# Tetrazine Responsive Self-Immolative Linkers

Kevin Neumann, Sarthak Jain, Alessia Gambardella, Sarah E. Walker, Elsa Valero, Annamaria Lilienkampf and Mark Bradley*[a]

***Abstract:*** *Molecules that undergo activation or modulation following the addition of benign external small molecule chemical stimuli have numerous applications. Here, we report the highly efficient "decaging" of a variety of moieties by activation of a "self-immolative" linker by application of a water soluble and stable tetrazine, including the controlled delivery of Doxorubicin in a cellular context.*

Stimuli responsive compounds that undergo molecular modulation, activation or that switch on a desired physical, chemical or biological function upon the addition of an external chemical or physical trigger such as pH, temperature or light, have enormous power and biomedical potential.[1],[2] Light in particular has been used, as a stimulus, for various applications ranging from polymers containing azobenzene units that undergo reversible cis/trans photoisomerisation resulting in controllable polymer modulation,[3] to nanoparticles that reversibly contract (150 to 40 nm) upon photo-triggering and light-mediated antibody activation.[4] Numerous polymer architectures have been generated where pH can reversibly alter polymer topography by changes in protonation state,[5] or irreversibly via bond cleavage (resulting in cargo liberation).[6],[7]

Many chemical moieties have been developed that respond to a range of molecular triggers. For example, boronate functionalised polymers have been designed to react with glucose thereby mediating insulin release[8] and hydrogels that respond to the presence of specific antigens via swelling and cargo liberation have been synthesised.[9] Li reported the caging of the catalytic lysine residue in OspF with a propargyloxycarbonyl group, which was cleaved by Pd catalysis switching on the protein function.[10] A key feature of many of these activation systems is the integration of a "self-immolative safety-catch"[11] type linker, whereby remote functional group activation leads to subsequent 1,6-elimination and target/cargo liberation. This includes the work of Urano who used 500 nm light to liberate BODIPY-caged histamine[12] whereas Springer applied this approach to generate carboxypeptidase activated prodrugs of Doxorubicin (Dox).[13] Numerous other examples exist where the 1,6-elimination process has been used as a response to a variety of analytes and redox states leading to polymer "depolymerisation".[14],[15],[16] Even though these triggers and materials show huge potential there are still major challenges, including problems associated with light stimulated materials due to high tissue absorbance, neurotoxicity for common acrylate and acrylamide-based thermally responsive materials,[17] and the lack of tissue specificity with respect to pH.

Tetrazines have recently been exploited in a variety of inverse electron-demand Diels–Alder reactions ($DA_{INV}$) as a means of conjugating reporters (*e.g.* fluorophores[18],[19],[20] and PET isotopes[21],[22]) to a variety of biological entities such as DNA,[23] targeting peptides,[24] and antibodies.[25],[26] Typically, these bioconjugations are performed between a tetrazine and strained dienophile, such as *trans*-cyclooctene (TCO), resulting in a fast $DA_{INV}$ reaction.[27] Thus Chen developed bioorthogonal protein activation chemistry, with protection of the catalytic lysine residue in firefly luciferase with TCO rendering the protein inactive and treatment with a tetrazine restoring the protein function.[28] Tetrazine ligation with TCO has also been utilised for prodrug activation using Doxorubicin conjugated via a carbamate linkage to the allylic position of TCO, with $DA_{INV}$ liberating the free drug.[29]

In addition to the widely applied reactivity with strained alkenes and alkynes, tetrazines have been shown to rapidly react with cyclopentanone morpholine enamines and *N*-vinyl pyrrolidinones to liberate amines and amides, respectively.[30] Recently, it was shown that tetrazines react with phenyl vinyl ethers resulting in liberation of a phenol moiety.[31] This reactivity directed our attention to the application of tetrazines not only in the decaging of phenols but also to 1,6-elimination chemistry via a self-immolative linker approach. Herein, we demonstrate the selective release of both

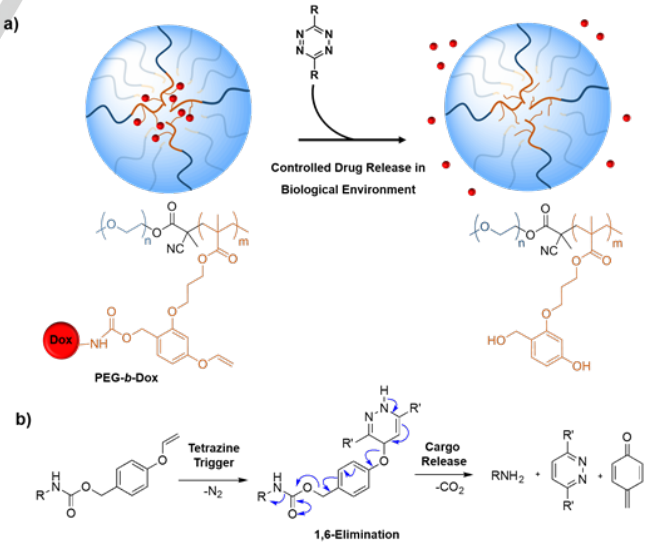


**Figure 1.** a) Nanoparticles with an average diameter of 35 nm were fabricated from the amphiphilic block-*co*-polymer PEG-*b*-Dox, with the methacrylate–Doxorubicin conjugated segment forming the hydrophobic core. Upon reaction with tetrazine, doxorubicin (red spheres) is liberated, driven by the 1,6-elimination reaction of the self-immolative linker. b) The mechanism of tetrazine mediated vinyl ether decaging and cargo liberation (here $RNH_2$).

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caged fluorophores and the anticancer drug Doxorubicin by a tetrazine-mediated vinyl ether based dienophile, which upon a reaction with a $DA_{INV}$ reaction (Figure 1). The designed system comprised a tetrazine (the stimulus) decages a phenol(ate) prompting the subsequent release of Doxorubicin via 1,6-elimination. Additional functionalisation of the linker with a methacrylate allowed the formation of amphiphilic PEG-*b*-Dox co-polymer nanoparticles that, upon reaction with tetrazine, released Doxorubicin resulting in the "switch-on" of cytotoxicity. In the absence of the stimulus, these PEG-*b*-Dox nanoparticles have low cytotoxicity and therefore have the potential to improve drug efficacy. Vinyl groups have been shown to be good dienophiles in $DA_{INV}$ reactions and have been used to efficiently label and subsequently image 5-vinyl-2'-deoxyuridine modified DNA.[32] Our hypothesis was that vinyl ethers could act as masking groups for phenols and that tetrazine-mediated activation via the incorporation of a self-immolative liker would allow the "switch-on" of fluorophores as well as enabling targeted drug release. The phenolic groups of fluorescein **1** and resorufin **2** were readily converted to vinyl ethers using the vinyl boronic anhydride pyridine complex reported by O'Shea[33] to give the quenched fluorophores bis-*O*-vinyl fluorescein **3** and *O*-vinyl resorufin **4** (Figure S1–S2). Incubation with dipyridyl tetrazine **5** allowed efficient removal of the vinyl groups via the $DA_{INV}$ with the reaction being readily monitored by $^1$H NMR and regeneration of fluorescence, giving a 23-fold increase in fluorescence with bis-*O*-vinyl fluorescein **3** and 99-fold increase with *O*-vinyl resorufin **4** (Figure 2, Figure S3–S4).

To broaden this approach, a tetrazine responsive self-immolative linker was designed consisting of a 4-hydroxymethyl phenyl vinyl ether scaffold, thereby allowing cargo conjugation (such as fluorophores or drugs) via carbamate formation,

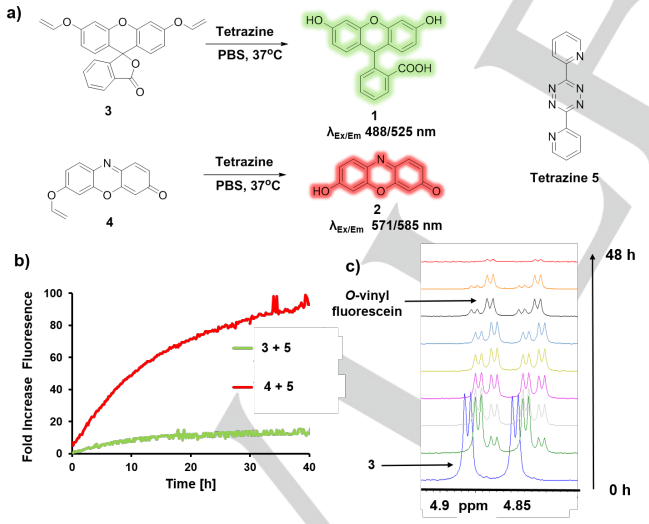


**Figure 2**. a) Tetrazine **5** triggered decaging of bis-*O*-vinyl fluorescein **3** and *O*-vinyl resorufin **4** at 37 °C in PBS (pH 7.4) led to fluorescence "switch on". b) $DA_{INV}$ reaction between tetrazine **5** (200 µM) and **4** (35 µM) led to a 99-fold increase in fluorescence ($\lambda_{Ex/Em}$ 530/590 nm) whereas the same reaction with **3** (35 µM) led to a 23-fold increase ($\lambda_{Ex/Em}$ 485/528 nm). c) Monitoring of the reaction between *bis*-*O*-vinyl fluorescein **3** and tetrazine **S1** (See Figure S5) in DMSO by $^1$H NMR showed the initial formation of the mono *O*-vinyl fluorescein with full conversion to **1** after 48 h at 37 °C.

with remote activation (based on a 1,6-elimination reaction) following liberation of the phenolate by the tetrazine-mediated removal of the vinyl ether group (Figure 1b). Furthermore, the phenyl ring contained a C3 spacer linked to a methacrylate moiety for polymerisation chemistries. The tetrazine responsive self-immolative linker **6** was synthesised by selective *para* tetrahydropyranyl (THP) ether protection of 2,4-dihydroxybenzaldehyde **7** (to give compound **8**) and subsequent *ortho* etherification using 3-bromopropyl methacrylate to give **9** (Figure 3a). Following THP deprotection, vinylation of the free phenol was achieved using the vinyl boronic anhydride pyridine complex[31] as described above. Finally, aldehyde **10** was reduced with $NaBH_4$ and the resulting linker **6** transformed into the nitrophenyl activated carbonate **11**. Nile Blue was coupled to the linker to give the Nile Blue carbamate **12** with quenched fluorescence (Figure 3b), which upon reaction with tetrazine **5** under aqueous conditions underwent vinyl group removal with subsequent 1,6-elimination, loss of $CO_2$ and switch-on of fluorescence of Nile Blue **13** (Figure 3, Figure S6).

To further demonstrate the potential of external small-molecule controlled cargo release, the activated linker **11** was conjugated to the anti-cancer agent Doxorubicin (Figure 4a), a DNA interchelating anthracycline antibiotic used for the treatment of malignancies including breast and ovarian tumours, sarcomas, and acute leukemias.[34] The Doxorubicin monomer **14** showed >90% conversion to the free drug after 5 day incubation with tetrazine **5** (Figure 4b). The methacrylate moiety of **14** was

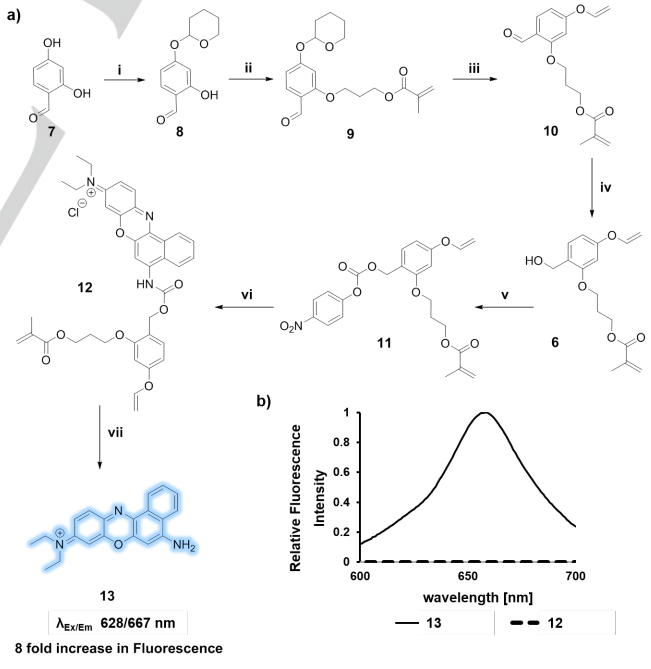


**Figure 3**. Synthesis and activation of the tetrazine cleaved self-immolative linker. a) i) 2,4-Dihydro-2H-pyran, PPTS, DCM, 60 %; ii) 3-Bromopropyl methacrylate, $Cs_2CO_3$, DMF, 50 °C, 77 %; iii) (1) 1M HCl (aq.), MeOH, (2) $Cs_2CO_3$, vinyl boronic anhydride pyridine complex, $Cu(OAc)_2$, DCM, 55 %; iv) $NaBH_4$, MeOH, quant.; v) Phenylchloroformate, $Et_3N$, 83 %.; vi) Nile Blue **13**, $Et_3N$, DCM/THF, rt, 16 %; vii) $DA_{INV}$ of **12** (40 µM) and tetrazine **5** (100 µM) led to an 8-fold increase in fluorescence in PBS (pH 7.4) at 37 °C (see Figure S6). b) Fluorescence spectra of Nile Blue **13** and the fully quenched Nile Blue carbamate **12** ($\lambda_{Ex/Em}$ 590/645 nm).

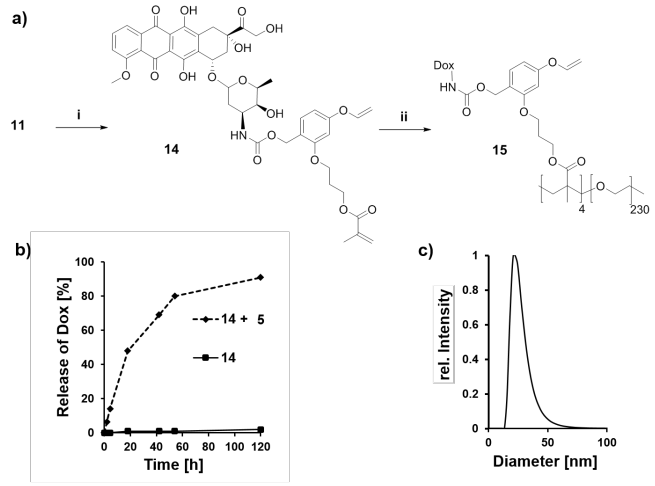


**Figure 4**. Synthesis and characterisation of Doxorubicin conjugated nanoparticles (PEG-*b*-Dox). a) i) Doxorubicin hydrochloride, $Et_3N$, 51 %; ii) PEG CTA, APS, TMEDA, DMSO, 30 °C. b) Release profile of Dox from monomer **14** (2.3 mM) by tetrazine **5** (23 mM) at 37 °C in PBS/ACN as monitored by HPLC ($\lambda_{Abs}$ 495 nm). c) Size analysis (by dynamic light scattering) of the PEG-*b*-Dox derived nanoparticles showing an average diameter of 35 nm in PBS (pH 7.4) at 37 °C.

polymerised with the RAFT reagent PEG CTA ($M_n$ 10,000 g mol$^{-1}$) using APS/TMEDA as the redox initiator to give the amphiphilic PEG-*b*-Dox *co*-polymer **15** ($M_n$ 13,000 g mol$^{-1}$). These mild reaction conditions were required as thermally or UV initiated polymerisations led to co-reaction of the vinyl ether groups. Once placed in water, the PEG-*b*-Dox co-polymer **15** formed nanoparticles with a diameter of 35 nm (Figure 4c,

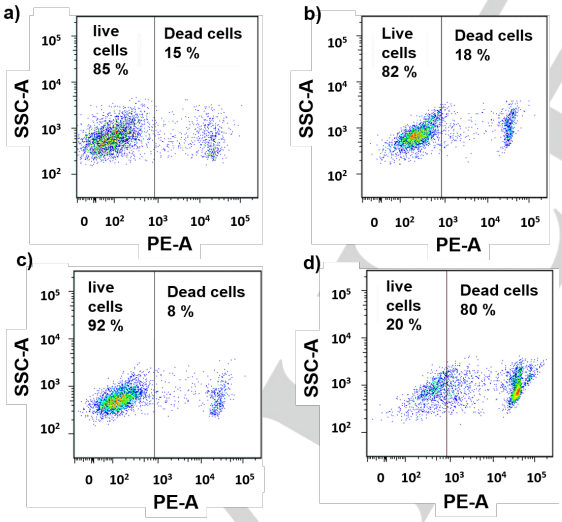


**Figure 5**. Tetrazine triggered release of Doxorubicin. HEK273T cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % FBS. Nanoparticles, tetrazine **5** and/or free Doxorubicin were incubated with cells at 37 °C with 5 % $CO_2$ for 48 h. a) Control (just cells); b) PEG-*b*-Dox **15** nanoparticles (1 µM equiv. of Dox); c) Tetrazine **5** (35 µM); d) Tetrazine **5** (35 µM) and PEG-*b*-Dox **15** nanoparticles (1 µM). The samples were stained with propidium iodide (2 µM) and analysed by flow cytometry ($\lambda_{Ex}$ 488 nm with 500–554 nm broad pass filter). Forward versus side scatter (SSC-A) profiles were used to gate intact cellular materials and determine membrane integrity (PI).

Figure S7), with the hydrophobic core of each particle, consisting of four Doxorubicin units (determined by $^1$H NMR) linked to the methacrylate backbone, surrounded by a hydrophilic PEG shell.

Tetrazine-mediated controlled drug release was explored using HEK273T cells. The PEG-*b*-Dox nanoparticles (loading equivalent to 8 µM of Dox) showed no cytotoxicity after 48 h (MTT assay), whereas 1 µM "free" Doxorubicin resulted in complete cell death (Figure S8–S9). Tetrazine **5** showed no toxicity at a concentration of 35 µM. When the cells were treated with 1 µM PEG-*b*-Dox nanoparticles (equivalent to 4 µM of Dox), the addition of tetrazine **5** (35 µM) triggered cytotoxicity with 80 % cell death after 48 h incubation (Figure 5, Figure S10). Comparable results were obtained with prostate cancer cell line PC3, with cytotoxicity of PEG-*b*-Dox triggered only in combination with tetrazine (Figure S10). This indicates that the nanoparticles underwent efficient tetrazine triggering, even in a complex cellular environment, leading to a controlled switch-on of cytotoxicity.

In summary, we have demonstrated the practicality and application of a tetrazine-activated self-immolative linker, which allows the controlled release of fluorophores and drugs within a complex biological milieu. Nanoparticles containing multiple covalently attached Doxorubicins (attached via a 4-hydroxymethyl phenyl vinyl ether linker) demonstrated efficient tetrazine-mediated switch-on of cytotoxicity via 1,6-elimination driven release. In the absence of a tetrazine stimulus, the PEG-*b*-Dox nanoparticles display low cytotoxicity and inherently have EPR targeting abilities. This novel approach offers new opportunities in the field of targeted and controlled drug delivery

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1 M. A. C. Stuart, W. T. S. Huck, J. Genzer, M. Müller, C. Ober, M. Stamm, G. B. Sukhorukov, I. Szleifer, V. V. Tsukruk and M. Urban, F. Winnik, S. Zauscher, I. Luzinov and S. Minko, *Nat. Mater.*, **2010**, *9*, 101.

2 S. Mura, J. Nicolas and P. Couvreur, *Nat. Mater.,* **2013**, *12*, 991.

3 H. Zeng, P. Wasylczyk, C. Parmeggiani, D. Martella, M. Burresi and D. S. Wiersma, *Adv. Mater.,* **2015**, *27*, 3883.

4 S. Thompson, A. C. Self and C. H. Self, *Drug Discov. Today,* **2010**, *15*, 468.

5 E. S. Lee, K. Na and Y. H. Bae, *J. Control. Release,* **2003**, *91*, 103.

6 F. Zhan, W. Chen, Z. Wang, W. Lu, R. Cheng, C. Deng, F. Meng, H. Liu and Z. Zhong, *Biomacromolecules,* **2011**, *12*, 3612.

7 R. Tomlinson, J. Heller, S. Brocchini and R. Duncan, *Bioconjug. Chem.,* **2003**, *14*, 1096.

8 K. Kataoka, H. Miyazaki, M. Bunya, T. Okano and Y. Sakurai, *J. Am. Chem. Soc.,* **1998**, *120*, 12694.

9 T. Miyata, N. Asami and T. Uragami, *Nature,* **1999**, *399*, 766.

10 J. Li, J. Yu, J. Zhao, J. Wang, S. Zheng, S. Lin, L. Chen, M. Yang, S. Jia and X. Zhang and P. R. Chen., *Nat. Chem.,* **2014**, *6*, 352.

11 I. Ojima, *Acc. Chem. Res.,* **2008**, *41*, 108.

12 N. Umeda, H. Takahashi, M. Kamiya, T. Ueno, T. Komatsu, T. Terai, K. Hanaoka, T. Nagano and Y. Urano, *ACS Chem. Biol.,* **2014**, *9*, 2242.

13 I. Niculescu-Duvaz, D. Niculescu-Duvaz, F. Friedlos, R. Spooner, J. Martin, R. Marais and C. J. Springer, *J. Med. Chem.,* **1999**, *42*, 2485.

14 G. I. Peterson, M. B. Larsen and A. J. Boydston, *Macromolecules,* **2012**, *45*, 7317.

15 C. A. Blencowe, A. T. Russell, F. Greco, W. Hayes and D. W. Thornthwaite, *Polym. Chem.,* **2011**, *2*, 773.

16 E. Sella, A. Lubelski, J. Klafter and D. Shabat, *J. Am. Chem. Soc.,* **2010**, *132*, 3945.

17 P. Bawa, V. Pillay, Y. E. Choonara, L. C. du Toit, *Biomed. Mater.,* **2009**, *4*, 022001.

18 J. C. T. Carlson, L. G. Meimetis, S. A. Hilderbrand and R. Weissleder, *Angew. Chem. Int. Ed.,* **2013**, *52*, 6917.

19 N. K. Devaraj, R. Weissleder and S. A. Hilderbrand, *Bioconjug. Chem.,* **2008**, *19*, 2297.

20 G. Lukinavičius, K. Umezawa, N. Olivier, A. Honigmann, G. Yang, T. Plass, V. Mueller, L. Reymond, I. R. Corrêa Jr, Z. Luo, C. Schultz, E. A. Lemke, P. Heppenstall, C. Eggeling, S. Manley and K. Johnsson, *Nature Chemistry*, **2013**, *5*, 132.

21 C. Denk, D. Svatunek, S. Mairinger, J. Stanek, T. Filip, D. Matscheko, C. Kuntner, T. Wanek and H. Mikula, *Bioconjug. Chem.,* **2016**, *27*, 1707.

22 O. Keinänen, X.-G. Li, N. K. Chenna, D. Lumen, J. Ott, C. F. M. Molthoff, M. Sarparanta, K. Helariutta, T. Vuorinen and A. D. Windhorst and A. J. Airaksinen, *ACS Med. Chem. Lett.,* **2016**, *7*, 62.

23 H. Bußkamp, E. Batroff, A. Niederwieser, O. S. Abdel-Rahman, R. F. Winter, V. Wittmann and A. Marx, *Chem. Commun.,* **2014**, *50*, 10827

24 R. Selvaraj, B. Giglio, S. Liu, H. Wang, M. Wang, H. Yuan, S. R. Chintala, L.-P. Yap, P. S. Conti, J. M. Fox. And Z. Li, *Bioconjug. Chem.,* **2015**, *26*, 435.

25 N. K. Devaraj and R. Weissleder, *Acc. Chem. Res.,* **2011**, *44*, 816.

26 K. Lang, L. Davis, J. Torres-Kolbus, C. Chou, A. Deiters and J. W. Chin, *Nat. Chem.,* **2012**, *4*, 298.

27 M. L. Blackman, M. Royzen and J. M. Fox, *J. Am. Chem. Soc.*, **2008**, *130*, 13518.

28 J. Li, S. Jia and P. R. Chen, *Nat Chem Biol.,* **2014**, *10*, 1003.

29 R. M. Versteegen, R. Rossin, W. ten Hoeve, H. M. Janssen and M. S. Robillard, *Angew. Chem. Int. Ed.,* **2013**, *52*, 14112.

30 D. L. Boger, R. P. Schaum and R. M. Garbaccio, *J. Org. Chem.,* **1998**, *63*, 6329.

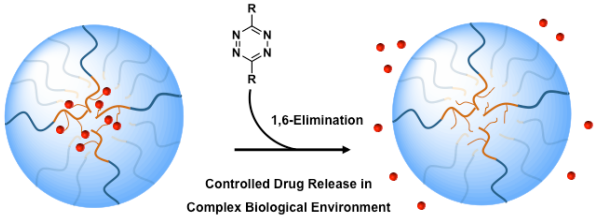
31 H. Wu, S. C. Alexander, S. Jin, and N. K. Devaraj, *J. Am. Chem. Soc.*, **2016**, *138*, 11429.

32 U. Rieder and N. W. Luedtke, *Angew. Chem. Int. Ed.,* **2014**, *53*, 9168.

33 N. F. McKinley and D. F. O'Shea, *J. Org. Chem.,* **2004**, *69*, 5087.

34 T. L. Jackson, *J. Theor. Biol.,* **2003**, *220*, 201.

# COMMUNICATION

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