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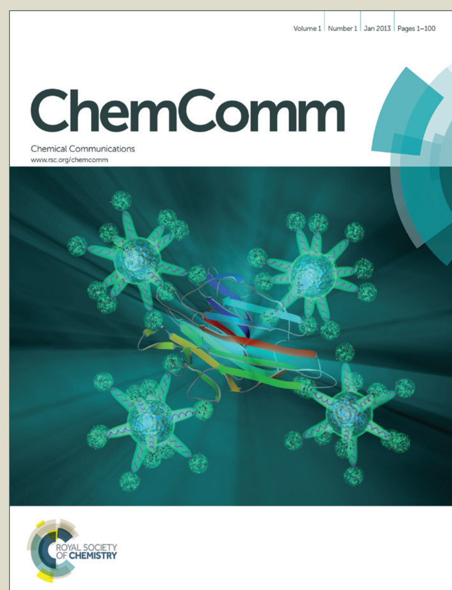

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COMMUNICATION

Enzyme-responsive-based polymer-substituted pillar[5]arene amphiphiles: synthesis, self-assembly in water, and application in controlled drug release

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An enzyme-responsive drug delivery system was constructed from a pillar[5]arene-based polyethyleneglycol-substituted amphiphile which self-assembles into micelles in water. These micelles exhibit superior drug encapsulation capability, and display drug release behaviour in response to enzyme catalysis, in partiular to L-asparaginase. Doxorubicin-loaded micelles show significant cytotoxicity against MCF-7 cancer cells.

Supramolecular assemblies that respond to external stimuli (e.g. pH, temperature, magnetic fields, light, redox processes), including biological initiators, such as glucose or enzymes have received growing attention due to their potential applications as nanocarriers for drug delivery.[1] These responsive drug carriers can be intelligently designed to control drug action in terms of timing, location and dose based on the inherent properties of the target site such as pH, presence of specific enzymes or tissue-specific markers. Up to now, most reported 'smart' assemblies are designed to respond to pH variation, temperature change, light irradiation or their combinations.[2] Apart from these approaches towards the stimuli-triggered drug release, enzyme-responsive assemblies have emerged as an elegant biocompatible method to play a complementary, but important role in this field. The enzyme-catalyzed reactions are highly sensitive and selective even under mild conditions.[3] Although substantial advantages can be achieved by employing enzymes as the triggers, the field of enzyme-responsive systems is still in its infancy stage as compared to extensively investigated conventional stimuli-responsive structures.[3,4] For example, cathepsin B, a lysosomal papain-family cysteine protease is frequently overexpressed in malignant tumors and premalignant lesions at the mRNA and protein level. Cathepsin B is involved in the cellular metabolism responsible for tumour progression and metastasis.[5] Gu and coworkers reported that doxorubicin (DOX) conjugated to PEGylated dendrons through the Gly-Phe-Leu-Gly oligopeptide (GFLG) linker could efficiently deliver DOX into breast tumor 4T1 cells and kill the cells, since the GFLG linker is responsive to the abundant intracellular cathepsin B.[6] The enzyme-stimulus could be a promising therapeutic target for the inhibition of tumor progression and metastasis.

Pillararenes are a kind of macrocyclic hosts with a symmetrical pillar-like architecture and electron-rich inside cavity walls. They have attracted considerable attention since 2008, as they can be easily functionalised by a broad variety of substituents on the benzene rings for different purposes.[7,8] The design and fabrication of macrocyclic pillararenes amphiphiles have attracted more and more interest due to their intriguing topological structures and potential use in the construction of multidimensional and hierarchical assemblies, which are essential for future applications in materials science, biomedicine and molecular electronics.[7e-h] In a previous study, Huang and coworkers reported that one kind of low-molecular-weight macrocyclic amphiphile could successfully encapsulate calcein, which is released in response to decreased pH.[9] Although the aggregates assembled from the low-molecular-weight macrocyclic amphiphiles were demonstrated to possess the ability to release guests, these nanoaggregates could not protect their cargo with long-time stabilization. It should be noted that polymeric assemblies with hydrophilic shells such as poly(ethylene glycol) (PEG) more effectively protect hydrophobic cores against the physiological conditions and extend their retention time in the blood by decreasing non-specific interactions with endogenous components and macrophages.[1e] Macrocyclic pillararenes amphiphiles possess plenty of advantages, such as their facile synthesis, convenient attachment of hydrophilic and hydrophobic chains on the two sides, easy construction of well-defined superstructures and tunable properties toward external stimuli. Here, we report a facile and efficient synthesis of a novel enzyme-responsive delivery system constructed from a pillar[5]arene-based polymer-substituted macrocyclic amphiphile (Fig. 1). This amphiphile self-assembles into micelles in water and encapsulates hydrophobic drug molecules with high loading capabilty. These nanoaggregates dissociate quickly in the presence of amide-cleaving enzymes which cleave off the hydrophilic PEG shell from the pillar[5]arene framework. The specifically enzyme-responsive micellar nanosystems are a promising platform for targeted and efficiently intracellular anticancer drug release.

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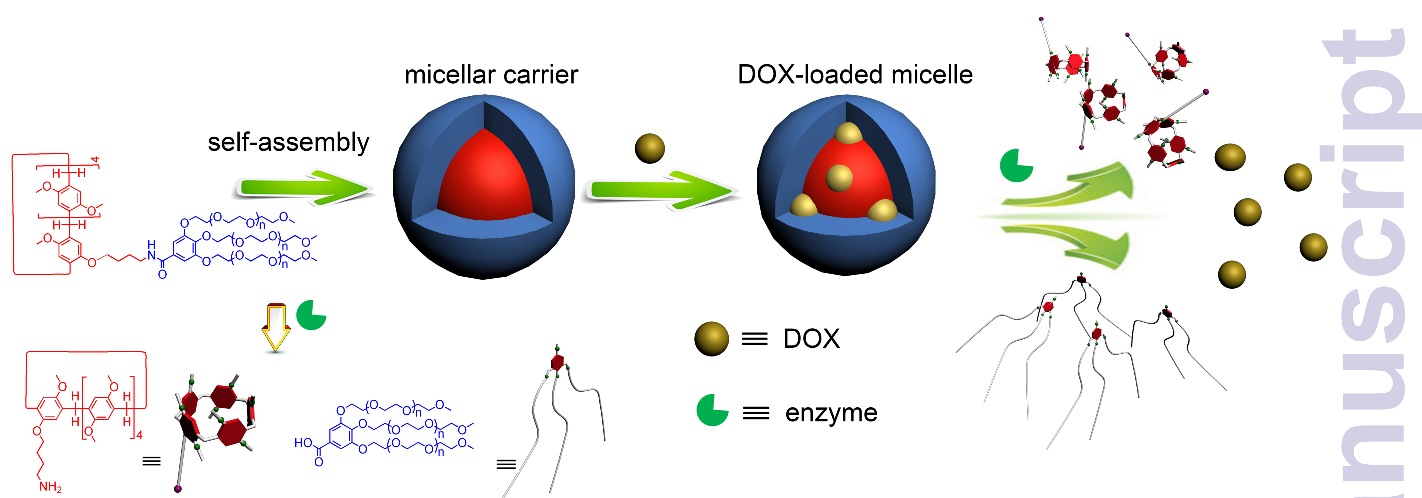
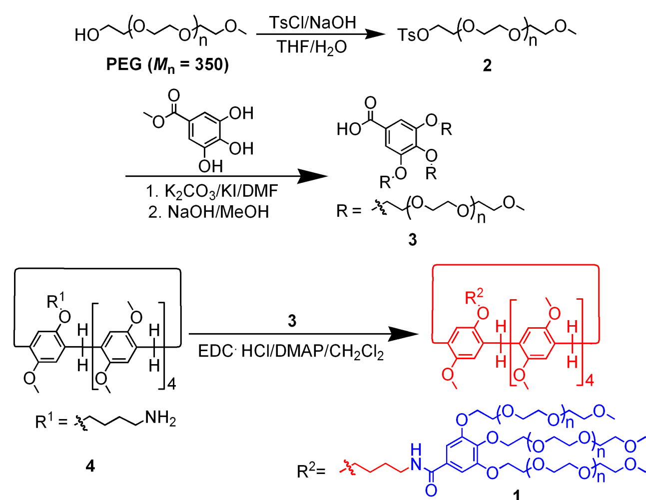


Fig. 1 Schematic representation of the encapsulation of hydrophobic guests in the hydrophobic core of a smart micellar nanocarrier. Upon enzymatic cleavage of the hydrophilic PEG chains, the nanocarrier disassembles and the guest molecules are released.

The synthetic route to amphiphilic pillar[5]arene **1** is shown in Scheme 1. First, the literature-known amino-substituted copillar[5]arene **4** was synthesized.[8d] Next, the PEG chains (M_n = 350, Figs. S1 and S2, ESI†) were added as the solublising groups and hydrophilic compound **3** was obtained. Then **3** and **4** were covalently linked by amide coupling to afford **1** as a yellow oil.[8d]



Scheme 1. Synthesis of the polymer-substituted pillar[5]arene amphiphile under study.

The amphiphilic monomer **1** spontaneously forms micelles **M1** when dissolved in water or aqueous phosphate buffer containing saline (PBS, pH 7.4, 10 mM). The ^{1}H NMR spectrum of **1** in deuterium oxide showed only the proton resonances of PEG segments without any signals of hydrophobic pillar[5]arene, which was mostly due to the formation of core-shell architectures (Fig. S5, ESI†).[3d] The size and morphology of **M1** were characterized by dynamic light scattering measurement (DLS) and transmission electron microscopy (TEM). As shown in Fig. 2a, spherical micelles with diameters of 60–100 nm were observed by TEM. These values are in excellent agreement with the 58-105 nm size distribution obtained from DLS measurements (mean diameter: 78 nm). Furthermore, the assembly of **1** in water was evaluated by DLS derived count rate which indicated that **1** formed micelles with a low CMC in the range of 0.05-0.06 μM (Fig. S6, ESI†). Moreover, it was found that **M1** was stable enough against the external physiological conditions and the presence of 5% fetal calf serum (FBS) for 24 h.

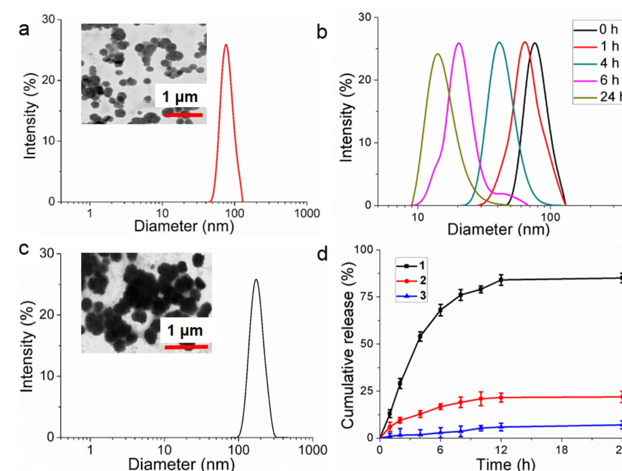


Fig. 2 (a) TEM image and DLS size distribution of **M1**; (b) DLS size distribution of 1 after the addition of L-ASP (0.5 U/mL) at different time (0, 1, 4, 6, 24 h); (c) TEM image and DLS size distribution of **DM1**; (d) *In vitro* release profile of DOX from **DM1** [(1) in the presence of L-ASP (0.5 U/mL), (2) in the presence of BChE (0.5 U/mL) and (3) in the absence of enzyme].

Due to the enzyme-cleavable amide bond in **1**, L-asparaginase (L-ASP), which can catalyse the hydrolysis of asparagine to aspartic acid, is used as a model enzyme to mimic the intracellular cathepsin B to investigate the hydrolysis of amide bond. As shown in Fig. 2b, the DLS study of **M1** revealed the gradual disappearance of the large micellar aggregates in the presence of L-ASP over time. After 24 h, new peaks correlated to

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the smaller sizes of non-assembled PEG chains and the enzyme could be observed. These results demonstrated **M1** to show enzymatic responsiveness ability. The enzyme-responsive disassembly was further supported by direct observation of the solution (sample concentration: 5 mg/mL); a white flocculent precipitate gradually appeared after the addition of L-ASP (the inset photos in Fig. S8b, ESI†). The white precipitate was isolated and examined by ^{1}H NMR spectroscopy which indicated that it was composed of the pillar[5]arene frame (Fig. S7, ESI†) and can be traced back to the enzymatic cleavage of the amide. Finally, the water-insoluble pillar[5]arene precipitated.

Pillararenes possess a hydrophobic cavity that binds a variety of hydrophobic agents in water solution, so that **M1** was anticipated to encapsulate hydrophobic guest molecules within the pillararene cavities. DOX as a hydrophobic chemotherapeutic drug widely used in cancer treatment, was thus chosen as a model drug to investigate the ability of these assemblies to act as drug delivery systems. A DOX solution in DMSO was added to freshly prepared aqueous solution of **M1** and sonicated for 5 minutes to get a clear solution. The unloaded free DOX was removed by glucan gel column chromatography (Fig. S8b, ESI†). DOX was successfully loaded into the interior of the micelles formed by **1** as evidenced by the change of the solution from colorless to dark purple (inset picture in Fig. S8a, ESI†). The DOX-loaded **M1** (**DM1**) were confirmed by UV/Vis measurements, which clearly demonstrated a new broad absorption peak from 426 to 580 nm correlating to the absorption of DOX (Fig. S8a, ESI†). According to the calculation of drug loading content (DLC) and drug loading efficiency (DLE), **DM1** exhibited good DOX loading levels with the DLE ranging from 72.0 to 82.0% at theoretical DLC of 16.7, 33.3 and 50 wt.% (Table S1, ESI†). The morphology and size distribution of **DM1** were characterized by DLS and TEM. As shown by the DLS result given in Fig. 2c, after loading DOX, the average size of the assemblies increased from 78 nm (Fig. 2a) to 200 nm still with a quite narrow size distribution. TEM image showed that the morphologies of **DM1** were spherical particles with diameters of around 100–200 nm (inset picture in Fig. 2c), which was in good agreement with the DLS result above.

As **M1** dissociates when treated with L-ASP, UV/Vis measurements were further carried out to monitor the controlled release behaviour of **DM1** by employing an enzyme under physiological conditions (PBS buffer, pH 7.4). First, we examined the stability of **DM1** in the absence of enzyme. As shown in Fig. 2d, **DM1** was found to be stable with less than ca. 12% of DOX release in 24 h (blue line). As anticipated, DOX was gradually released from the hydrophobic core of the micelles upon their disassembly in the presence of L-ASP, and about 85% DOX was released within 24 h (dark line). Moreover, the enzyme selectivity of the system was evaluated. BChE (a non-specific cholinesterase enzyme that hydrolyses many different choline esters) was introduced into the micellar aqueous solution. From the release profile, we could see that only ca. 22% DOX was released within the time scale (red line), far below that obtained in the presence of L-ASP. This indicates that the loaded drug could only be efficiently released upon the addition of an amidase. These results demonstrate the drug molecules to be preserved in the core at physiological conditions while released quite quickly upon the addition of suitable enzymes.

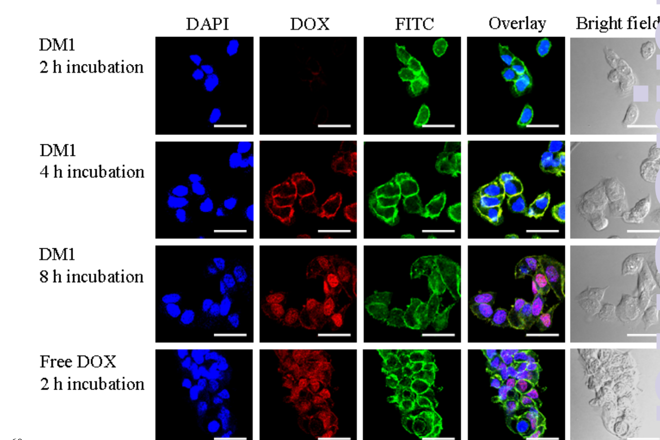

Fig. 3 CLSM images of MCF-7 cells recorded after 2, 4 or 8 h incubation with **DM1** and free DOX (5 μg/mL). The images show for each panel from left to right cell nuclei stained by DAPI (blue), DOX fluorescence in cells (red), early endosome labeled by antibody-FITC (green), and overlays of three fluorescent images and bright field images. The scale bars correspond to 25 μm in all images.

Subsequently, to demonstrate that DOX can be efficiently released from the micelles after cellular uptake and is subsequently internalized into the cell nucleus, we investigated the intracellular DOX release from **DM1** in MCF-7 cells using confocal laser scanning microscopy (CLSM). As shown in Fig. 3, it was found that little DOX could be observed in the cells during the first two hours, which is likely due to the low cellular uptake of nanoparticles with PEG-shielding. At longer incubation times, significant DOX fluorescence was found in the cytoplasm and the perinuclei region of MCF-7 cells. DOX can thus be efficiently released from micelles once they enter into the cells. This is most likely due to the abundant cathepsin B in these cancer cells, which exists not only in perinuclear vesicles and vesicles throughout the cytoplasm, but also at the cell periphery. The amide bond in the micellar system is then cleaved by cathepsin B following cellular uptake and further induced the release of DOX into the nuclei. The localization of DOX in the cell nuclei is crucial because DOX has to intercalate with DNA to induce cell death.

Then, the *in vitro* cytotoxicity of the blank micelles **M1** and DOX-loaded micelles **DM1** were evaluated *via* the MTT assay. As shown in Fig. 4a, the viabilities of MCF-7 and DOX-resistant MCF-7 cells (MCF-7/ADR cells) treated with different concentrations of **1** were over 98% after 48 h incubation, which suggested that **M1** had low cytotoxicity and could be safely used as a biocompatible carrier for drug delivery under specific concentration. The tumor cell toxicity incubated with **DM1** or free DOX was further investigated using MCF-7 and MCF-7/ADR cells. As shown in Fig. 4b, the addition of **DM1** into the cell culture led to a rapid decrease in relative cell viability, and had a low half maximal inhibitory concentration (IC_{50}). Moreover, it was found that **DM1** exhibited much improved cytotoxicity to MCF-7/ADR cells after 48 h culture in comparison with free

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DOX under the same conditions, supporting efficient internalization of **DM1** into MCF-7/ADR cells as well as rapidly intracellular drug release to effectively reverse the drug resistance in MCF-7/ADR cells.

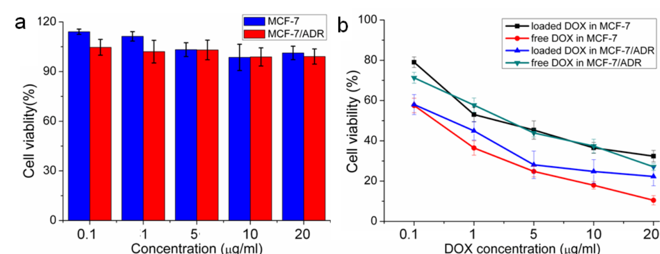

Fig. 4 (a) *In vitro* cytotoxicity of **1** determined by MTT assay against MCF-7 and MCF-7/ADR cells after 48 h incubation; (b) The viability of MCF-7 and MCF-7/ADR cells after being cultured for 48 h with **DM1** (here free DOX was used as the control).

In conclusion, we have successfully prepared a pillar[5]arene-based polymer-substituted macrocyclic amphiphile which self-assembles into enzyme-responsive micelles in water with a low CMC. These micelles had a high loading capability for hydrophobic drugs such as doxorubicin within their interiors and enzyme-triggered fast drug release behaviour in the presence of specific active enzymes, such as L-asparaginase. Moreover, cytotoxicity studies revealed that drug-free micelles were biocompatible with little toxic effect on the MCF-7 and MCF-7/ADR cell proliferation while DOX-loaded micelles exhibited significant cytotoxicity even for the MCF-7/ADR cells.

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† Electronic Supplementary Information (ESI) available: Synthetic procedures, characterizations. See DOI: 10.1039/c0xx00000x.

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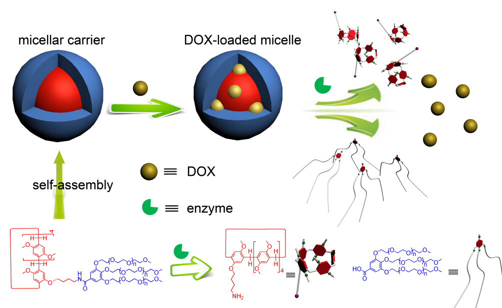

Pillar[5]arene-based PEG-substituted amphiphiles form enzyme-responsive micelles in water useful for drug-delivery.

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