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Rapid Release of Entrapped Contents from Multi-Functionalizable, Surface Cross-Linked Micelles upon Different Stimulation

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Abstract: Hydrophobic guests such as pyrene could be readily trapped inside the micelles of an alkynylated surfactant in the presence of an azide-functionalized cross-linker using the click reaction. The cross-linker was designed to contain cleavable bonds such as geminal diol, disulfide, and acetal. The resulting pyrene-containing water-soluble nanoparticle was under electrostatic stress when diluted below the CMC of the surfactant. Extremely rapid (<1 min) release of the hydrophobic content was observed when the cross-linker was cleaved. This method combines the ease of physical entrapment and the precision of chemical ligation, and potentially is highly useful in the delivery and controlled release of pharmaceutical agents.

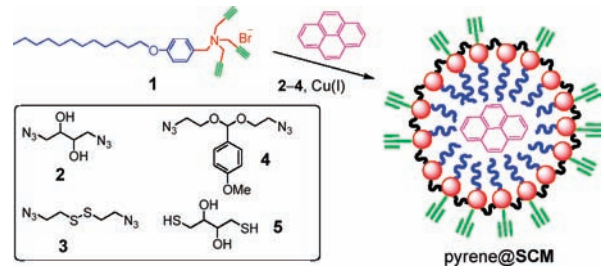
Approximately half of potential drug candidates identified in high throughput screening have poor solubility in water and thus are often denied further chance of development.[1] Although surfactant micelles can solubilize hydrophobic agents in water, their usage in drug delivery is hampered by the high critical micelle concentration (CMC), low thermodynamic stability, and the exceedingly dynamic nature of the assembly.

Polymeric micelles represent significant improvements over surfactant micelles because macromolecular amphiphiles tend to aggregate at concentrations orders of magnitude lower than their small molecule counterparts and produce micelles with greater thermodynamic stability.[2] A hydrophobic drug may be physically trapped inside the hydrophobic core of a polymeric micelle[3] or covalently attached to the polymer.[4] The latter approach enables the controlled release of drugs by specific stimuli and is more effective at preventing premature drug release than physical entrapment—features of particular importance in the delivery of drugs with high cytotoxicity. It has been reported, for example, that physically entrapped anticancer drugs display as high cytotoxicity as the small molecule versions.[5] Nonetheless, covalent linking between the drug and the delivery vehicle puts severe constraints on the structure of both components and adds considerable complexity to the production and formulation of the therapeutic package.

We recently reported a simple method to capture the micelles of alkynylated surfactants such as **1** by covalent cross-linking (Scheme 1).[6] Cross-linking was readily achieved by the highly efficient alkyne−azide click reaction[7] in the presence of 1 equiv of **2** and a catalytic amount of Cu(I).[8] The resulting surface-cross-linked micelles (SCMs), 8−10 nm in diameter, had numerous residual alkynes on the surface. Multivalent postmodification was conveniently accomplished via the same click reaction by adding desired azide-functionalized polymers or ligands after the cross-linking.

In this communication, we report the surprising discovery that these SCMs can release entrapped contents extremely rapidly

Scheme 1. Preparation of the Pyrene-Containing SCM



(<1 min) upon cleavage of the cross-linkages. Because of the outstanding tolerance of the click reaction to functional groups, we can prepare SCMs with a variety of cross-linkers and, as a result, employ different environmental stimuli to trigger the release.

We chose pyrene as a mock hydrophobic drug because of its environmentally sensitive fluorescence.[9] Its five vibronic bands respond to environmental polarity differently. The intensity ratio between the third (~384 nm) and the first band (~372 nm) is particularly sensitive to environmental changes. As shown by Figure 1a, the emission spectrum of pyrene was dependent upon the concentration of **1**. According to I_3/I_1, pyrene was in a more hydrophobic microenvironment in the 1 mM aqueous solution of **1** than in 0.05 mM. The CMC of the surfactant was determined to be $\sim 1.5 \times 10^{-4}$ M by monitoring I_3/I_1 at different concentrations of the surfactant (Figure 1b). The number compares favorably with the 1.4×10^{-4} M obtained by surface-tension measurement.[6]

Pyrene-containing SCMs were prepared according to Scheme 1. An aqueous solution containing 74 μM pyrene and 10 mM **1** was combined with a cross-linker (**2**−**4**) and $CuCl_2$/sodium ascorbate. Because the solubility limit of pyrene in water is 0.67 μM,[10] the majority of the dissolved pyrene resided within the surfactant micelles.[11] The reaction was generally allowed to continue for 24 h at room temperature.

Entrapment of pyrene was confirmed by fluorescence spectroscopy. When the pyrene-containing SCMs were diluted in water so that the concentration of (the cross-linked) **1** was below its CMC,

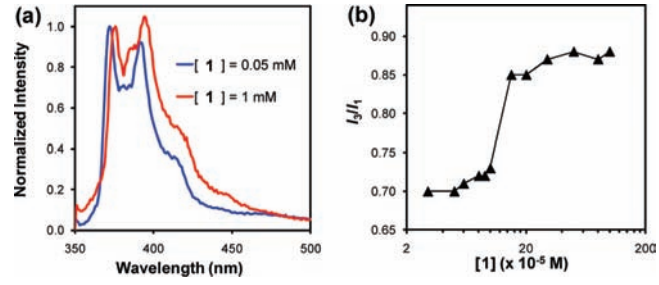


Figure 1. (a) Normalized emission spectra of pyrene in the presence of surfactant **1** in water. (b) Pyrene I_3/I_1 ratio as a function of [**1**]. [pyrene] = 0.1 μM.

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I_3/I_1 of pyrene remained unchanged at 0.84−0.85, the same as that above the CMC of uncross-linked **1** (Figure 1b). Importantly, the pyrene emission showed no change over at least a period of six months, suggesting that the hydrophobic guest was physically trapped inside the nanoparticle and could not escape.

As soon as periodic acid (HIO_4) was added to the mixture to cleave the 1,2-diol group in the cross-linker, however, I_3/I_1 dropped quickly (Figure 2a).[12] To our amazement, release of pyrene was so rapid that, by the time HIO_4 was added and the solution was mixed by gentle vortexing (<1 min), the change in I_3/I_1 was complete. The end I_3/I_1 value was somewhat dependent on the amount of HIO_4 added. 1 equiv of the cleaving agent (20 μM) reduced the I_3/I_1 to 0.74−0.75, higher than the 0.70 observed in the uncross-linked surfactant below the CMC (Figure 1b). Quite likely, not all the 1,2-diol groups in the SCMs were cleaved when an equivalent amount of the cleaving agent was added. Release seemed to be complete in the presence of 10 or 100 equiv of HIO_4, as the final I_3/I_1 was similar or even slightly lower than 0.70 in the uncross-linked micelles below the CMC.[13]

The outstanding functional group compatibility of the click reaction enabled us to incorporate cross-linkers sensitive to different stimuli. Diazide **3**, for example, contains a disulfide bond cleavable under reducing conditions, e.g., upon addition of **5**. Release of pyrene once again was found to occur extremely rapidly (Figure 2b). Excess **5** was no longer needed to reduce the I_3/I_1 ratio to 0.70. It seems that the entrapped pyrene was completely released even with just 1 equiv or 20 μM of **5**.

The specificity of the release was demonstrated by the control experiments.[14] Considering the low concentration of the pyrene-containing SCMs[15] and the cleaving agent (HIO_4 or **5**), the release was remarkably efficient. Cleavage of disulfide bonds in cross-linked polymers was reported to take hours to days to complete and often requires millimolar concentrations of reducing thiols.[16] Why did the SCMs expel pyrene so rapidly? Cationic surfactants, such as **1**, form micelles under two opposing forces. Hydrophobic interactions among the hydrocarbon tails favor micellization, and electrostatic repulsion among the headgroups disfavors it. Once captured by covalent cross-linkages, the SCMs cannot disassemble. Because the stability of the SCMs is maintained by covalent bonds, these nanoparticles are properly described as under "electrostatic stress" below the CMC. It is reasonable to expect, as soon as the covalent constraint is removed, the nanoparticle would "explode" like an "electrostatic bomb". Of course, not all cross-linkages have to be cleaved and "partial explosion" might be sufficient to release the pyrene. It is also likely that the electrostatic stress of the SCMs may accelerate the cleaving reaction. After all, any stress in the starting materials of a reaction, whether steric, conformational, or, in this case, electrostatic, should raise the ground-state energy of the system and lower the activation energy.

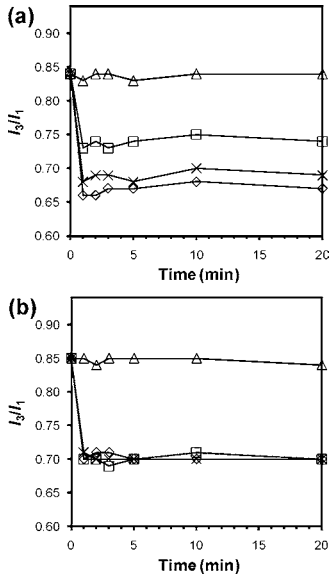

Figure 2. Change of pyrene I_3/I_1 ratio after addition of 0 (△), 1 (□), 10 (◇), and 100 equiv (×) of cleaving agent to the pyrene-containing SCMs in deionized water at ambient temperature. (a) Cross-linker = **2**, cleaving agent = HIO_4. (b) Cross-linker = **3**, cleaving agent = **5**. [**1**] = 20 μM.

We also encapsulated pyrene within SCMs using acetal-containing **4** as the cross-linker, with the intention that the guest would be released under acidic conditions. Acid-triggered release is important in many delivery applications. Endosomes and lysosomes are more acidic than cytosols.[17] Successful delivery via endocytosis thus often requires acid-triggered release. Cancerous and inflammatory tissues are also known to be more acidic than normal tissues.[18]

To our dismay, the pyrene-containing SCMs prepared with **4** only showed a slight change in I_3/I_1 from 0.81 to 0.77 at pH = 5 over a period of 96 h, although treatment with 0.01 M HCl did decrease the number to 0.7 (data not shown). The result was initially very puzzling to us as the SCMs were surrounded by acidic water and the *p*-methoxybenzyl acetal group in **4** was highly prone to hydrolysis. The compound, for example, underwent partial hydrolysis during aqueous workup in our hands.

Considering that pyrene fluorescence did not *directly* monitor what happened to the nanoparticles, we turned to dynamic light scattering (DLS), which correlates the scattered light with the diffusion coefficient of a particle in solution. Figure 3 shows the change in the scattering intensity for various pyrene-containing SCMs. Disintegration of the nanoparticles was once again found to be extremely rapid for SCMs prepared with **2** or **3** as the cross-linker (△ and □). The scattering intensity dropped to 15 and 24% of the original value, respectively, when 1 equiv of HIO_4 or **5** was added to the corresponding SCMs. On the other hand, the change in scattering intensity was clearly slower in the acid-triggered disassembly. The nanoparticles prepared with **4** as the cross-linker disintegrated gradually over a period of 60 min at 37 °C, although the majority of the change occurred in the first 20 min.[19] In contrast, the nanoparticles at pH = 7 displayed negligible changes in scattered light over the same period of time (×).

Why did the acid-triggered disassembly have a different reaction profile? These SCMs are positively charged on the surface. Anionic periodate should be attracted electrostatically to the surface of the nanoparticles. Dithiol **5** at least would not be repelled. For the acetal cross-linkers to be hydrolyzed, however, not only is the reaction catalyzed by proton but the intermediate is also a carbocation. Neither prefers to stay in a positively charged microenvironment. Moreover,

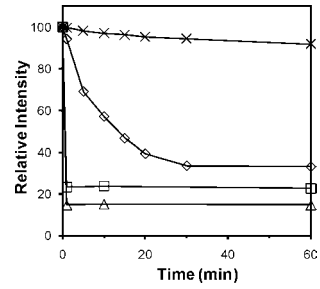

Figure 3. Relative intensity of scattered light for the pyrene-containing SCMs upon different stimulation: 1 equiv of HIO_4 for SCMs cross-linked with **2** (△), 1 equiv of **5** for SCMs cross-linked with **3** (□), and pH 5 (◇) and 7 (×) acetate buffer at 37 °C for SCMs cross-linked with **4**. [**1**] = 20 μM.

given its hydrophobicity, the acetal may be located in a relatively hydrophobic region of the SCM and thus is unlikely to be fully solvated by water. All the above factors would slow down the hydrolysis.[20]

What could be the reason for the small change of pyrene emission in the acid-sensitive SCMs? According to Figure 3, the SCMs were only partially disintegrated in our experiments, as uncross-linked surfactants are too small to scatter light at 20 μM. The I_3/I_1 value for the SCMs prepared with **2** and **3** was 0.84−0.85 (Figure 2), the same as that for the uncross-linked micelles above the CMC, but only 0.81 for those prepared with **4**. Thus, the former had higher cross-linking density than the latter.[21] As soon as some surfactants become free and escape from the SCM, pyrene would not be protected by the remaining surfactants if their hydrocarbon tails cannot adjust themselves around the guest; this is most likely to be the case when the initial SCM is highly cross-linked. If, however, the initial cross-linking density is low, the nanoparticles would be less rigid. As some surfactants are removed, the remaining structure can reconfigure itself easily and might still be able to protect its hydrophobic guest. As long as the binding affinity is sufficiently high between the partially hydrolyzed SCM and pyrene, the guest would want to stay inside the hydrophobic particle.

As demonstrated by our previous research,[6] multivalent surface functionalization was extremely simple in the alkynyl-terminated SCMs. If a combination of several cross-linkers is used, the stability of these nanoparticles should be tailored readily for specific applications. Our finding that the SCMs can eject entrapped hydrophobic content rapidly is significant. The hydrophobic guest is apparently trapped in a high energy state when the surface is heavily cross-linked. During cleavage, if the hydrocarbon tails cannot rearrange to accommodate the loss of surfactants, the remaining structure could not bind the guest very well. The result is much higher sensitivity toward stimulation; even partial cleavage can cause complete ejection of the guest. Overall, our entrapment−release strategy combines the ease of physical entrapment and the precision of chemical ligation and, as a result, requires no covalent modification of the entrapped agents. With additional benefits of multivalent surface modification, outstanding tolerance of the click reaction for functional groups, and tunable surface charge (e.g., by using anionic, nonionic, or zwitterionic surfactants with multiple alkynyl or azide groups), the SCMs may become very useful in the delivery and controlled release of pharmaceutical agents.

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Supporting Information Available: Experimental details for the synthesis and additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Lipinski, C. A. *J. Pharmacol. Toxicol. Methods* **2000**, *44*, 235–249. (b) Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. *Adv. Drug Delivery Rev.* **2001**, *46*, 3–26.
(2) Kwon, G. S. *Colloids Surf., B* **1994**, *2*, 429–434.
(3) Kabanov, A. V.; Chekhonin, V. P.; Alakhov, V. A.; Batrakova, E. V.; Lebedev, A. S.; Melik-Nubarov, N. S.; Arzhakov, S. A.; Levashov, A. V.; Morozov, G. V.; Severin, E. S.; Kabanov, V. A. *FEBS Lett.* **1989**, *258*, 343–345.
(4) Yokoyama, M.; Kwon, G. S.; Okano, T.; Sakurai, Y.; Seto, T.; Kataoka, K. *Bioconjugate Chem.* **1992**, *3*, 295–301.
(5) Bae, Y.; Kataoka, K. *Adv. Drug Delivery Rev.* **2009**, *61*, 768–784.
(6) Zhang, S.; Zhao, Y. *Macromolecules* **2010**, *43*, 4020–4022.
(7) (a) Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2002**, *41*, 2596–2599. (b) Kolb, H. C.; Finn, M. G.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2001**, *40*, 2004–2021. (c) Finn, M. G.; Kolb, H. C.; Fokin, V. V.; Sharpless, K. B. *Prog. Chem.* **2008**, *20*, 1–4.
(8) The popular $CuSO_4$−sodium ascorbate combination produced a precipitate from a micellar solution of **1**, possibly because the divalent sulfate anion affects the solubility of the cationic surfactant. $CuCl_2$ (5 mol %) and sodium ascorbate (25 mol %) were found to work well in the cross-linking.
(9) Kalyanasundaram, K.; Thomas, J. K. *J. Am. Chem. Soc.* **1977**, *99*, 2039–2044.
(10) (a) Abraham, M. H.; Le, J. *J. Pharm. Sci.* **1999**, *88*, 868–880. (b) Futoma, D. J. *Polycyclic aromatic hydrocarbons in water systems*; CRC Press: Boca Raton, FL, 1981; pp 14−22.
(11) Approximately one-third of the micelles contained pyrene in our experiment. The ratio of [**1**]/[pyrene] was ∼130 in our experiments. Our previous work (ref 6) estimated the micelle aggregation number of **1** to be 40−50. Thus, on average, every three micelles contained one pyrene molecule. The emission spectra of pyrene showed the absence of pyrene excimer, indicating that few (if any) micelles contained more than one pyrene.
(12) The releasing experiments were performed in triplicates. The relative error in I_3/I_1 was <5%.
(13) Periodic acid at high concentration (2×10^{-3} M) was found to quench the fluorescence of pyrene significantly. This could be the reason why the I_3/I_1 ratio was slightly lower that the 0.70 observed for pyrene below the CMC of **1**. Possibly, not all the vibronic bands were quenched to the same extent.
(14) SCMs prepared with **2** only released pyrene in the presence of periodic acid, but not with dithiol **5** or at pH = 5; those prepared with **3** released pyrene only in the presence of **5**, not with periodic acid or at pH = 5. SCMs prepared with an uncleavable cross-linker, *p*-xylyl diazide, on the other hand, showed no release in the presence of 1 equiv of periodic acid, **5**, or at pH = 5. The corresponding figures are given in the Supporting Information (Figures 1S−3S).
(15) The concentration of the cross-linked **1** and the cleaving agent was 20 μM. The aggregation number of **1** was estimated to be 40−50 by DLS. The concentration of the SCM was thus 0.4−0.5 μM.
(16) (a) Sun, K. H.; Sohn, Y. S.; Jeong, B. *Biomacromolecules* **2006**, *7*, 2871–2877. (b) Koo, A. N.; Lee, H. J.; Kim, S. E.; Chang, J. H.; Park, C.; Kim, C.; Park, J. H.; Lee, S. C. *Chem. Commun.* **2008**, 6570–6572. (c) Zhang, L.; Liu, W.; Lin, L.; Chen, D.; Stenzel, M. H. *Biomacromolecules* **2008**, *9*, 3321–3331.
(17) Mellman, I.; Fuchs, R.; Helenius, A. *Annu. Rev. Biochem.* **1986**, *55*, 663–700.
(18) (a) Helmlinger, G.; Sckell, A.; Dellian, M.; Forbes, N. S.; Jain, R. K. *Clin. Cancer Res.* **2002**, *8*, 1284–1291. (b) Trevani, A. S.; Andonegui, G.; Giordano, M.; López, D. H.; Gamberale, R.; Minucci, F.; Geffner, J. R. *J. Immunol.* **1999**, *162*, 4849–4857. (c) Engin, K.; Leeper, D. B.; Cater, J. R.; Thistlethwaite, A. J.; Tupchong, L.; Mcfarlane, J. D. *Int. J. Hyperthermia* **1995**, *11*, 211–216.
(19) The scattering intensity dropped to 15% of the original value after 48 h.
(20) Overall, the reaction was still quite fast. Hydrolyses of similar benzaldehyde acetals at comparable pH were reported to take hours to days to complete. See: Jensen, J. L.; Herold, L. R.; Lenz, P. A.; Trusty, S.; Sergi, V.; Bell, K.; Rogers, P. *J. Am. Chem. Soc.* **1979**, *101*, 4672–4677.
(21) It is difficult to know exactly how many cross-links an SCM contained. Digestion of the diol-cross-linked SCMs in our previous work (ref 6) indicated that the cross-linking density was in line with the stoichiometry of the reagents when a water-soluble cross-linker (i.e., **2**) was employed. For water-insoluble cross-linkers, such as **3** and **4**, the SCMs were expected to have a lower cross-linking density, as the cross-linkers were not completely consumed at the end of the cross-linking reaction. SCMs prepared with **4** had lower cross-linking density probably because the cross-linker underwent hydrolysis during cross-linking.

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