratio of peak intensity at 450 nm over time, which was not observed in FAM independent of DMTMM in the same time period, suggesting that DMTMM is undergoing chemical modification and that multiple products may be formed over time (Figures 7 and S4).[17−19] For NMR analysis, a solution was prepared with 100 mM FAM and 100 mM DMTMM in deuterated DMSO, and $^1$H NMR measurements were taken over a 24 h period. For comparison, a solution was prepared with 100 mM pyronin Y and 100 mM DMTMM in deuterated DMSO, and similar measurements were taken over a 24 h period. NMR analysis showed several new peaks across both DMTMM- and FAM-associated regions, suggesting a chemical reaction between DMTMM and FAM (Figures S5−S14). In comparison, very little change is observed in the dye-associated regions of the reaction of pyronin Y and DMTMM (Figures S15−S17). In comparison, very little change is observed in the dye-associated regions of the spectra for the reaction of pyronin Y and DMTMM (Figures S15−S17). Interestingly, we do observe differences in DMTMM-associated regions between the two dyes, which may be due to the differential interaction with these dyes. For example, DMTMM in the presence of FAM has chemical shifts of 4.3 (d), 4.05 (s), 3.95 (d), and 3.45 (s), while DMTMM in the presence of pyronin Y appears at 3.80 (s), 3.7 (t), 3.6 (t), and 3.05 (s). Given this observation, the reaction of DMTMM with the carboxylic acid moiety on FAM is likely the cause of the quenched fluorescence, though it is then interesting that Rhodamine 110 is not significantly quenched despite having this same functionality. Through time-lapse $^1$H NMR, conducted on a reaction solution of 50 mM FAM and 50 mM DMTMM in deuterated DMSO, we can see changes over the course of 24 h in both the FAM- and DMTMM-associated regions (Figures S18−S21). As these additional peaks appear independent of isolated FAM or DMTMM solutions, we suspect that a covalent modification occurs within the first hour of reaction. Changes following this hour may then be the result of degradation of our reactants during the 24 h period (Figures S5−S14).[20,21] Furthermore, MS experiments at comparable time points were performed, which suggest the formation of a collection of new products, which falls in line with our observations from NMR experiments (Figures S22−S51). Of note, an adduct appears to form between FAM and dimethoxytriazine (FAM: 348.08, $[M + H]$ + $C_{20}H_{14}NO_5^+$, FAM-DMT adduct: 487.12, $[M + H]$ + $C_{25}H_{19}N_4O_7^+$). Further studies, however, would be needed to elucidate the specific structure of this adduct.

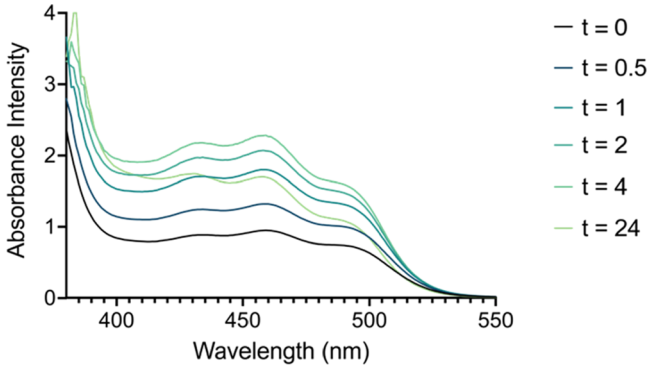


**Figure 7.** UV−vis measurement of 50 $\mu$M FAM and 50 $\mu$M DMTMM solution over 24 h.

To probe whether fluorescence depletion is specific to the DMTMM coupling reagent, a comparative study was performed to observe the fluorescence behavior of FAM in the presence of different coupling agents. In this, 1 $\mu$M FAM was exposed to varying concentrations of DMTMM, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and *N*-hydroxysuccinimide (NHS) ranging from 10 $\mu$M to 1 M. A calibration curve was generated for each of the dyes, and fluorescence measurements were taken over 24 h to observe the kinetics of depletion. Similar considerations, including


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photobleaching and calibration, were considered, and raw fluorescence values were compared to the calibration curve and normalized to initial fluorescence intensity. Unlike with DMTMM, FAM fluorescence remains unchanged after 24 h when exposed to NHS and increases in a dose-dependent fashion with EDC, as can be observed in Figure 8.

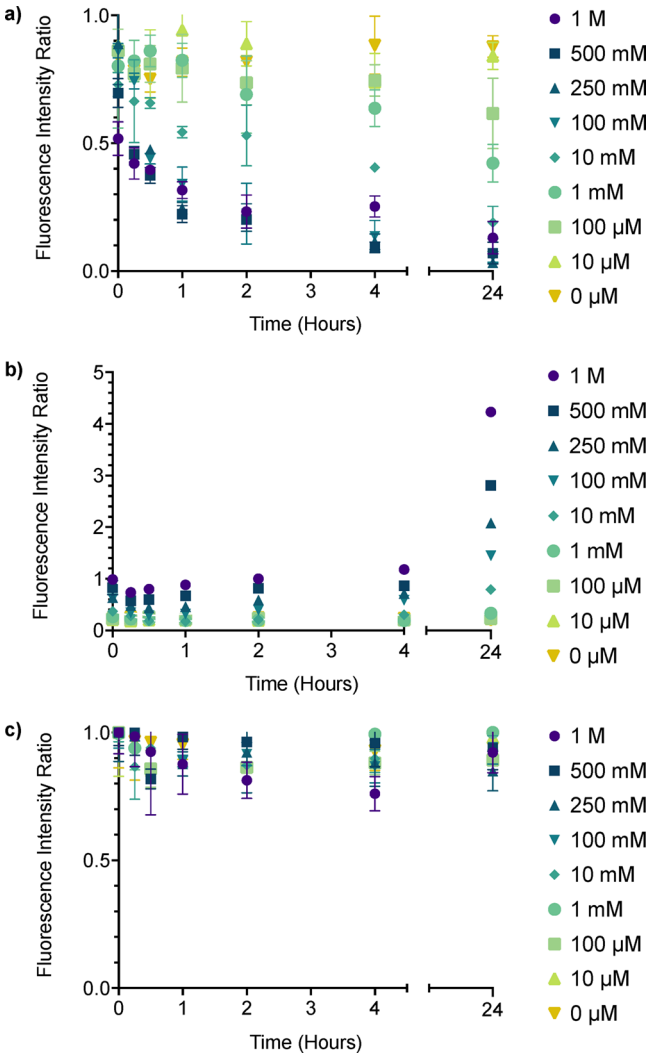


**Figure 8.** Fluorescence depletion of FAM upon exposure to varying concentrations of (a) DMTMM, (b) EDC, and (c) NHS. All fluorescence experiments were performed at $N = 6$, and error bars represent standard deviation.

While DMTMM is relatively stable in water for up to 3 h, after an extended period of time, it may degrade into several byproducts, including compounds such as 2-chloro-4,6-dimethoxy-1,3,5-triazine and *N*-methylmorpholine.[22] The multiple products observed in NMR are potentially the result of interactions of such byproducts with xanthene dyes, and additional NMR analysis and incubation with known DMTMM byproducts can be used to discern this effect in further studies. However, we do note that the majority of quenching is observed within the first hour of reaction, suggesting that FAM and similarly structured dyes are modified at least in part directly by DMTMM.

As noted earlier, the interconversion between the closed, non-fluorescent forms and the opened, carboxylic acid forms of many of the xanthene dyes studied is sensitive to environmental conditions. EDC functions as a coupling agent by activating available carboxylic acid groups to form an o-acylisourea intermediate, which prepares compounds for coupling with amines.[23] Our data suggests that EDC may react with the open carboxylic acid form of FAM in a manner that forces it to maintain its fluorescent state, accounting for the increased fluorescence observed after 24 h. In comparison, NHS has no inherent reactivity with carboxylic acids, and thus, it is not surprising that it exerts no detectable influence over fluorescence.[24] Interestingly, while DMTMM activates carboxylic acids in a manner similar to EDC, we observe a decrease in fluorescence rather than an increase. Additionally, if the change in fluorescence upon reaction with DMTMM were only attributable to the formation of an activated ester, then this effect would be expected to be reversible over time after the removal of DMTMM. Thus, our data suggests that further reaction or rearrangement may occur to generate irreversible byproducts. Moreover, even if the reaction with DMTMM locks xanthene dyes in the open form, the presence of the triazine may serve to still quench fluorescence through an electron transfer mechanism. In a similar context, for instance, quenching of nanoparticles has been observed while using EDC as a coupling agent, and this was attributed to the net positive charge of the chemical as opposed to its functionality.[25,26] Further studies of these detailed pathways could reveal new insights into the reactivity of DMTMM and xanthene fluorophores. However, the primary purpose of our current work is to provide a cautionary tale to researchers seeking to use DMTMM as a coupling reagent for bioconjugation reactions involving fluorophores.

## CONCLUSIONS

In summary, we explore the impact of DMTMM on the fluorescence activity of xanthene and cyanine dyes, with a specific focus on FAM, given its widespread use in biological applications. A survey of different dye structures reveals that xanthene dyes are significantly more susceptible to fluorescence quenching by DMTMM than cyanine dyes, and we offer guidance as to the relative effect of DMTMM on the various fluorophores. We also surveyed the effect of buffer conditions and found that while a range of conditions typically used for amide bond formation do give rise to significant quenching, this seems to be exacerbated at higher pH. Furthermore, using a FAM-labeled aptamer, we demonstrate that the fluorescence depletion observed upon exposure to DMTMM is not reversible. Given the wide use of DMTMM for bioconjugation and the prevalence of xanthene fluorophores in these applications, this highlights a key challenge in bioconjugation that researchers should be aware of. We present this breadth of information to highlight a challenge we encountered in our bioconjugation process, in the hopes of providing a pool of data to the public so that other researchers conducting similar bioconjugation experiments can plan their experiments accordingly and ideally avoid some of the challenges we experienced. With this observed, Cy3 and other cyanine dyes present themselves as viable substitutes to xanthene dyes in bioconjugation reactions using DMTMM, especially when fluorescence intensity will be used as a means of quantification or will serve as a critical function in subsequent experiments. If xanthene dyes are to be used in bioconjugation contexts, utilizing DMTMM judiciously with regard to concentration


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and reaction time may serve to minimize this fluorescence quenching effect.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c03085.

Materials, methods, DNA sequences, fluorescence quenching experiments, characterization of reactivity via variation of solvent and pH conditions, and surveys of reversibility and product formation via UV−vis spectroscopy, NMR, and mass spectrometry (PDF)

## ■ AUTHOR INFORMATION


### Corresponding Author

**Jennifer M. Heemstra** − *Department of Chemistry, Emory University, Atlanta, Georgia 30322, United States; Department of Chemistry, Washington University in St. Louis, St. Louis, Missouri 63130, United States;* orcid.org/0000-0002-7691-8526; Email: heemstra@wustl.edu, jen.heemstra@emory.edu

### Authors

**Kristen N. Patterson** − *Department of Chemistry, Emory University, Atlanta, Georgia 30322, United States;* orcid.org/0000-0002-7887-3077

**Misael A. Romero-Reyes** − *Department of Chemistry, Emory University, Atlanta, Georgia 30322, United States; Department of Chemistry, Hanover College, Hanover, Indiana 47243, United States;* orcid.org/0000-0003-4614-4512

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.2c03085


### Author Contributions

All authors have given approval to the final version of the manuscript.

### Notes

The authors declare no competing financial interest.

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