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# Bioorthogonal Drug Release from Nanometric Micelles in Living Cells

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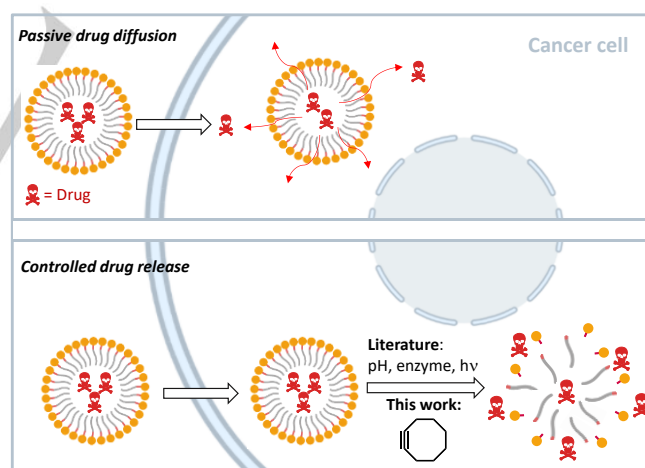
Supporting information for this article is given via a link at the end of the document.

**Abstract:** We explored a bioorthogonal approach to release drugs from stimuli-responsive micelles inside tumor cells. The concept relies on syndnonimine-based micelles that undergo quantitative cleavage in presence of cyclooctynes, hence releasing their content within living cells. Four cleavable micelles were developed to allow massive burst release of Entinostat, a potent histone deacetylase inhibitor, following their internalization inside cancer cells. A comparative study on the influence of the bioorthogonal-mediated versus passive drug release from micelles was carried out. The results indicated that a fast release of the drug triggered a stronger antiproliferative activity on tumor cells compared to the passive diffusion of the drug from the micelles core. These findings may be of great interest for the development of new nanomedicines.

## Introduction

Nanomedicine has been a source of great hope as it promises to solve a number of crucial issues associated with conventional therapeutic strategies. Over the past decades, remarkable progresses have been made in the design of engineered nanoparticles able to transport and deliver effective drugs to treat pathologies more effectively. Therefore, many nanoparticles with optimal sizes, shapes and surface properties have been successfully developed to modify the biodistribution, prolong the circulation half-life and reduce the side effects of drugs.<sup>[1]</sup> Moreover, the enhanced permeability and retention effect (EPR)<sup>[2]</sup> and the addition of specific ligands to the surface of nanoparticles allowed the development of efficient targeted therapies especially in the field of cancer.<sup>[3]</sup> Biodegradable materials designed for the selective delivery of drugs have been particularly investigated for various biomedical applications.<sup>[4]</sup> However, although nanotechnology-based targeted delivery has shown promising results in preclinical models, their transfer to the clinic remains problematic. Among the possible reasons are uncontrolled release of the drug from nanocarriers and lack of specificity of targeted strategies. One alternative to this problem involves on-demand processes that allow for tailored drug release with spatial, temporal and dosage control.<sup>[5]</sup> On-demand drug delivery has become feasible through the design of stimuli-responsive nanocarriers that react in a dynamic way in the targeted

microenvironment through either enzymatic,<sup>[6]</sup> pH<sup>[7]</sup> or physical external stimuli such as temperature, light or ultrasounds for example (Figure 1).<sup>[8]</sup> Self-immolative nanoparticles, in particular dendrimers, have been also successfully developed.<sup>[9]</sup> All of these strategies are based on cleavable linkers<sup>[10]</sup> introduced at the right place in the structure of the nanoparticles. However, the difficulties to control endogenous triggers like pH or enzymes in healthy versus pathological tissues, and the lack of focusing and tissue-penetration depth of external stimuli remains major challenges in the field. A promising alternative strategy is the use of bioorthogonal reactions to control the selective cleavage of the nanoparticles and the subsequent release of their content encapsulated inside their core (Figure 1).



**Figure 1.** Strategies for micelle payloads delivery in cancer cells.

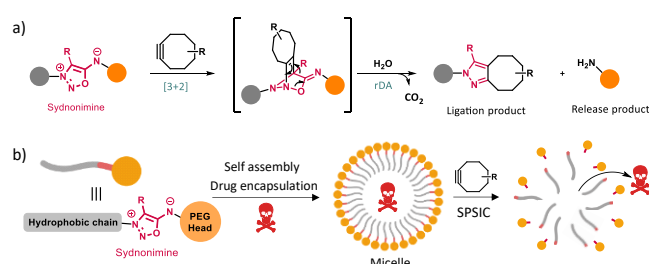
One main advantage of such an approach is the possibility to apply complementary targeted strategies to both nanoparticles and trigger in order to concentrate them in one precise location while limiting their concomitant presence in other areas of the body. In 2019, our teams validated this approach for the first time using bioorthogonally cleavable micelles that enabled the release of fluorophores in cells as well as in mice upon activation by an enzyme-sensitive trigger.<sup>[11]</sup> Based on this preliminary work, we propose in this article to evaluate the interest of this approach for

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the controlled delivery of an anticancer drug from micelles in tumor cells.

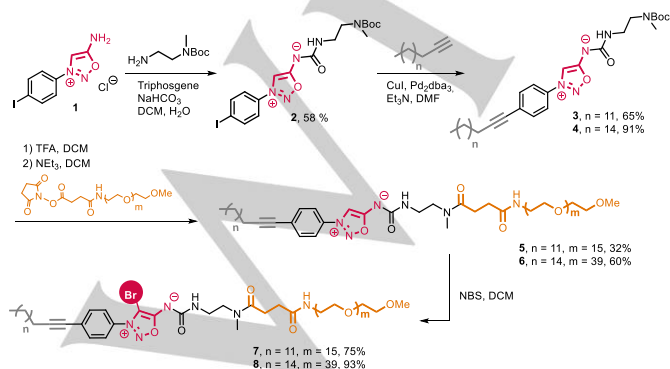
## Results and Discussion

We designed amphiphilic structures bearing a hydrophobic aliphatic chain connected to a PEG moiety by a sydnonimine spacer. Sydnonimines were described by our group as bioorthogonal linkers, which can be cleaved in presence of cyclooctyne triggers through the Strain-Promoted Sydnonimine-Cycloalkyne cycloaddition reaction (SPSIC, Figure 2a).<sup>[12]</sup> In the case of the designed amphiphiles, SPSIC offers the possibility to separate the PEG head from the hydrophobic core inducing the disassembling of the micelles (Figure 2b).



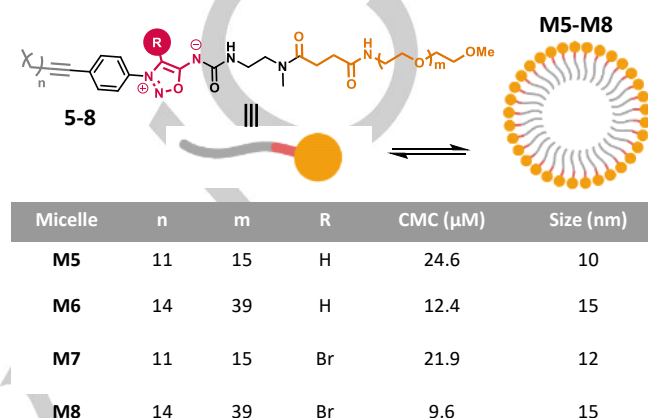
**Figure 2.** a) SPSIC reaction, b) Design of amphiphiles cleavable by SPSIC reaction. rDA = retro Diels-Alder.

The four surfactants **5-8** differing by their size and the presence or absence of a bromine atom on the sydnonimine core were designed and synthesized (Figure 3). Previous finding from our group showed the superior reactivity of bromo-sydnonimines toward the SPSIC reaction.<sup>[13]</sup> Micelles constructed with these cleavable linkers should therefore be destroyed faster in presence of cyclooctynes. Surfactants **5-8** were prepared in 4 to 5 steps starting from iodo-sydnonimine **1** prepared according to known procedures (see supporting information). Sydnonimine **2** was first obtained from salt **1** by reaction with the isocyanate prepared in situ from Boc-*N*-methyl-diethylamine. Sonogashira coupling between **2** and fatty alkynes allowed the formation of compounds **3** and **4** in good yields. Surfactants **5** and **6** were then obtained after deprotection of the secondary amine and subsequent peptide coupling with a carboxylic PEG chain. Finally, bromination on C4 position of the sydnonimine core was achieved using *N*-bromosuccinimide to give surfactants **7** and **8** (Figure 3).



**Figure 3.** Synthesis of amphiphiles **5-8**.

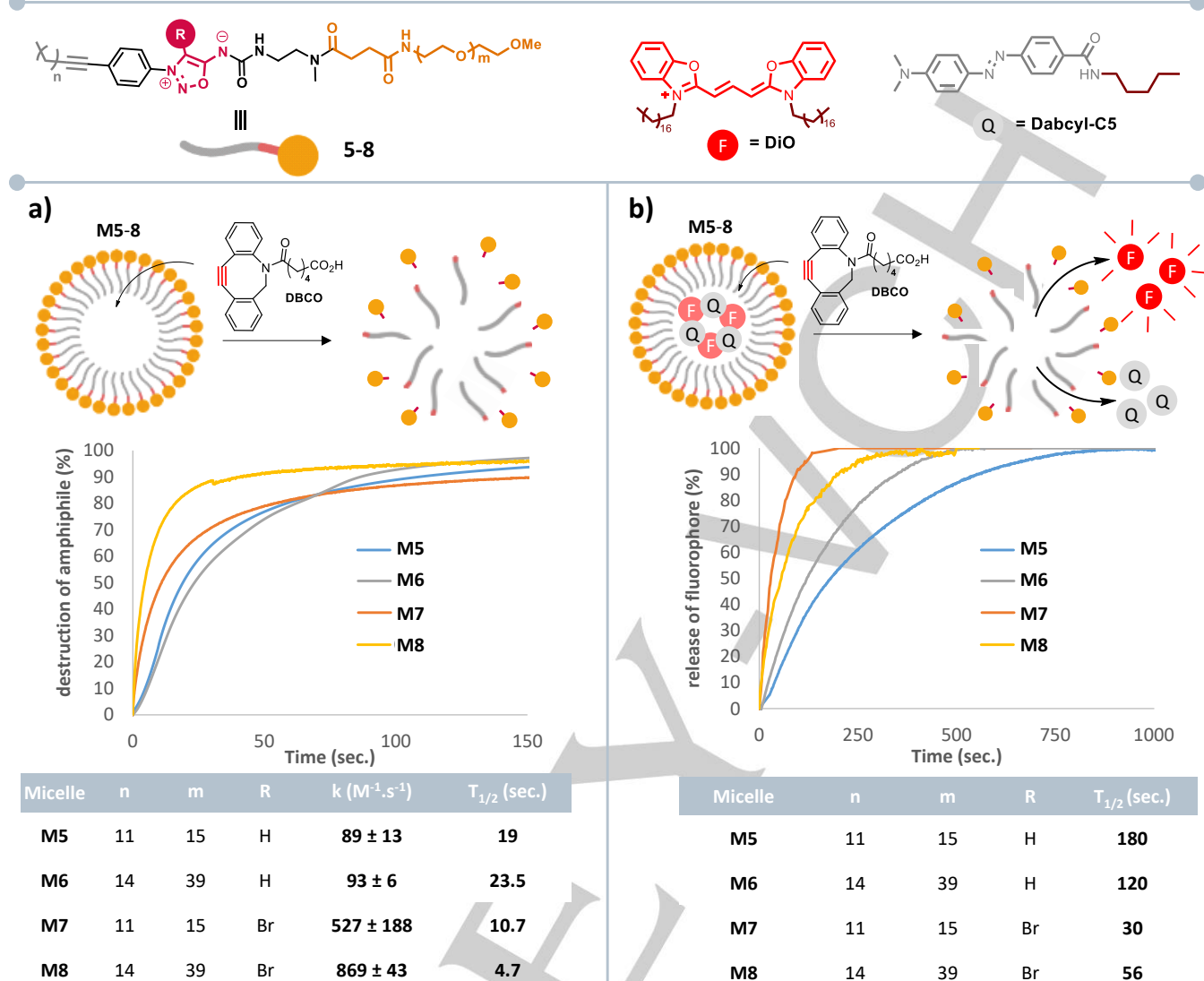
Physicochemical characterizations proved that each amphiphiles **5-8** are able to self-assemble in aqueous solutions into micelles **M5-8** of 10 to 15 nm size according to DLS analysis (Figures S1-4). Surface tension measurements showed low values of critical micellar concentration (CMC) ranging from 10 to 25  $\mu\text{M}$  (Figure 4). The larger micelles **M6** and **M8** showed the lowest CMC indicating improved stabilities of the self-assembly. The presence of the bromine atom has apparently no impact on the micelle formation and stability. Control experiments indicated complete stability of these micelles for more than 10 days at pH 7.4 (Figure S10).



**Figure 4.** Formation and characterization of micelles **M5-8**. CMC and size were determined using surface tension and DLS respectively.

We then investigated the SPSIC reaction of these micelles with the cyclooctyne **DBCO**. In addition to be highly reactive, this cyclooctyne is quite hydrophobic and thus should concentrate inside the hydrophobic core of micelles. Local concentrations of **DBCO** and sydnonimine linkers would then be very high, increasing the kinetics of the SPSIC reaction. A comparative kinetic study was carried out with micelles **M5-8** confirming this hypothesis (Figure 5a). Second order rate constants were globally more than 100-fold higher with micelles than with non-assembled amphiphiles (Table S1). In addition, rate constants  $k$  increased from around  $90 \text{ M}^{-1} \cdot \text{sec}^{-1}$  for **M5** and **M6** to more than  $500 \text{ M}^{-1} \cdot \text{sec}^{-1}$  for **M7** and  $800 \text{ M}^{-1} \cdot \text{sec}^{-1}$  for **M8** highlighting the high beneficial effect of the presence of the bromine atom in the sydnonimine structure. With these high kinetic values, the SPSIC reaction occurring inside micelles provokes depegylation and complete disassembly of the nanostructure in less than one minute. Complete destruction of the micelle nanostructures was confirmed by DLS measurements. Molecules encapsulated into these micelles should therefore be rapidly released after reaction with **DBCO**. To confirm this hypothesis, we encapsulated hydrophobic fluorophore **DiO** and quencher **Dabcyl-C5** into the four micelles and monitored **DiO** release following addition of **DBCO** by measurement of the fluorescence signal enhancement. As shown in Figure 5b, the full release of **DiO** from micelles was completed in only 3 to 12 minutes after addition of **DBCO**, depending on the micelle structure. As expected, micelles **M7** and **M8**, constructed with bromo-sydnonimines, are the fastest to release their content allowing 100% release of **DiO** in 3 and 5 minutes respectively. The higher speed of bioorthogonal release observed with micelle **M7** is probably due to its smaller size and lower strength of self-assembly compared to **M8**.

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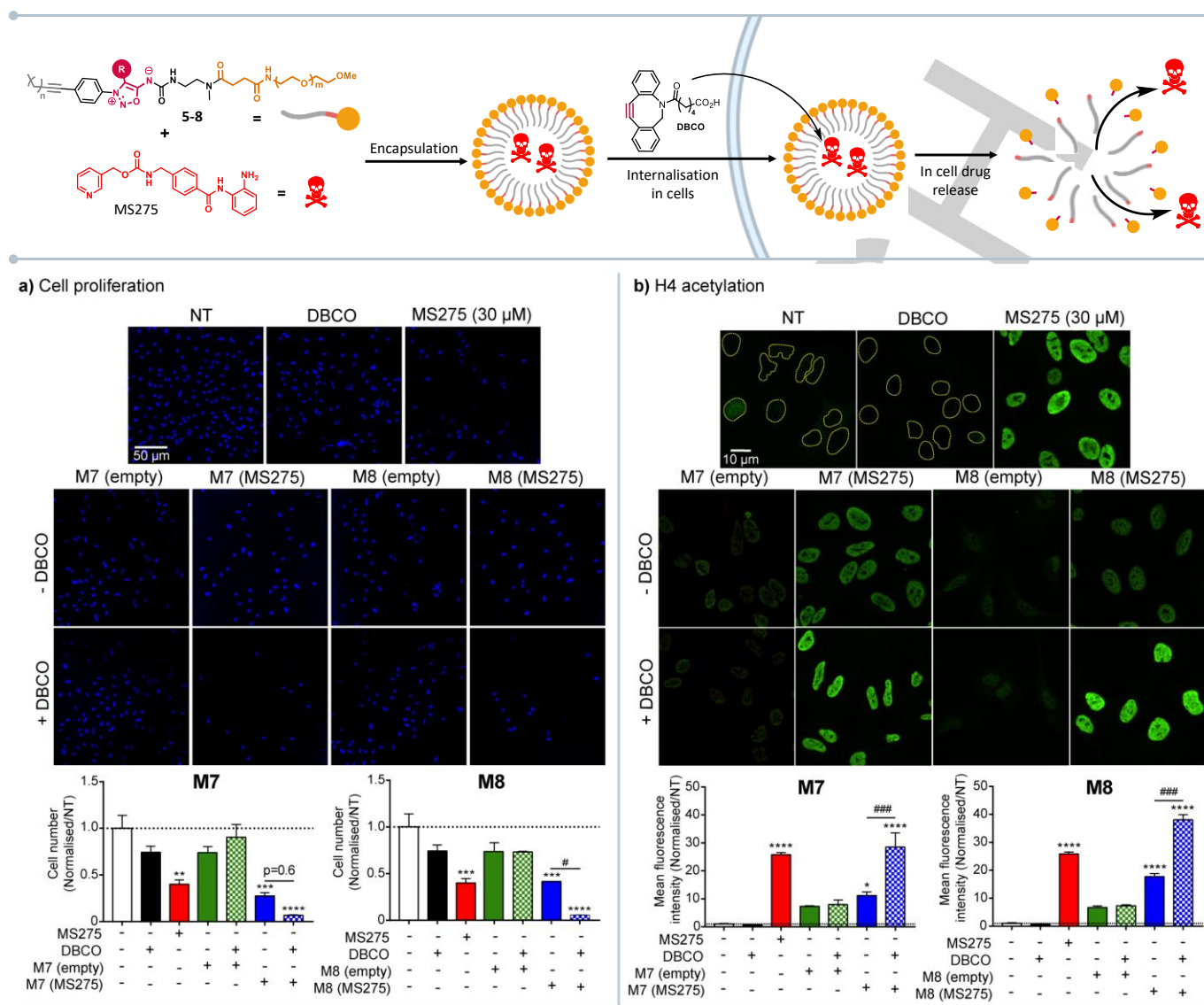
**Figure 5.** Reactivity of micelles towards **DBCO**. a) Influence of amphiphile structure on the kinetics of micelle cleavage. Reactions were conducted with 200  $\mu\text{M}$  micelles and 300  $\mu\text{M}$  **DBCO** at 25  $^{\circ}\text{C}$  in water and monitored by following the absorbance of the sydnimine moiety. b) Influence of the amphiphile structure on the release of compounds encapsulated inside micelles. Fluorophore **DiO** and quencher **Dabcyl-C5** were encapsulated during micelle preparation. Reactions were run with 200  $\mu\text{M}$  of micelles and 300  $\mu\text{M}$  of **DBCO** at 25  $^{\circ}\text{C}$  in water and monitored by following the appearance of fluorescence signal of **DiO** ( $\lambda_{\text{ex}} = 484 \text{ nm}$ ,  $\lambda_{\text{em}} = 502 \text{ nm}$ ).

Globally, these results demonstrated the possibility to release the totality of the molecules encapsulated inside these micelles with high speed upon addition of **DBCO**.

These results in hands, we pursued the evaluation of micelles in the context of drug delivery within cancer cells. We were particularly interested in the influence of the bioorthogonal-mediated versus passive drug release on micelles biological activity. Indeed, such micelles that can be activated "on demand" offer the opportunity to evaluate whether the massive and rapid release of the drug triggered by **DBCO** is beneficial for the killing of tumor cells compare to the slow passive drug diffusion process. Within this framework, we encapsulated Entinostat (MS275, Table S2) into the bioorthogonal stimuli-responsive micelles **M7-8** that exhibited the highest rate constants. Entinostat is a well-known synthetic histone deacetylase (HDAC) inhibitor that targets selectively class I and IV HDAC enzymes.<sup>[14]</sup> Along the past decade, this compound has been evaluated in the clinic for treating solid tumors and hematological cancers.<sup>[15]</sup> More recently,

Entinostat showed promise in combination with immunotherapy treatments for the therapy of advanced tumors.<sup>[16]</sup> The micelles **M7-8** loaded with Entinostat were first assessed for their antiproliferative activity on A549 tumor cell line (human bronchial carcinoma cells). In this experiment, micelles were first incubated with the cells at a concentration of 40  $\mu\text{M}$  equivalent of Entinostat for 2 h. The cells were then washed in order to remove non-internalized micelles. The effect of micelles on cell proliferation was recorded after 48 h treatment by counting cell number using Hoechst staining (Figure 6a). As expected, incubation of the micelles **M7-8** reduced significantly the number of A549 tumor cells compared to untreated control conditions. A similar effect was observed upon treatment with Entinostat (30  $\mu\text{M}$ ). In contrast, non-loaded micelles slightly decreased cell proliferation although not significantly compared to the control experiment. These results suggested that the antiproliferative activities measured with loaded micelles **M7** and **M8** were the consequence of the passive release of the drug within tumor cells.





On the other hand, when micelles containing Entinostat were treated by **DBCO** after their internalization by the cells, a strong inhibition of cell proliferation was recorded (Figure 6a; 93 and 94 % inhibition with micelles **M7** and **M8**, respectively). Under these conditions, the number of cells was 4- and 7-fold lower than when loaded micelles **M7** and **M8** were incubated without **DBCO**. These outcomes provided evidence that the SPSIC reaction occurred successfully inside living cells. Furthermore, our results demonstrated the benefit brought by the bioorthogonal-mediated release of Entinostat compared to passive drug diffusion from micelles on the inhibition of cancer cell proliferation.

In order to confirm that the biological activity of micelles **M7-8** on tumor cells arose from the release of Entinostat, we examined the

inhibition of HDAC by measuring histone H4 acetylation levels using immunofluorescence assays. As shown on Figure 6b, a significant increase on histone H4 acetylation was observed only when A549 cancer cells were treated with Entinostat or the loaded micelles **M7-8**. However, the treatment of these latter by **DBCO** led to a higher HDAC inhibition than that recorded with micelles alone. Together with cell proliferation assays, these experiments highlighted that the active release of the drug, via the SPSIC reaction-mediated micelle destruction, produced a superior biological effect on A549 tumor cells than passive drug diffusion. Overall, the results of this study pointed out that the rate of drug release from such nanocarriers could impact significantly their efficacy for cancer therapy.

## Conclusion

In conclusion, we described four different bioorthogonal stimuli-responsive micelles that can be activated via the SPSIC reaction for releasing their content. Kinetic experiments demonstrated that the decomposition of such micelles occurred at different speeds in the presence of DBCO, depending on the structure of the sydnonimine included in the surfactant. Within this framework, we showed that the introduction of a bromine atom on the sydnonimine core of the amphiphiles accelerated micelles disassembly upon bioorthogonal activation. These micelles allowed encapsulation of an anticancer agent such as Entinostat highlighting their potential as drug nanocarriers. When triggered by the SPSIC reaction, the micelles led to the efficient release of the drug within cancer cells. This active process of drug release produced superior biological activities compared to the passive drug diffusion from the nanocarrier. This suggested that the rate of drug release can have a significant impact on the therapeutic efficacy of such nanodevices. We think this finding may be important for the design of the next generation of nanocarriers dedicated to the selective delivery of anticancer drugs.

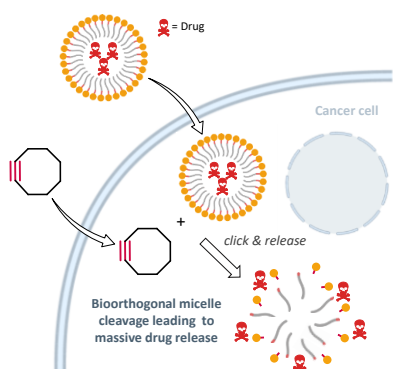
## Experimental Section

For details of synthetic procedures, chemical characterizations, kinetics as well as biological results, see the Supporting Information.

**Keywords:** Micelles • biorthogonal reactions • drug release • sydnonimines • nanoparticles

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## Entry for the Table of Contents



Micelles constructed with sydonimines are cleaved upon addition of cyclooctynes in a biorthogonal manner, leading to the massive release of an anticancer drug in living cells. Fast release of the drug triggered a stronger antiproliferative activity on tumor cells compared to the passive diffusion from the micelles core.