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Accelerating Strain-Promoted Azide–Alkyne Cycloaddition Using Micellar Catalysis

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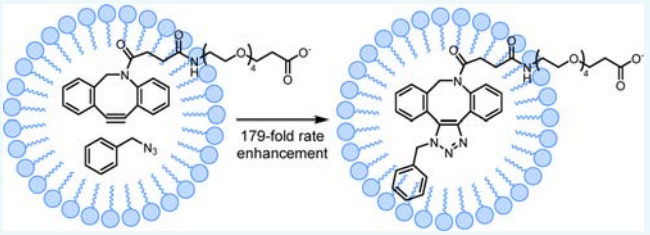
Supporting Information

ABSTRACT: Bioorthogonal conjugation reactions such as strain-promoted azide–alkyne cycloaddition (SPAAC) have become increasingly popular in recent years, as they enable site-specific labeling of complex biomolecules. However, despite a number of improvements to cyclooctyne design, reaction rates for SPAAC remain significantly lower than those of the related copper-catalyzed azide–alkyne cycloaddition (CuAAC) reaction. Here we explore micellar catalysis as a means to increase reaction rate between a cyclooctyne and hydrophobic azide. We find that anionic and cationic surfactants provide the most efficient catalysis, with rate enhancements of up to 179-fold for reaction of benzyl azide with DIBAC cyclooctyne. Additionally, we find that the presence of surfactant can provide up to 51-fold selectivity for reaction with a hydrophobic over hydrophilic azide. A more modest, but still substantial, 11-fold rate enhancement is observed for micellar catalysis of the reaction between benzyl azide and a DIBAC-functionalized DNA sequence, demonstrating that micellar catalysis can be successfully applied to hydrophilic biomolecules. Together, these results demonstrate that micellar catalysis can provide higher conjugation yields in reduced time when using hydrophobic SPAAC reagents.



INTRODUCTION

Strain-promoted cycloaddition between a cyclooctyne and an azide was first reported in 1953,[1] and more recently, the power of this reaction for use in complex biological milieu has become widely recognized.[2−4] A number of creative strategies have been employed to generate modified cyclooctynes having increased reactivity due to steric or electronic effects.[5−8] However, despite these modifications, the rates of strain-promoted azide–alkyne cycloaddition (SPAAC) reactions remain lower than those of other commonly used bioconjugation reactions, such as the copper-catalyzed azide–alkyne cycloaddition (CuAAC) reaction (Figure 1).[9] Seeking methods to further increase the rate of SPAAC reactions, we recognized that the most reactive cyclooctynes are extremely hydrophobic, and thus we hypothesized that micellar catalysis could potentially provide dramatic increases in reaction rate between these cyclooctynes and hydrophobic azide molecules.

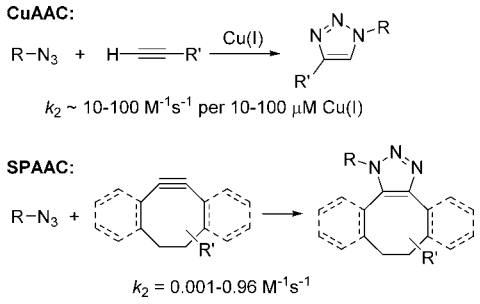


Figure 1. Despite improvements in cyclooctyne reactivity, rate constants for SPAAC remain lower than those of CuAAC.

Micelles are spherical macromolecular architectures that form as a result of phase-driven assembly of amphiphilic surfactant molecules.[10] Surfactants comprise a hydrophilic portion and a hydrophobic portion, and in aqueous or other polar media, they assemble to shield the hydrophobic portion of each molecule from the polar solvent. In addition to their ability to undergo self-assembly, micelles are also capable of sequestering hydrophobic guest molecules, as these molecules are better solvated in the nonpolar interior of the micelle than in the aqueous bulk solution. Upon sequestration, the guest molecules experience a higher local concentration than they would in the bulk solution, and thus micelles have been successfully utilized as catalysts capable of increasing reaction rate by colocalizing molecules having complementary reactive functionalities.[11] When we initiated our research in this area, the use of micellar catalysis to enhance the rate of bioorthogonal reactions such as SPAAC had yet to be explored. However, while our project was underway, Huber and co-workers reported that *n*-dodecyl β-D-maltoside (DM) micelles could be used to accelerate SPAAC between a genetically encoded *p*-azido-phenylalanine residue and a cyclooctyne-functionalized fluorophore, providing

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impressive rate enhancements of up to 1000-fold.[12] Here we present a complementary study quantifying the effect of commonly used surfactants on reaction rate between azadibenzobicyclooctyne (DIBAC) and a hydrophilic or hydrophobic azide, and explore this catalysis in the context of a DIBAC-functionalized DNA sequence. We investigate anionic, cationic, and nonionic surfactants, and show that anionic and cationic micelles are the most effective catalysts, providing up to 179-fold enhancement of reaction rate. We also demonstrate that micellar catalysis provides dramatically different reaction rates for hydrophilic and hydrophobic azides, which may contribute an additional dimension of orthogonality to the SPAAC reaction. Finally, we demonstrate that micellar catalysis can be achieved even when DIBAC is attached to a hydrophilic biomolecule such as DNA. We anticipate that these results will enable researchers to obtain higher conjugation yields in reduced time when using hydrophobic SPAAC reagents attached to molecules that are compatible with low concentrations of surfactants.

RESULTS AND DISCUSSION

To quantify the effect of micellar catalysis on SPAAC reactions, we employed DIBAC, as this is among the most reactive and hydrophobic cyclooctynes.[6] Additionally, DIBAC is widely used for bioconjugation reactions, in part because a number of analogues are commercially available.[13] Specifically, we employed DIBAC-PEG$_4$-CO$_2$H (**1**, Figure 2), as PEG-linkers

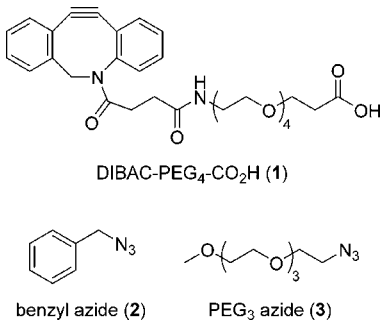

Figure 2. Chemical structure of cyclooctyne and azide reagents used in kinetics studies.

are often utilized in bioconjugation reactions to prevent interference of the attached label with the target molecule. For the azide reaction partner, we explored both benzyl azide (**2**) and a PEG$_3$-azide (**3**), with the hypothesis that the hydrophobicity of **2** would make it an excellent substrate for micelle catalysis, whereas **3** would be well-solvated in the aqueous solution, and thus would not experience significant rate enhancement in the presence of micelles.

Second-order rate constants were obtained by carrying out all reactions under pseudo-first-order conditions using an excess of the azide. DIBAC conveniently undergoes a decrease in absorbance at 309 nm upon reaction with an azide to produce a triazole product. Thus, UV spectrophotometry was utilized to monitor reaction progress. Figure 3a shows a representative plot of absorbance at 309 nm as a function of time, and these data can be fit to the integrated rate law to provide k_{obs} (Figure 3b), which can in turn provide the second-order rate constant (k_2) using eq 1

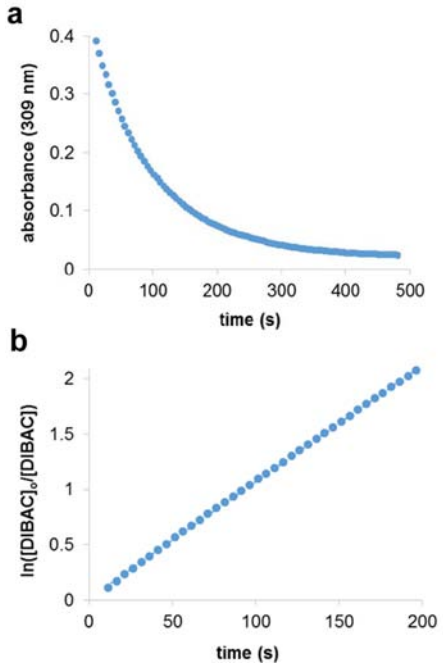

Figure 3. Representative plot of (a) absorbance at 309 nm over the course of a reaction, and (b) fitting of absorbance data to the integrated first-order rate law. Data are from reaction of **1** + **3** in the presence of SDS.

$$k_2 = \frac{k_{obs}}{[\text{azide}]} \quad (1)$$

For each reaction, we calculated k_{obs} and k_2 using at least three different concentrations of azide. While micellar catalysis systems can show saturation behavior at high concentrations of reactants,[14] we observed that the values of k_2 were independent of azide concentration over the range of concentrations tested, indicating that the reactions were still operating under second-order kinetics (see Supporting Information).

To explore the relationship between surfactant structure and catalytic efficiency, we tested a variety of anionic (SDS), cationic (DTAB, CTAB), and nonionic (Tween 80, Triton X-100) surfactants. For each surfactant, we measured the second-order rate constant for reaction of **1** with **2** or **3** in aqueous solution with the surfactant present at a concentration 2-fold higher than the reported critical micelle concentration (CMC). As a control, we also measured k_2 for the same reactions in water. As shown in Table 1, hydrophobic azide **2** shows 2.7-fold slower reaction rate than hydrophilic azide **3** in water, likely due

Table 1. Effect of Micellar Catalysis on Second-Order Rate Constant for SPAAC Reactions Using Either a Hydrophobic or Hydrophilic Azide[a]

surfactant	k_2 (**1** + **2**, M^{-1} s^{-1})	k_2 (**1** + **3**, M^{-1} s^{-1})
none	0.439 ± 0.040	1.18 ± 0.09
Tween 80	2.79 ± 0.97	1.07 ± 0.11
Triton X-100	4.67 ± 0.54	1.07 ± 0.06
SDS	40.4 ± 2.0	4.05 ± 0.53
DTAB	40.4 ± 2.7	1.26 ± 0.28
CTAB	78.7 ± 3.5	1.53 ± 0.16

[a]Error represents standard deviation of at least three independent trials.

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to aggregation of the benzyl azide. As a point of calibration, we do note that our rate constant of 0.439 M^{-1} s^{-1} for reaction of **1** with benzyl azide is similar to the rate constant of 0.31 M^{-1} s^{-1} reported in the literature for reaction of a DIBAC analogue with benzyl azide in methanol.[6] Upon the addition of surfactant, we were excited to observe that reaction rates for **2** increase dramatically, while reaction rates for **3** remain largely unaffected. Nonionic surfactants Tween 80 and Triton X-100 provide modest rate enhancements of 6.4- and 11-fold, respectively, for reaction of **1** with **2**. In contrast, SDS, DTAB, and CTAB provide rate enhancements of 92-, 92-, and, 179-fold, respectively. Interestingly, because surfactants increase the rate of reaction for **1** + **2**, but not **1** + **3**, micellar catalysis provides selectivity for reaction of the hydrophobic azide over the hydrophilic azide. This selectivity is greatest with CTAB, as we observe 51-fold faster reaction of **2** relative to **3**.

To validate that the observed rate enhancements result from micellar catalysis, we selected the most efficient catalyst, CTAB, and measured the second-order rate constant as a function of surfactant concentration. As shown in Figure 4, we observe that

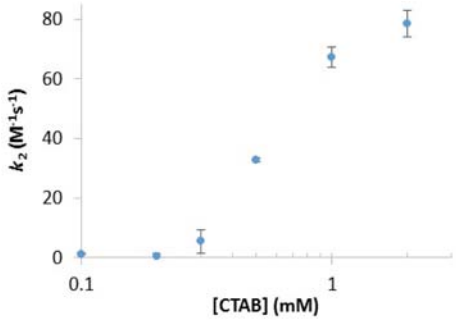

Figure 4. Effect of CTAB concentration on reaction rate. Error bars represent the standard deviation of at least three independent trials.

the reaction rate is highly dependent upon surfactant concentration, with a dramatic decrease in k_2 observed below the CMC value of 1 mM.[15] However, we still observe a statistically significant level of catalysis using 0.5 mM CTAB, despite the fact that this is below the CMC of the surfactant. It has been reported that hydrophobic small molecules can act to template the assembly of micelles, resulting in a shift in CMC value.[16] Thus, we hypothesize that the hydrophobic cyclooctyne and benzyl azide molecules act to promote transient assembly of micelles at 0.5 mM CTAB, producing the observed rate enhancement.

We were intrigued to observe that different surfactants produced dramatically different levels of catalysis, but propose that this can be explained by considering the chemical structures of the surfactants (Figure 5). The nonionic surfactants have bulky hydrophilic segments that are largely composed of PEG polymer, whereas the anionic and cationic surfactants have compact hydrophilic head groups. We hypothesize that because cyclooctyne **1** is itself amphiphilic, it would be expected to bind to the charged micelles with higher affinity, as the carboxylate group could be incorporated into the hydrophilic corona of the micelle. In contrast, upon binding to the nonionic surfactants, the carboxylate group of **1** must be solvated by the PEG polymers, which is expected to be disfavored relative to solvation by the bulk aqueous medium. We do find it interesting that SDS and DTAB show identical rate enhancements, as cationic DTAB would be expected to

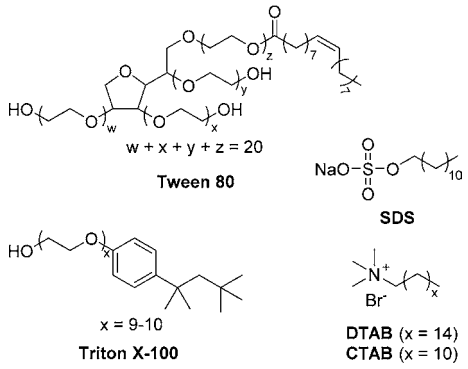

Figure 5. Chemical structures of surfactants employed for micellar catalysis.

bind the anionic DIBAC molecule with higher affinity due to electrostatic complementarity. However, it is possible that the counterions present with each surfactant electrostatically screen the charged head groups, minimizing electrostatic interactions between the surfactant and cyclooctyne **1**. It is also interesting that we observe a dramatic difference in rate enhancement between DTAB and CTAB, as these surfactants are both cationic and have similar chemical structures. We hypothesize that the four additional methylene groups in the aliphatic chain of CTAB provide a larger hydrophobic core that is able to more efficiently sequester the DIBAC and benzyl azide, thus providing more efficient catalysis. This hypothesis is also consistent with the similar rate enhancements observed for SDS and DTAB, as these surfactants have identical C_{12} aliphatic chains.

We were curious as to whether DIBAC would still be capable of benefiting from micellar catalysis when appended to a more highly charged biomolecule. To investigate this question, we chose DNA as a challenging test case, as the high charge density of DNA makes it extremely hydrophilic, reducing the driving force for DIBAC to bind to the micelles. Additionally, DIBAC containing phosphoramidites are commercially available, making SPAAC an increasingly common strategy for functionalization of nucleic acids. We reacted the DNA sequence $^{5\prime}$DIBAC-GTA GAT CAC T$^{3\prime}$ with benzyl azide in water or 2 mM CTAB, and measured k_2 values of 2.66 and 28.1 $M^{-1}s^{-1}$, respectively. Given the hydrophilicity of DNA, we were not surprised to observe a decrease in the efficiency of micellar catalysis. However, we were surprised to observe a significant increase in the reaction rate in water. We hypothesize that the benzyl azide may be capable of weakly intercalating with the aromatic nucleobases of the DNA, providing an enhanced effective molarity. Despite these effects, the presence of CTAB micelles still provides an 11-fold rate enhancement for SPAAC with DIBAC-functionalized DNA, which is anticipated to be of significant utility for nucleic acid functionalization.

CONCLUSIONS

In conclusion, we demonstrate that micellar catalysis can be used to dramatically accelerate the reaction of cyclooctynes with hydrophobic azides. We find that anionic and cationic surfactants provide the most efficient catalysis, with rate enhancements of up to 179-fold for reaction of benzyl azide with DIBAC cyclooctyne. In the context of a DIBAC-functionalized DNA sequence, we observe a more modest, but still substantial, 11-fold rate enhancement upon addition of CTAB, demonstrating that micellar catalysis can be successfully

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applied to hydrophilic biomolecules. Importantly, micellar catalysis increases the reaction rate of SPAAC such that it is on par with that of the CuAAC reaction, but retains the benefit of not requiring the use of a copper catalyst. In the case of nucleic acids, the presence of surfactants is anticipated to be universally well-tolerated, but proteins can be susceptible to denaturation in the presence of high concentrations of surfactants. We anticipate that this will not pose a significant limitation, however, as the concentration of surfactants required to achieve catalysis is relatively low. For example, in the case of SDS, 0.05 wt % of surfactant is sufficient to provide the observed 92-fold rate enhancement, and many proteins are able to tolerate this concentration of SDS at room temperature without denaturing.[17−19] One other possible limitation to our method is the need to purify the resulting reaction products from the surfactant. However, in the case of large biomolecules, this can generally be achieved using methods such as size exclusion filtration. In addition to increasing reaction rate, we also demonstrate that surfactants can provide selectivity for reaction of a hydrophobic azide over a hydrophilic azide. We envision that this could be utilized to generate an additional dimension of orthogonality in bioconjugation reactions, as the presence of micelles could enable parallel orthogonal reactions between hydrophobic and hydrophilic cyclooctyne-azide reactant pairs.

EXPERIMENTAL PROCEDURES

General. All chemicals were purchased from commercial sources and used without further purification. DIBAC-functionalized DNA was purchased from the University of Utah DNA/peptide core facility, where it was synthesized using phosphoramidites from Glen Research, including DIBAC phosphoramidite (Glen catalog # 10−1941). Stock solutions of surfactants, DIBAC, and PEG azide **3** were prepared using distilled water, then combined with additional water to provide the desired concentration for each reaction. For experiments using benzyl azide **2** with surfactants, the azide stock solution was prepared in the presence of the appropriate surfactant. UV measurements were acquired at room temperature (21−24 °C) using either a Shimadzu UV-1800 or a PerkinElmer Lambda 25 UV/vis spectrophotometer with a quartz cuvette having a 1 cm path length. Data analysis was carried out using *Microsoft Excel*.

Kinetics Measurement. In all experiments, the concentration of DIBAC was held constant at 50 μM (**1**) or 20 μM (DIBAC-DNA), and the concentration of azide was varied from 0.2 to 2.0 mM. This large excess of azide enabled calculation of reaction rates using the pseudo-first-order assumption. For each reaction, a mixture was prepared containing DIBAC and surfactant, then transferred to a quartz cuvette. The appropriate volume of azide was added, the cuvette capped, and the solution mixed by inversion to initiate the reaction. The time between addition of azide and the start of data acquisition was noted. Absorbance at 309 nm was recorded at intervals of 1−10 s for a total time of 5−10 min. For reactions that proceeded to completion within <20 min, the absorbance at 309 nm was monitored until no change was observed, and this value was used as the final absorbance. For reactions that did not proceed to completion within this time, one trial of the reaction was monitored for 2−4 h to ensure completion, and the final A_{390} value was used for all other trials under that reaction condition.

For each reaction condition, 3−4 independent trials were carried out using varying concentrations of azide, as this enabled validation that the reactions were functioning under second-order kinetics. For each independent reaction, the first 4−6 data points were extrapolated over the recorded delay time to provide the initial absorbance (A_0). The final absorbance (A_f) was obtained as described above. The absorbance data over the course of the reaction (A) were used to calculate the fraction of starting material remaining using eq 2

$$\frac{[\mathrm{DIBAC}]}{[\mathrm{DIBAC}]_0} = \frac{A - A_f}{A_0 - A_f} \quad (2)$$

in which $[\mathrm{DIBAC}]_0$ is the initial concentration of DIBAC and $[\mathrm{DIBAC}]$ is the concentration remaining at a given time point. In accordance with the integrated first-order rate law, we then plotted $\ln([\mathrm{DIBAC}]_0/[\mathrm{DIBAC}])$ vs time, and obtained k_{obs} as the slope of this plot up to the point of 50% conversion. Finally, we converted k_{obs} into k_2 using eq 1. For each of the reaction conditions, the k_2 values from the 3−4 independent trials were averaged to provide the values reported in Table 1.

ASSOCIATED CONTENT

Supporting Information

Surfactant CMC values and comprehensive kinetics data. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00274.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

SPAAC strain-promoted azide−alkyne cycloaddition; CuAAC copper-catalyzed azide−alkyne cycloaddition; DIBAC dibenzobicyclooctyne; SDS sodium dodecyl sulfate; DTAB dodecyltrimethylammonium bromide; CTAB cetyltrimethylammonium bromide

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