COMMUNICATION

Hierarchical Self-assembled Photo-Responsive Tubisomes from Cyclic Peptide-Bridged Amphiphilic Block Copolymer

Jie Yang,[a] Ji-Inn Song,[a] Qiao Song,[a] Julia Y. Rho,[a] Edward DH Mansfield,[a] Stephen C. L. Hall,[a] Megan Sambrook,[a] Feihe Huang[b*] and Sébastien Perrier[a*]

Abstract: Typically, the morphologies of the self-assembled nanostructures from block copolymers are limited to spherical micelles, wormlike micelles and vesicles. Herein, we report a new generation of materials with unique shape and structures, cylindrical soft matter particles (tubisomes), obtained from the hierarchical self-assembly of cyclic peptide-bridged amphiphilic diblock copolymers. Additionally, the capacity of obtained photo-responsive tubisomes as potential drug carriers is evaluated. The supramolecular tubisomes described here paves an alternative way for fabricating polymeric tubular structures, and will expand the toolbox for the rational design of functional hierarchical nanostructures.

Nature provides a large array of fascinating architectures with unique functions from the self-assembly of elementary building blocks, such as amino acids, lipids and nucleic acids, mediated by synergistic non-covalent interactions (*e.g.* hydrophobic interactions, electrostatic interactions, hydrogen bonding, π-π stacking, and so on).[1,2] In spite of the fact that the biological self-assembly is extremely complicated, much work has been devoted to unveil and further employ these principles in the design of elaborate synthetic materials.[3,4] Polymeric nanoparticles, which exhibit better stability and durability compared to small-molecule aggregates, are ones of the most prominent examples of this approach and have received great attention due to their widely applications in nanotechnology and medical technologies.[5-8] Therefore, various synthetic methodologies have been exploited in the preparation of non-spherical nanostructures by ether controlling the thermodynamic or kinetic factors of block copolymer self-assembly, such as polymerization-induced self-assembly (PISA) and crystallization-driven self-assembly (CDSA). However, the limited solvophilic monomer (solvophobic when polymerized) and exquisite polymerization control of PISA greatly restrict its practical applications especially in bio-related fields.[9] Similarly, a crystalline or semi-crystalline polymer is essential for CDSA approach, such a transformation of the energetically favored symmetric shapes into anisotropic nanostructures still remains a big challenge.[10] In this respect, broadening the dimensionality of polymeric nanoparticles through a well-defined process is particularly important.

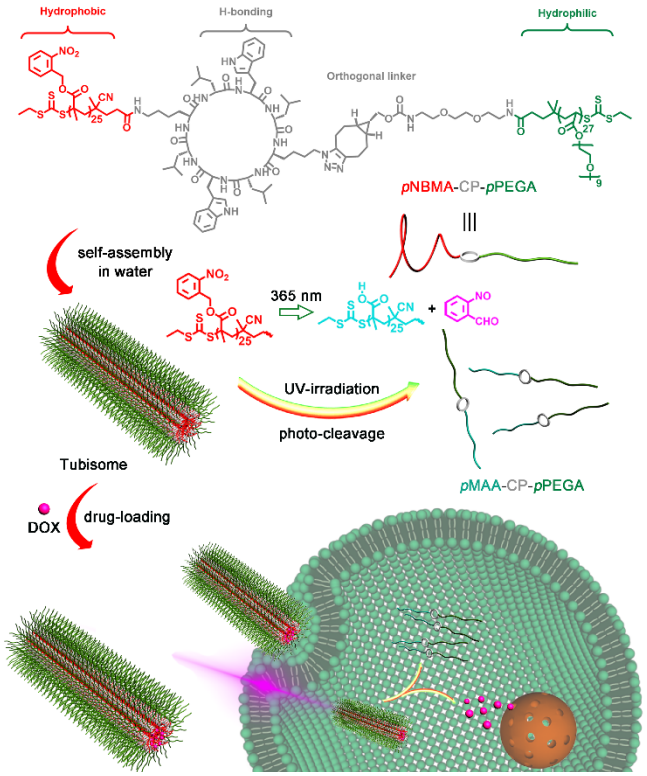
Cyclic peptides (CP), a type of flat ring-like configurations consisted of an even number of alternating D- and L-amino acids, can stack through anti-parallel *β*-sheet hydrogen bonding to afford rigid tubular structures.[11–13] These peptides have been used as building blocks to construct structure-predictable nano-objects based on tubular brush supramolecular polymers.[14,15] Our previous work has focused on polymer conjugates based on water-soluble homopolymers, such as poly(ethylene glycol), poly(*N*-acryloylmorpholine) and poly(*N*,*N*-dimethyl acrylamide).[16–20] It's anticipated that introducing both hydrophobic and hydrophilic homo-polymers onto cyclic peptides could afford amphiphilic diblock copolymers and may result in more complicated self-assembly nanostructures with the help of hydrophobic interactions. Very recently, we reported the secondary self-assembly of supramolecular nanotubes (made by the CP core) into cylindrical micelles termed "tubisomes" by introducing both hydrophobic and hydrophilic polymers on a cyclic peptide.[21] The special properties of tubisomes, such as unique morphology, high internal surface area and accessible functionalisation, make them versatile polymeric materials.[22]

In this study, we expand the versatility and applications of tubisomes by introducing systems that respond to light, an exciting trigger for ist time and space controllable localization and modulable operation.[23,24] Herein, by utilizing the cyclic peptide building blocks, we have designed and prepared the first photo-responsive tubisome nanoparticles. As depicted in Scheme 1, both hydrophilic (poly(PEG acrylate), *p*PEGA) and photosensitive hydrophobic (poly(2-nitrobenzyl methacrylate), *p*NBMA) polymers were selectively conjugated onto opposite sides of cyclic peptide. Driven by the synergistic hydrogen bonding interactions and hydrophobic effects, hierarchical tubisomes were obtained in aqueous solution. Subjecting the nano-objects to UV irradiation (365 nm) can lead to the switch of the hydrophobic core to hydrophilic, resulting in the disassembly of the tubisomes. The tubisomes shown great biocompatibility and have the potential to be loaded by an active agent. Here, we show that by loading the tubisomes with doxorubicin, they have the capacity to be used as drug carriers, as shown *in vitro* on human breast cancer MDA-MB-231 cells.

[a] Dr. J. Yang, J.-I. Song, Dr. Q. Song, J. Rho, Dr. E. D. Mansfield, M. Sambrook, Prof. S. Perrier
Department of Chemistry
University of Warwick
Coventry CV4 7AL, United Kingdom
E-mail: s.perrier@warwick.ac.uk

[b] Prof. F. Huang
Department of Chemistry
Zhejiang University
Hangzhou 310027, China
E-mail: fhuang@zju.edu.cn

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



Scheme 1. Chemical structure of *p*NBMA$_{25}$-CP-*p*PEGA$_{27}$ and cartoon illustration of cellular uptake of DOX-loaded tubisomes and photo triggered intracellular drug release.

For the ligation of both hydrophobic and hydrophilic polymers to the cyclic peptide, rapid chemical reactions with high specificity, mild reaction conditions and quantitative yields are essential.[25] With these requirements in mind, a cyclic peptide was designed with azido and amino groups on opposite sides, to provide a platform for an orthogonal azide-alkyne 'click' reaction and amidation reaction.[26,27] *N*-hydroxysuccinimide (NHS) functionalized polymer *p*NBMA with controlled molecular weight was synthesized by reversible addition−fragmentation chain-transfer (RAFT) polymerization and conjugated in slight excess to the cyclic peptide by mild amidation reaction.[28] The product was easily purified by precipitation in dichloromethane/diethyl ether mixture to remove the excess *p*NBMA. Then a *p*PEGA with bicyclo-[6.1.0]-nonyne (BCN) as chain-end was obtained by modifying the NHS-*p*PEGA with a commercially available strained alkyne, and further conjugated, in slight excess, to the cyclic peptide by a catalyst free strain promoted azide-alkyne cycloaddition. *p*NBMA$_{25}$-CP-*p*PEGA$_{27}$ was obtained by precipitation in methyl *tert*-butyl ether to remove unreacted BCN-*p*PEGA.[29] The orthogonal ligation and convenient purification methods used here provide a convenient approach for the preparation of versatile cyclic peptide bridged block copolymers with tailored functions.

Transmission electron microscopy (TEM) and scanning electron microscope (SEM) were used to verify the morphology of the cyclic peptide-induced self-assembly of the conjugates. Unlike the corresponding block copolymer *p*NBMA$_{25}$-*b*-*p*PEGA$_{27}$, which self-assembles into typical spherical micelles with a diameter of 27 ± 2 nm driven by hydrophobic-hydrophilic interactions in water (Fig. S11). *p*NBMA$_{25}$-CP-*p*PEGA$_{27}$ self-assembled into distinctly different nanostructures. By introducing a cyclic peptide to link each block, the morphology of the aggregates changes dramatically into cylindrical structures with an average length of 129 ± 23 nm and width of 25 ± 3 nm (Fig. 1c, 1d). However, some spherical nanoparticles can still be observed. We assumed that these "spheres" are short tubisomes since the self-assembly process is dynamic and the size distribution of the tubisomes is polydispersed. To get further insights of the nanostructures observed from TEM and SEM, fitting the scattering data collected from small angle neutron scattering (SANS) showed the system was assembling as hairy cylindrical micelle models (Fig. 1b), which is in good agreement with the observed morphology from TEM and SEM. Remarkably, the diameter of the core of the fitted cylindrical structure is 5.9 nm which exceeds the size of cyclic peptide based single tube (a diameter of 0.9 nm for eight amino acids CP), suggesting a secondary self-assembly from single tubular brush polymers to tubisomes.[30] The distinctive nanostructure from cyclic peptide bridged diblock polymer demonstrates the important role of the cyclic peptide in directing the self-assembly of block copolymers. Indeed, instead of forming a thermodynamically favourable spherical micelles morphology, the robust and directional hydrogen bonding interactions between cyclic peptides drive the self-assembly of block copolymer into hierarchical superstructures-tubisomes with synergistic hydrophobic interactions.

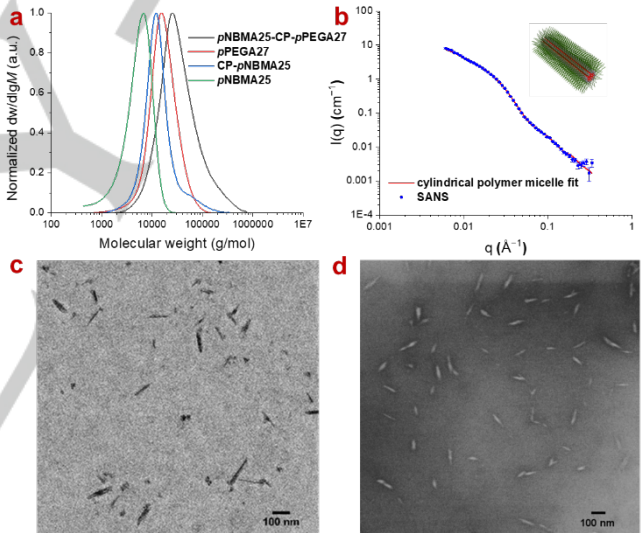


Figure 1. a) GPC traces (DMF + 0.1% LiBr) of the free polymers and conjugates; b) SANS plots for the cyclic bridged block polymer *p*NBMA$_{25}$-CP-*p*PEGA$_{27}$. The scattering profile was best fitted with a cylindrical polymer micelle model (red line); c) TEM image of *p*NBMA$_{25}$-CP-*p*PEGA$_{27}$; d) SEM image of *p*NBMA$_{25}$-CP-*p*PEGA$_{27}$.

The *o*-nitrobenzyl ester group is a typical photo-cleavable group that has been widely employed in the biomedical field.[31] The hydrophobic *p*NBMA block can be switched to hydrophilic poly(methacrylic acid) (*p*MAA) after photo irradiation (UV light, 365 nm). UV-*vis* spectroscopy was first employed to monitor the photo-cleavage process of the self-assembled tubisomes (Fig. 2a). Upon irradiation with UV light, the gradually decrease of the characteristic absorption (320 nm) for *o*-nitrobenzyl ester group was observed, accompanied by the increase of absorbance at 360 nm (characteristic absorption for *o*-nitrosobenzaldehyde), demonstrating the photo-stimulated cleavage of the *p*NBMA segment.

In order to study the photo-responsive self-assembly behavior of tubisomes, SANS, TEM and DLS were employed. As

indicated from TEM, all the tubular structures disappeared after irradiation with 365 nm UV light for 20 minutes, indicating the disassembly of the tubisomes (Fig. S12). From DLS, there is a significant decrease in the size of the aggregates from 100 nm to 10 nm which is in good agreement with TEM (Fig. 2b). In addition, fitting the data collected from SANS after irradiation revealed that the nanotube structures had disappeared and only Gaussian chains remain in solution (Fig. S10). The reason for the complete disassembly of tubisomes can be explained by the negatively charged *p*MAA block obtained from light irradiation – since the pK_a of *p*MAA is about 5.0, at neutral pH the methacrylic acid groups are almost entirely deprotonated.[32] As shown in Fig. S14, by gradual photo irradiation, the zeta potential decreased rapidly and reached about −6 mV at pH 7.4, indicating a negative charged surface, due to the deprotonated *p*MAA. The steric hindrance of *p*PEGA and electrostatic repulsion of charged *p*MAA greatly inhibit the stacking of cyclic peptides, leading to the disassembly of tubisomes into stretched polymer chains.

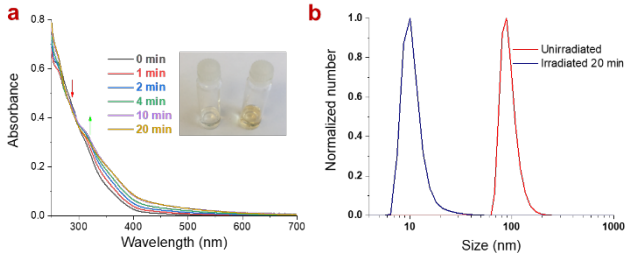


Figure 2. a) UV-*vis* spectra of *p*NBMA$_{25}$-CP-*p*PEGA$_{27}$ upon irradiation with UV light (365 nm); b) Number-weighted size distribution of *p*NBMA$_{25}$-CP-*p*PEGA$_{27}$ before (red) and after (blue) UV irradiation.

The light-responsive self-assembly of the cyclic peptide amphiphilic conjugates provides great potential as a drug delivery system. The toxicity of *p*NBMA$_{25}$-CP-*p*PEGA$_{27}$ was investigated by 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT) assay on diffrent cell lines, including both healthy cells and cancer cells. As indicated in Figure S22 and S23, even at the concentration of *p*NBMA$_{25}$-CP-*p*PEGA$_{27}$ of 200 μg/mL, there is no obvious cytotoxicity to all of the cell lines, thus suggesting good biocompatability of the tubisomes. Furthermore, XTT also showed that the UV light and the side-products obtained from light irradiation are not toxic to cells. Indeed, after irradiation with UV light for 20 minutes, the cell viability is comparable to the viability of cells incubated with *p*NBMA$_{25}$-CP-*p*PEGA$_{27}$ (Fig. S23).

Finally, doxorubicin (DOX), a classical hydrophobic anticancer drug, was selected as the model candidate to explore the drug encapsulation and controlled release property of the tubisomes. Upon deprotonating doxorubicin hydrochloride with trimethylamine, DOX was successfully encapsulated inside tubisomes by co-self-assembly in water, and the drug loading content of tubisomes was calculated to be 10.3 wt.% (Fig. S15-Fg.S18). A similar protocol with the amphiphilic block copolymers led to a drug loading of 6.8 wt.%. This loading difference suggests that the tubular structures are better vectors for cargo transport. Furthermore, DLS and TEM were performed to study the morphology change of the tubisomes by loading DOX inside. As shown in Fig. S20, the tubisome nanostructures could be still observed. From DLS (Fig. S21), the average size of DOX-loaded tubisomes is about 107 nm which increased slightly when compared to free tubisomes, demonstrating negligible influence on the morphology and size of the tubisomes by co-self-assembly with DOX. The light-stimulated dissociation of tubisomes was then exploited to realize the controlled release of DOX. As indicated in Fig. 3a, less than 13% of DOX release was observed without photo stimulus after 24 h incubation, indicating a good load-retention capability of the tubisome carriers. In contrast, a much faster release of DOX was observed after photo-irradiating the solution for 20 minutes. The release increased to 60% within 24 h, demonstrating the photo-triggered disassembly of the tubisomes indeed accelerated the release of DOX. *In-vitro* experiments also supported this conclusion. Upon incubation with human breast cancer MDA-MB-231 cells, DOX-loaded tubisomes were taken up efficiently (Fig. S26) and, upon UV irradiation, showed enhanced DOX release into the cytoplasm, leading to eradication of the cancer cells. As shown in Fig. 3b, the half-maximal inhibitory concentration (IC_{50}) of DOX-loaded tubisomes without UV irradiation could not be fitted out, due to the much lower toxicity, while the IC_{50} of DOX-loaded tubisomes after UV irradiation was estimated to be 9.7 μg/mL, which is very close to the value obtained for free DOX (7.4 μg/mL), indicating the photo irradiation indeed accellerate the release of DOX.

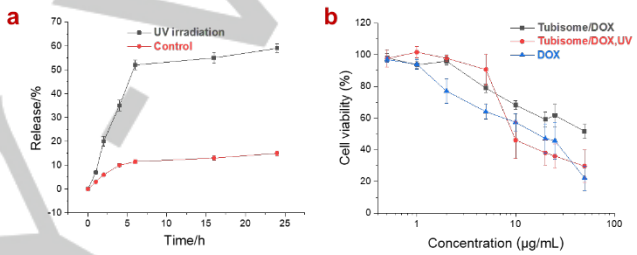


Figure 3. a) Time-dependent drug release behavior of DOX-loaded tubisomes with or without photo irradiation in PBS (pH 7.4); b) Cytotoxicity of free DOX and DOX-loaded tubisomes with or without photo irradiation in MDA-MB-231 cells by XTT assay (mean ± s.d., n = 3, P < 0.05, Student's t-test).

In summary, we have developed an efficient approach for fabricating polymeric tubular nanostructures in aqueous solution. By simply employing cyclic peptide as the linker between a hydrophobic and hydrophilic block within a copolymer, highly directional hydrogen bonding interactions could be introduced to drive the self-assembly into hierarchical superstructures, tubisomes, where hydrogen bonds and hydrophobic effect act in synergy. Additionally, the self-assembled tubisomes show good biocompatibility, high drug loading content and rapid drug release upon UV irradiation. The anticancer drug Doxorubicin loaded tubisomes were stable under physiological conditions, with burst release achieved upon UV irradiation. Our finding paves an alternative way for fabricating polymeric tubular structures and expands the toolbox for the rational design of functional hierarchical nanostructures.

Acknowledgements

This work was supported by the Royal Society Wolfson Merit Award (WM130055; S.P.), the European Research Council (TUSUPO647106; S.P.), the National Natural Science Foundation of China (21861130352), the Newton Advanced Fellowships of The Royal Society (NAF\R1\180066), and the Marie Sklodowska-Curie action (TSPBNTM; J.Y.).

Keywords: tubisomes • nanotubes • peptide–polymer conjugates • drug loading • photo-responsive

References

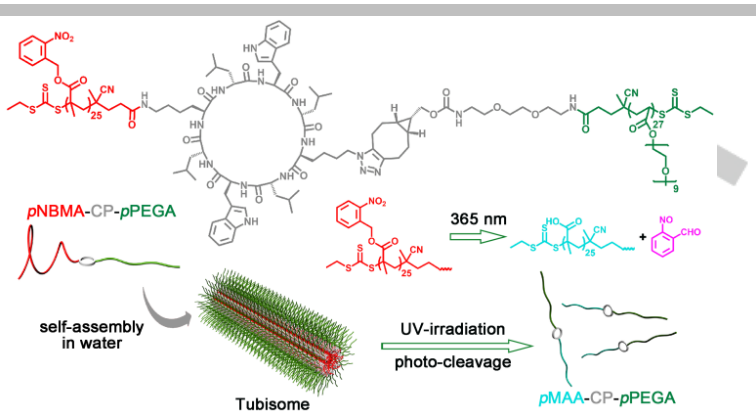
[1] C.-I. Branden, J. Tooze, *Introduction to Protein Structure* edn. 2 (Garland, New York, 1999).
[2] M. Sarikaya, C. Tamerler, A. K.-Y. Jen, K. Schulten, F. Baneyx, *Nat. Materials* **2003**, *2*, 577–585.
[3] J. W. Szostak, D. P. Bartel, P. L. Luisi, *Nature* **2001**, *409*, 387−390.
[4] T. Aida, E. W. Meijer, S. I. Stupp, *Science* **2012**, *335*, 813−817.
[5] Y. Y. Mai, A. Eisenberg, *Chem. Soc. Rev*. **2012**, *41*, 5969−5985.
[6] H.-A. Klok, S. Lecommandoux, *Adv. Mater*. **2001**, *13*, 1217−1229.
[7] M. Elsabahy, K. L. Wooley, *Chem. Soc. Rev*. **2012**, *41*, 2545−2561.
[8] A. Walther, A. H. E. Müller, *Chem. Rev*. **2013**, *113*, 5194−5261.
[9] B. Karagoz, L. Esser, H. T. Duong, J. S. Basuki, C. Boyer, T. P. Davis, *Polym. Chem.* **2014**, *5*, 350−355.
[10] Z. Hudson, C. Boott, M. Robinson, P. Rupar, M. Winnik, I. Manners, *Nature Chem*. **2014**, *6*, 893–898.
[11] M. R. Ghadiri, J. R. Granja, R. A. Milligan, D. E. McRee, N.Khazanovich, *Nature* **1993**, *366*, 324−327.
[12] M. R. Ghadiri, J. R. Granja, L. K. Bühler, *Nature* **1994**, *369*, 301−304.
[13] S. Fernandez-Lopez, H. S. Kim, E. C. Choi, M. Delgado, J. R. Granja, A. Khasanov, K. Kraehenbuehl, G. Long, D. A. Weinberger, K. M. Wilcoxen, M. R. Ghadiri, *Nature* **2001**, *412*, 452−455.
[14] M. Li, M. Ehlers, S. Schlesiger, E. Zellermann, S. K. Knauer, C. Schmuck, *Angew. Chem., Int. Ed*. **2016**, *55*, 598−601.
[15] J. Montenegro, M. R. Ghadiri, J. R. Granja, *Acc. Chem. Res.* **2013**, *46*, 2955−2965.
[16] M. Danial, C. M. N. Tran, K. A. Jolliffe, S. Perrier, *J. Am. Chem. Soc*. **2014**, *136*, 8018−8026.
[17] S. C. Larnaudie, J. C. Brendel, K. A. Jolliffe, S. Perrier, *ACS Macro Lett*. **2017**, *6*, 1347−1351.
[18] J. Y. Rho, J. C. Brendel, L. R. MacFarlane, E. D. Mansfield, R. Peltier, S. Rogers, M. Hartlieb, S. Perrier, *Adv. Funct. Mater.* **2018**, *28*, 1704569−1704579.
[19] S. C. Larnaudie, J. Sanchis, T. H. Nguyen, R. Peltier, S. Catrouillet, J. C. Brendel, C. J. Porter, K. A. Jolliffe, S. Perrier, *Biomaterials*, **2018**, *178*, 570−582.
[20] Q. Song, J. Yang, J. Y. Rho, S. Perrier, *Chem. Commun.* **2019**, *55*, 5291-529.
[21] J. C. Brendel, J. Sanchis, S. Catrouillet, E. Czuba, M. Z. Chen, B. M. Long, C. Nowell, A. Johnston, K. A. Jolliffe, S. Perrier, *Angew. Chem., Int. Ed.* **2018**, *57*, 16678−16682.
[22] J. C. Brendel, S. Catrouillet, J. Sanchis, K. A. Jolliffe, S. Perrier, *Polym. Chem*. **2019**, DOI: 10.1039/c9py00179d.
[23] H. Zhao, E. S. Sterner, E. B. Coughlin, P. Theato, *Macromolecules* **2012**, *45*, 1723–1736.
[24] J. Yang, G. Yu, D. Xia, F. Huang, *Chem. Commun*. **2014**, *50*, 3993–3995.
[25] R. K. Iha, K. L. Wooley, A. M. Nyström, D. J. Burked, M. J. Kade, C. J. Hawker, *Chem. Rev*. **2009**, *109*, 5620−5686.
[26] M. Danial, C. My-Nhi Tran, P. G. Young, S. Perrier, K. A. Jolliffe, *Nat. Commun*. **2013**, *4*, 2780.
[27] D. M. Beal, L. H. Jones, *Angew. Chem., Int. Ed*. **2012**, *51*, 6320−6326.
[28] Y.-C. Hu, Y. Liu, C.-Y. Pan, *J. Polym. Sci Part A: Polym. Chem*. **2004**, *42*, 4862–4872.
[29] N. J. Agard, J. A. Prescher, C. R. Bertozzi, *J. Am. Chem. Soc*. **2004**, *126*, 15046–15047.
[30] M. R. Ghadiri, K. Kobayashi, J. R. Granja, R. K. Chadha, D. E. McRee, *Angew. Chem., Int. Ed*. **1995**, *34*, 93–95.
[31] A. Bansal, Y. Zhang, *Acc. Chem. Res.* **2014**, *47*, 3052– 3060.
[32] I. C. Kwon, Y. H. Bae, S. W. Kim, *Nature 1991*, 354, 291–293.

COMMUNICATION

Jie Yang, Ji-Inn Song, Qiao Song, Julia Y. Rho, Edward DH Mansfield, Stephen C. L. Hall, Megan Sambrook, Feihe Huang and Sébastien Perrier**

_____ Page – Page

Hierarchical Self-assembled Supramolecular Tubisomes for Photo Triggered Drug Release



Hierarchical supramolecular self-assembly of a cyclic peptide functional amphiphilic diblock copolymer into the first photo-responsive tubisomes in water is investigated. These tubisomes are employed as photo-controlled drug delivery vehicles for anticancer drug Doxorubicin to achieve enhanced intracellular DOX release and improved anticancer activity.