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GSH-Responsive Supramolecular Nanoparticles Constructed by β-D-Galactose-Modified Pillar[5]arene and Camptotecin Prodrug for Targeted Anticancer Drug Delivery

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Supramolecular construction of targeting and stimulti-responsive drug delivery system is still a challenging task. Herein, GSH-responsive supramolecular prodrug nanoparticles were constructed by the host-guest complexation between a β-D-galactose functionalized water-soluble pillar[5]arene (GalP5) and a disulfide bond containing camptothecin prodrug (G). The obtained prodrug nanoparticles were stable under physiological conditions, whereas efficient drug release was triggered in a simulated tumor environment with high GSH concentration. *In vitro* studies revealed that these prodrug nanoparticles preferentially entered asialoglycoprotein receptor over-expressing HepG2 cells due to the active targeting effect of galactose units, resulting in the maximization of anticancer efficacy while reducing the undesirable side effects to normal cells.

Stimuli-responsive drug delivery systems (DDSs) self-assembled from amphiphiles have received tremendous attention in cancer therapy due to various advantages, such as enhanced bioavailability, prolonged blood circulation, improved stability and so on.[1] Traditionally, nanocarriers entered into tumor tissues mainly relied on the enhanced permeability and retention (EPR) effect, which is also called passive targeting.[2a-b] However, many reports have pointed out that the impact of EPR effect is not as homogeneous as it was thought.[2c-d] In contrast to passive targeting, stimuli-responsive drug delivery systems with active targeting ability are able to efficiently reduce the undesirable side effects and systematical toxicity to normal tissues and cells.[3] Based on the unique cellular characteristics of malignant cells, targeting ligands such as peptides, folic acid, vitamins, sugars, and biotin have been adopted and introduced to nanocarriers for active cell-selective drug delivery by receptor-mediated endocytosis.[4] Among various reported targeting ligands, carbohydrates, which are outstanding hydrophilic motif with excellent biocompatibility,[5a] have attracted considerable attention in the development of targeting DDSs due to their unique ability to differentiate and recognize cells and the endocytic uptake resulting from specific carbohydrate-protein interactions.[5b-c] For example, HepG2 cancer cells overexpress asialoglycoprotein (ASGP-R, galactose receptor) which can be efficiently targeted by galactose-functionalized nanocarriers.[2c] Thus, it is highly desirable to develop stimuli-responsive DDSs with active targeting ability that can not only selectively recognize cancer cells, but also can efficiently release the anticancer drugs triggered by the tumor microenvironment.

Supramolecular amphiphiles have the ability to undergo reversible switching of structures, morphologies, and properties in response to various external stimuli[6] due to the non-covalent linkage by relatively weak and dynamic interactions, which endows them outstanding potential applications in fabricating stimuli-responsive DDSs. Pillar[n]arenes,[7] an emerging type of macrocyclic hosts, have been widely applied in constructing various interesting supramolecular systems,[8a-c] especially for fabricating supramolecular DDSs.[8d-f] However, most of these reported DDSs utilizes carboxylate-based pillararenes[8g-h] (**WP5** and **WP6**) as water-soluble hosts to build supramolecular nanocarriers. In order to enrich the pillar[n]arene family, improve their biocompatibility, and endow them with active targeting abilities, we intend to introduce β-D-galactose group to pillar[5]arene scaffold for targeting drug delivery. Furthermore, prodrug nanocarriers with drugs covalently conjugated to the carriers are especially promising due to neglectable drug leakage, high-loading capacity, and improved pharmaceutical

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† Electronic Supplementary Information (ESI) available: Experiments details, NMR spectra *etc*. See DOI: 10.1039/x0xx00000x

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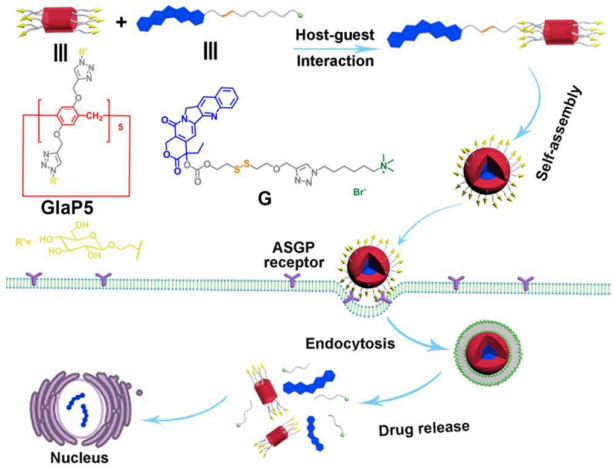
properties.[9] Herein, a targeting drug delivery system with glutathione (GSH)-responsiveness was rationally designed based on the host-guest interaction between a biocompatible *β*-D-galactose-modified water-soluble pillar[5]arene (**GalP5**) and a disulfide bond-containing camptothecin (CPT) prodrug (**G**) (Scheme 1). The formed supra-amphiphile **GalP5⊃G** could further self-assemble into supramolecular prodrug nanoparticles, and the anticancer drug could be efficiently released in a simulated tumor environment with high GSH concentration. Significantly, *in vitro* studies demonstrated that these prodrug nanoparticles exhibited excellent targeting ability to preferentially enter ASGP-R over-expressed HepG2 cells and led to significant drug accumulation in cancer cells *via* receptor-mediated endocytosis. Therefore, the therapeutic efficacy for cancer cells was retained, but the side effects to normal cells were remarkably reduced. This study provides a novel strategy for the construction of stimuli-responsive supramolecular DDSs with active targeting abilities, which may have great potential applications in cancer therapy.



Scheme 1. Schematic illustration of the construction of supramolecular prodrug nanoparticles and their applications in targeted intracellular drug delivery.

β-D-Galactose-based water soluble pillar[5]arene (**GalP5**) was synthesized by means of classic Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction between alkyne-substituted pillararene **1** and azido-functionalized galactose **2** in good yield (Scheme S1, ESI†). The CPT prodrug **G** was obtained by a four-step synthesis to introduce disulfide bonds and trimethylammonium groups as responsive units and binding sites, respectively (Scheme S2, ESI†). Although **G** was very difficult to dissolve in water, but with the presence of **GalP5**, distinct solubilization effect was observed. Moreover, the ^{1}H NMR spectroscopy of **GalP5⊃G** complex showed obvious negative field signals (Fig. S20, ESI†), indicating the binding behavior occurred between **GalP5** and **G**. In order to clearly investigate the host-guest interactions, a model compound $\mathbf{G_M}$ was synthesized for investigating the complexation of **GalP5⊃G**. Fig. 1 presented the ^{1}H NMR titration results of **GlaP5** with $\mathbf{G_M}$ in D_2O. It was found that all the resonance signals of $\mathbf{G_M}$ shifted upfield remarkably upon adding **GalP5** due to the inclusion-induced shielding effects. Simultaneously, the protons of H_1 and H_2 on **GalP5** exhibited slight downfield chemical shifts in the presence of $\mathbf{G_M}$. These results suggested that $\mathbf{G_M}$ was fully threaded into the hydrophobic cavity of **GalP5** to form a pseudorotaxane structure. Moreover, 2D NOESY experiment further confirmed the above conformation of such inclusion complex (Fig. S21, ESI†).

Subsequently, the stoichiometry of the **GalP5⊃$\mathbf{G_M}$** complex was further investigated by Job's plot method and the result showed a 1:1 binding stoichiometry (Fig. S22, ESI†). The association constant of **GalP5⊃$\mathbf{G_M}$** was further determined to be (1.22 ± 0.40) × 10^3 M^{-1} by using ^{1}H NMR titration (Fig. S23, ESI†). Combining the compound characteristic, we deduced the binding affinity for such **GalP5⊃G** inclusion complex might be mainly driven by the cooperative hydrophobic and CH-π interactions, which lead to the formation of a stable 1:1 amphiphilic inclusion complex.

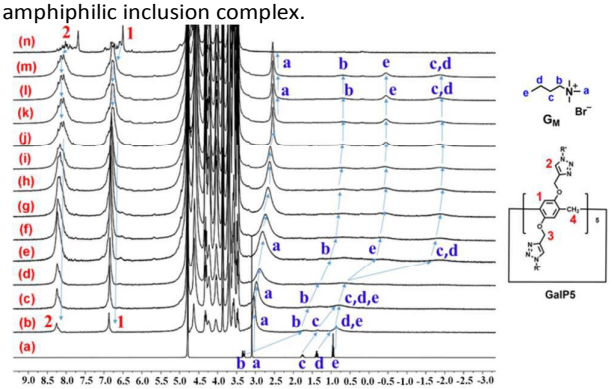


Fig. 1 ^{1}H NMR spectra (400 MHz, D_2O, 298 K) of $\mathbf{G_M}$ at a constant concentration of 4.0 mM with different concentrations of **GalP5** (mM): (a) 0.0, (b) 1.0, (c) 2.0, (d) 3.0, (e) 4.0, (f) 5.0, (g) 6.0, (h) 7.0, (i) 8.0, (j) 9.0, (k) 10.0, (l) 11.0, (m) 12.0, and (n) **GalP5** (4.0 mM).

The ability of such a supra-amphiphile to form higher-order supramolecular aggregates in water was further investigated. Initially, Dynamic light scattering (DLS) was utilized to estimate the self-aggregation behavior of free **G** in aqueous solution (8 × 10^{-5} M, containing 1 % DMSO), the results showed almost no measurable signal, indicating that no large-sized aggregates assembled under the examined conditions. However, when equimolar of **GalP5** was added to the above **G** solution, a light opalescence and clear Tyndall effect could be observed (Fig. 2a), suggesting the formation of abundant nanoparticles. Based on this host-guest molar ratio, the critical aggregation concentration (CAC) for **GalP5⊃G** was determined to be 6.17 × 10^{-6} M by measuring the concentration dependent optical transmittance of the solution (Fig. 2b). Subsequently, zeta-potential measurements showed that **GalP5⊃G** solution possessed positive ζ-potential (28.6 mV, Fig. S24, ESI†), meaning that the repulsive forces among nanoparticles exist, and the stability of the nanoparticle solution could be manifested.

The morphology and size of these aggregates were further investigated by transmission electron microscopy (TEM) and DLS. DLS results showed that, the aggregates formed by

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GalP5⊃G have an average hydrodynamic diameter of 217 nm (Fig. 2a). Meanwhile, TEM images indicated a spherical morphology with the diameters range from 130 nm to 210 nm, indicating that they formed spherical nanoparticles with a well-defined surface (Fig. 2c & 2d).

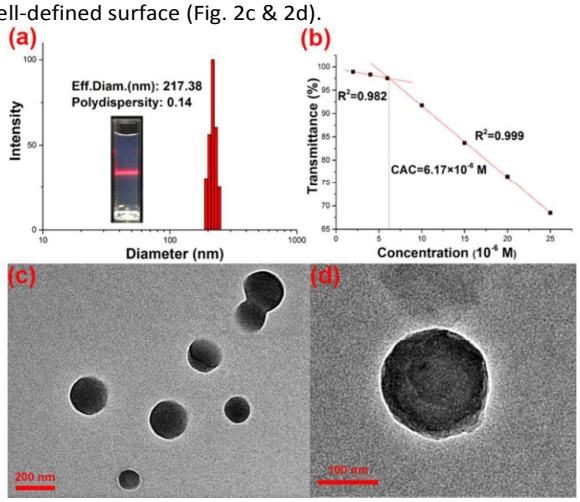

Fig. 2 (a) DLS data of the **GalP5⊃G** aggregates, Inset: Tyndall effect of **GalP5⊃G** solution; (b) Concentration dependent optical transmittance at 410 nm of the **GalP5⊃G** solutions with fixed molar ratio of [**GalP5**]/[**G**]=1:1; TEM images: (c) **GalP5⊃G** aggregates; (d) enlarged image of c.

It is well known that the disulfide bond was very sensitive to GSH,[10a] which might result in a favorable CPT release profiles from **GalP5⊃G** prodrug nanoparticles. Thus, the drug release profiles of **GalP5⊃G** nanoparticles under different GSH concentrations were evaluated. A control experiment without GSH showed negligible CPT leakage even for one month, suggesting these prodrug nanoparticles are very stable under physiological condition. In contrast, CPT was released rapidly in the presence of GSH. As shown in Fig. 3a, increasing the GSH concentration from 2 to 10 mM could trigger a gradual enhancement of cumulative CPT release from 55.4% to 87.1% within 200 min. Meanwhile, no nanoparticles could be detected from the TEM image (Fig. S25, ESI†). Moreover, MS and HPLC analysis of the above **GalP5⊃G** solution treated with GSH (10 mM) further verified the release of free CPT (Fig. 3b & c). The maximal drug loading efficiency and drug loading content were calculated to be 19.3% and 98.2%, respectively,

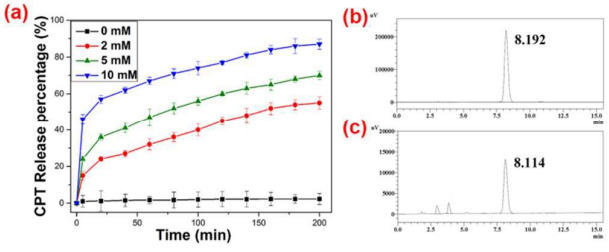

Fig. 3 (a) Time-dependent CPT release efficiency of **GalP5⊃G** nanoparticles with the presence of different concentrations of GSH; HPLC analysis of (b) pure CPT and (c) **GalP5⊃G** nanoparticles treated with GSH (10 mM) for 3 h.

which is particularly significant for developing efficient DDSs with high-loading capacity. In addition, the stability of **GalP5⊃G** prodrug nanoparticles in different medium was investigated by measuring their size changes based on DLS experiments (Fig. S26 & S27, ESI†). The results showed that these prodrug nanoparticles were stable in PBS and MEM cell cultrue medium, but they were not very good in fetal bovine serum, probably because pillararene-based drug nanocarriers could strongly bind to some metal ions that widely exist in fetal bovine serum.[11]

Subsequently, MTT assays were performed to evaluate the cytocompatibility for both **GalP5** and **GalP5⊃G** nanoparticles against MRC-5 normal cells. As shown in Fig. 4a, negligible influence on the relative cell viability was observed in the presence of **GalP5** at the tested concentrations ranging from 5 to 80 μM. With respect to the prodrug nanoparticles, although the relative cell viability was gradually reduced with increasing analytes concentrations, the cell viability in each treatment was still above 80%. The above results indicated low cytotoxicity for these supramolecular prodrug nanoparticles.

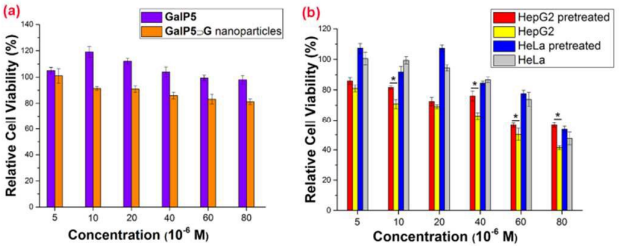

Fig. 4 Cytotoxicity of **GalP5** and **GalP5⊃G** prodrug nanoparticles against (a) MRC-5 cells and (b) HepG2 cells and HeLa cells with or without pretreatment with free galactose, respectively after 24 h incubation (*$p < 0.05$).

To confirm the presumed targeting specificity of galactose-decorated **GalP5⊃G** nanoparticles via ASGP-R receptor-mediated mechanism, HepG2 cells with a high ASGP-R expression level and HeLa cells with less expression of ASGP-R were used to investigate the anticancer efficiency. Meanwhile, HepG2 and HeLa cells pretreated with free galactose (a well-known inhibitor of ASGP-R) were used as negative controls.[10b] The relative cell viability in different groups was recorded after incubation for 24 h. As shown in Fig. 4b, the relative viability of HepG2 cells without pretreatment were always much lower than HeLa cells. Moreover, the cytotoxicity of **GalP5⊃G** nanoparticles towards HepG2 cells decreased by pretreatment with free galactose to block the receptors. However, no obvious changes of cell viability were observed for HeLa cells with or without pretreatment due to the lack of ASGP-R. In addition, based on the results in Figure 4b and Table S1 (ESI†), the IC50 values of **GalP5⊃G** nanoparticles for HepG2 and HeLa cells were calculated to be 50.5 and 87.9 μM, respectively. These results revealed that **GalP5⊃G** prodrug nanoparticles could efficiently targeted HepG2 cells through ASGP-R mediated interactions.

The ASGP-R mediated endocytosis was further verified by confocal laser scanning microscopy (CLSM) toward HepG2 cells.

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Initially, HepG2 cells with or without pretreatment by galactose were incubated with **GalP5⊃G** prodrug nanoparticles for 1 h and 4 h, respectively. As shown in Fig. 5, the blue fluorescence of CPT could be detected after 1h incubation, demonstrating that **GalP5⊃G** prodrug nanoparticles were successfully internalized by HepG2 cells. Upon extending the incubation time to 4 h, HepG2 cells showed intense intracellular blue fluorescence, indicating the efficient intracellular CPT accumulation with extended culture time. Furthermore, strong purple fluorescence that overlapped with the lysosome-labeled fluorescence was observed, suggesting the co-localization of **GalP5⊃G** prodrug nanoparticles with lysosomes. In contrast, HepG2 cells that were preincubated with free galactose showed very weak intracellular CPT fluorescence after 1 h and even 4 h incubation. Based on the above results, we concluded that **GalP5⊃G** prodrug nanoparticles could selectively target ASGP-R receptor over-expressed HepG2 cells via receptor-mediated endocytosis, and then efficiently released anticancer drug to kill cancer cells.

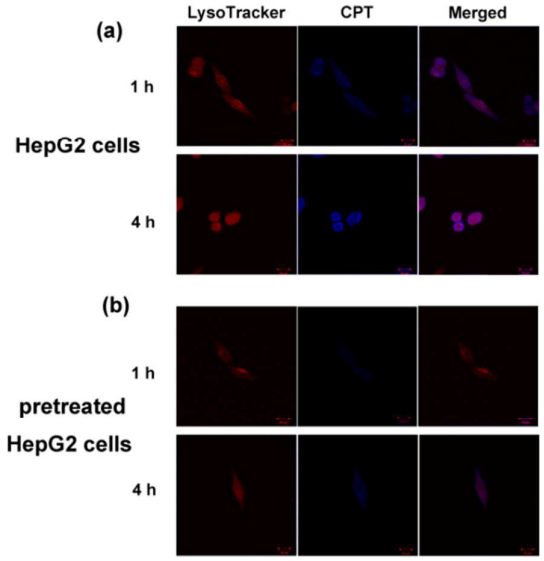

Fig. 5 CLSM images: (a) HepG2 cells incubated with **GalP5⊃G** prodrug nanoparticles (10 μM) for 1 h and 4 h, respectively; (b) HepG2 cells pretreated with free galactose for 15 min and then incubated with **GalP5⊃G** prodrug nanoparticles for 1 h and 4 h, respectively. (The scale bars correspond to 20 μm).

In summary, we have successfully developed a novel targeting drug delivery system based on the host-guest interaction between a *β*-D-galactose functionalized water-soluble pillar[5]arene (**GalP5**) and a CPT-based prodrug (**G**). The formed supramolecular amphiphile **GalP5⊃G** could form stable supramolecular nanoparticles under physiological conditions, but rapid CPT release could be achieved with the presence of high GSH concentration. Cytotoxicity experiments further revealed that **GalP5⊃G** prodrug nanoparticles could be selectively recognized by HepG2 cells and then efficiently kill cancer cells via receptor-mediated endocytosis, thus the side effects to normal cells were remarkably reduced. Significantly, CLSM experiments proved that these nanoparticles could lead to significant drug accumulation in HepG2 cancer cells. This study paves an alternative way to construct supramolecular targeting prodrug nanoparticles with stimuli-responsiveness, which have great potential applications in the fields of targeted drug delivery.

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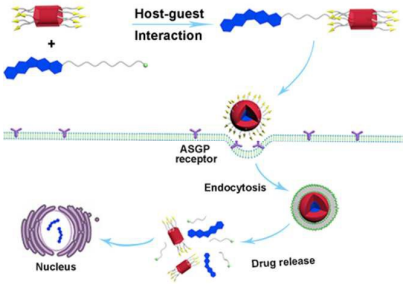

GSH-Responsive Camptothecin-based supramolecular prodrug nanoparticles with galactoce pendants were fabricated, which showed excellent targeting ability to HepG2 cancer cells.

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