ORIGINAL RESEARCH

A multifunctional supramolecular vesicle based on complex of cystamine dihydrochloride capped pillar[5]arene and galactose derivative for targeted drug delivery

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Background: Supramolecular vesicles are a novel class of nanocarriers that have great potential in biomedicine.

Methods: A multifunctional supramolecular vesicle (CAAP5G) based on the complex of CAAP5 and galactose derivative (G) assembled via host-guest interaction was constructed.

Results: Using Human embryonic kidney T (293T) cells as experimental models, the cytotoxic effects of CAAP5G was investigated to 0–50 μmol/L for 24 h. Notably, the CAAP5G vesicles revealed low-toxicity to 293T cells, it was critical to designing drug nano-carriers. Simultaneously, we have evaluated doxorubicin hydrochloride (DOX)-loaded CAAP5G vesicles anticancer efficiency, where DOX-loaded CAAP5G vesicles and free DOX incubated with Human hepatocellular carcinoma cancer cell (HpeG2 cells) and 293T cells for 24 h, 48 h, 72 h. It turned out that CAAP5G vesicles encapsulated anticancer drug (DOX) could decrease DOX side-effect on 293T cells and increase DOX anticancer efficiency. More importantly, the cysteamine as an adjuvant chemotherapy drug was released from CAAP5G vesicles in HepG2 cells where a higher GSH concentration exists. The adjuvant chemotherapy efficiency was evaluated, where free DOX and DOX-loaded CAAP5G vesicles incubated with DOX-resistance HepG2 cells (HepG2-ADR cells) for 24, 48, 72 h, respectively.

Conclusion: The results revealed that the DOX encapsulated by CAAP5G vesicles could enhance the cytotoxicity of DOX and provide insights for designing advanced nano-carriers toward adjuvant chemotherapies.

Keywords: supramolecular vesicles, cysteamine, responsive, adjuvant chemotherapies, targeted drug delivery

Introduction

Drug resistance of cancer cells and side effects to normal cells are major problems in cancer chemotherapy.[1–3] Nano-carriers (NC) are one of the most widely explored drug carriers in biomedicine with its distinctive properties.[4,5] Especially, targeted and stimuli-responsive nano-carriers (TSNC) can target cancer cells,[6–9] achieve a rapid release in the specific tumor microenvironments, enhance cytotoxicity and reduce adverse effect via new concepts or strategies based on nanotechnology.[10–13] Vesicles have been one of the most popular TSNC due to their unique cavity that have the potential to encapsulate and controllably release drugs in response to

specific stimuli.[14–16] Among them, supramolecular vesicles from amphiphilic supra-molecules via host-guest complexation, especially with targeted and stimuli-responsive units, are very important in the application of biotechnology and biomedicine.[17–20] Therefore, the construction of targeted and stimuli-responsive vesicles from multifunctional amphiphilic supra-molecule based on host-guest interaction between macrocyclic host molecule and guest molecule is of particular interest and importance in the progress of stimuli-responsive systems for targeted drug delivery.

Pillar[n]arenes, a new class of macrocyclic molecule, have received much attention in supramolecular chemistry.[21–26] Due to their intrinsic symmetrical and rigid structure, accessible modification and excellent properties in host-guest chemistry, pillar[n]arenes have been widely used to construct various interesting supramolecular vesicles for TSNC.[27–31] For example, our group developed two types of multifunctional supramolecular vesicles, by using ferrocene or tryptophan capped pillar[5]arene, which exhibited good responsiveness and significantly enhanced the cytotoxicity of DOX.[32,33]

The cystamine dihydrochloride (**CA**), an important pharmaceutical intermediate that contained a disulfide bond (sensitive to GSH) and amino groups (sensitive to pH), was investigated.[34–36] Most importantly, **CA** can produce the drug cysteamine in the presence of GSH for adjuvant chemotherapy.[37] Previous studies showed that the cysteamine not only inhibited the formation of gastric[38] and mammary tumors[39] that were induced chemically or after irradiation,[40,41] and also enhanced the cytotoxicity of DOX.[42] To the best of our knowledge, **CA** modified pillararene for constructing a dual-responsive amphiphilic host molecule have not yet been reported. We hypothesized that synthesis of **CA** capping pillararene would not only lead to a GSH-pH dual responsive amphiphilic host molecule, but also the cysteamine produced by the **CA** could enhance the cytotoxicity of DOX for adjuvant chemotherapy.

In this study, a **CA**-capped pillar[5]arene (**CAAP5**) amphiphilic host molecule was first synthesized, and thereafter the multifunctional supramolecular vesicles (**CAAP5G**) constructed from **CAAP5** and a galactose derivative guest molecule (**G**) based on host-guest interaction for targeted drug nanocarriers (Scheme 1), where the galactose residue on one end of **G** can act as the targeting ligand to asialoglycoprotein receptor (ASGP-R) overexpressing HepG2 cells via carbohydrate-protein interactions.[43]

Material and methods
Instrumentation and materials

All chemical reagents were of analytical reagent grade and used without further purification unless specified. Doxorubicin hydrochloride was purchased from Sangon Biotech. [1]NMR spectra were recorded on a Bruker 500 MHz Spectrometer, with working frequencies of 500 MHz for ^{1}H and 125 MHz for ^{13}C nuclei, respectively. Scanning electron microscope (SEM) images were obtained using an S-4800 instrument (Hitachi Ltd.) with an accelerating voltage of 10.0 kV. Negative-stained TEM images were recorded on an H-7650 instrument (Hitachi Ltd. 80 kV). Dynamic light scattering (DLS) measurements were performed on a Delsa[TM] Nano system (Beckman Coulter, U.S.A.). Water surface tension was recorded with a BZY-3B surface tension measurer (China). An AXIMA-CFR™ plus MALDI-TOF Mass Spectrometer (Kratos, UK) was used for mass analysis. UV-Vis spectra were recorded with a Shimadzu 1750 UV-Visible spectrophotometer (Japan) at 298 K. HepG2 cells and HepG2-ADR cells were obtained from the Type Culture Collection of the Chinese Academy of Science (Shanghai, China). 293T cells were obtained from the KeyGEN BioTECH Co. (Nanjing, China). Cell culture was carried out in an incubator with a humidified atmosphere of 5% CO_2 at 37 °C.

Methods
The preparation and characterization of the vesicles

2.46 mg (0.5 μmol) of **CAAP5** was dissolved in H_2O (0.5 mL) and stirred for 5 min. The solution was mixed 0.5 mL **G** (1 mM). The final mixture was ultrasonicated for 30 min, then left to stand for 6 h to obtain the vesicles of **CAAP5G**, which were characterized by SEM, DLS, and TEM, respectively.

DOX loading and release of CAAP5G vesicles

2.46 mg (0.5 μmol) of **CAAP5** was dissolved in H_2O (0.5 mL) and stirred for 5 min. To the solution were mixed 0.5 mL **G** (1 μmol) and 0.58 mg DOX (1 μmol). The mixture was ultrasonicated for 30 min and left to stand for 6 h, then subjected to dialysis (molecular weight cutoff 8,000–14,000) in distilled water. DOX-loaded vesicles were obtained when no DOX detected in the water outside the dialysis tube.[44]

The DOX release from DOX-loaded **CAAP5G** was studied at different pH buffer solution, with or without
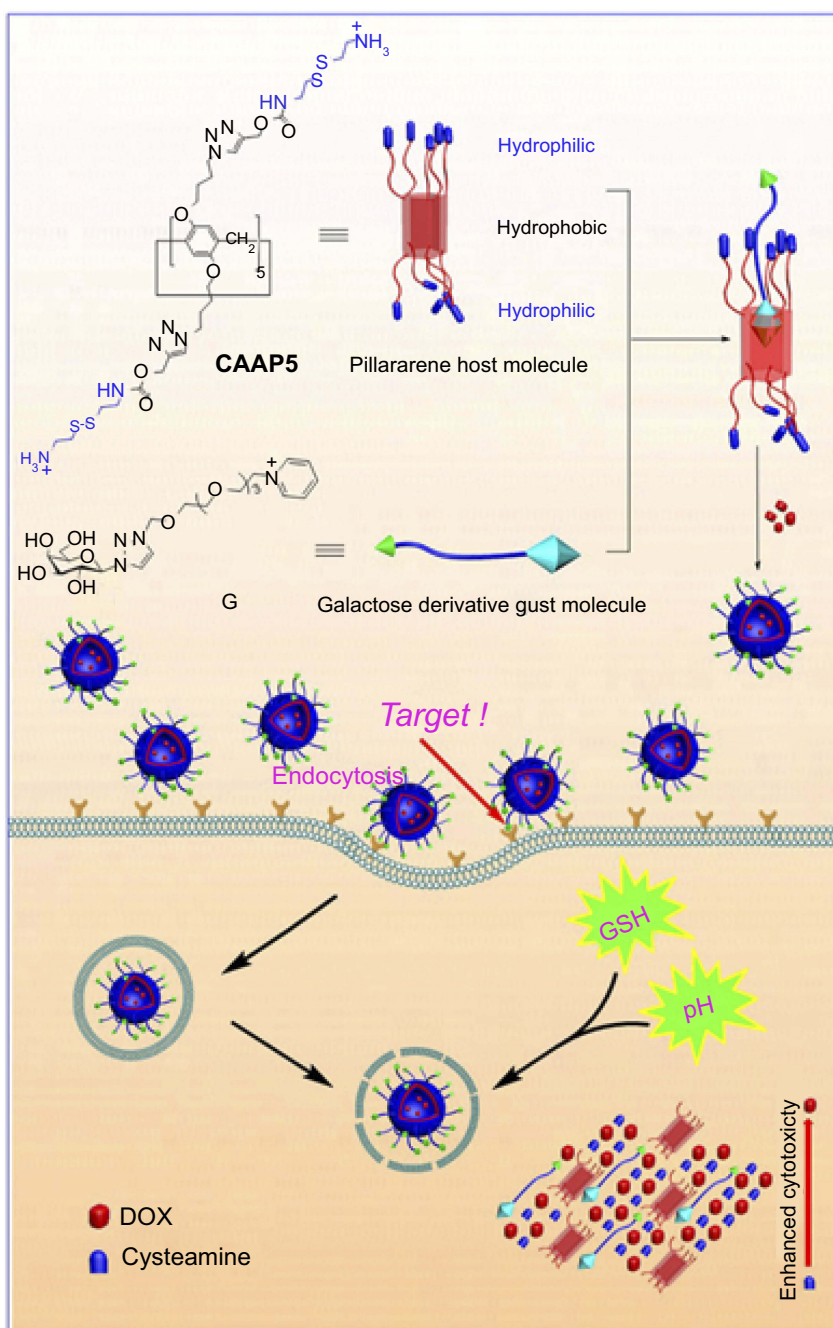
Scheme 1 Schematic illustration of the synthesis of **CAAP5**, the host-guest complexation with galactose derivatives (**G**), formation of vesicles (**CAAP5G**), and their GSH/pH dual-responsive drug release.

GSH (10 mM). 1 mL of DOX-loaded vesicles in a dialysis bag was added into 10 mL of corresponding release medium at room temperature. Within specified time intervals, 0.1 mL of the release medium was taken out for measuring the DOX released with an Epoch microplate spectrophotometer (Biotek).

Cell culture and cell viability

HepG2 cells and HepG2-ADR cells were cultured in RPMI 1640 containing 1% penicillin-streptomycin, 10% FBS, in a humidified atmosphere (5% CO_2) at 37 °C. 293T cells were cultured in DMEM medium containing 1% penicillin-streptomycin, 10% FBS, in a humidified atmosphere (5% CO_2) at

37 °C. The relative cytotoxicity of unloaded vesicles, DOX and DOX-loaded vesicles were evaluated in *vitro* by MTT assay, respectively. MTT assay procedures refer to our previous work.[44]

Results and discussion

To acquire **CAAP5**, azido-pillar[5]arene **H5** and alkynyl cystamine derivative **H4** were synthesized according to a published procedure, respectively.[45,46] The **CAAP5** can be synthesized through Cu-catalyzed azido-alkynyl click reaction. The synthetic details and characterization of **CAAP5** and **G** can be found in the Supporting Information (Schemes S1–S3, Figures S1–S11).

The host-guest interaction of **CAAP5** and **G** was investigated in D_2O via the ^{1}H NMR spectroscopy. The up field chemical shifts of the pyridine protons (H_{1-3}) of **G** were observed due to the shielding effect of the electron-rich cavities of **CAAP5** toward **G** (see in Figure S12). This result was consistent with that published,[21,32,33] demonstrating the pyridine moiety of **G** has been successfully included into the hydrophobic cavity of **CAAP5**.

To construct the targeting dual-responsive supramolecular vesicles (**CAAP5G**) based on the **CAAP5** and **G**

host-guest interaction, an aqueous solution of **CAAP5** and **G** was sonicated for 30 min. We observed a clear Tyndall effect (Figure 1A right), indicating the existence of abundant nanoparticles, which were further identified by scanning electron microscopy SEM (Figure 1B). TEM image demonstrated the cavity of the vesicles, which was used to loading-drug (Figure 1C). Further analysis with DLS showed that the average diameter of the vesicles was 158 nm (Figure 1D). The critical aggregation concentration (CAC) was found to be 22 μM by the water surface tension method (Figure S14).

In the following study, DOX was selected as a model drug to investigate the drug release behavior and encapsulation efficiency of the supramolecular vesicles. As showed in Figure 1A (left), a clear Tyndall effect was observed with DOX-loaded **CAAP5G** aqueous solution. DLS showed that the average diameter of the DOX-loaded **CAAP5G** vesicles was 203 nm (Figure S13), which were further identified by SEM (Figure 2A). The **CAAP5G** encapsulation efficiency was calculated 41 wt% by UV-vis spectroscopy. Subsequently, the DOX-loaded **CAAP5G** vesicles released DOX was studied in an acidic solution (pH 4.0), neutral solution (pH 7.4), and with GSH
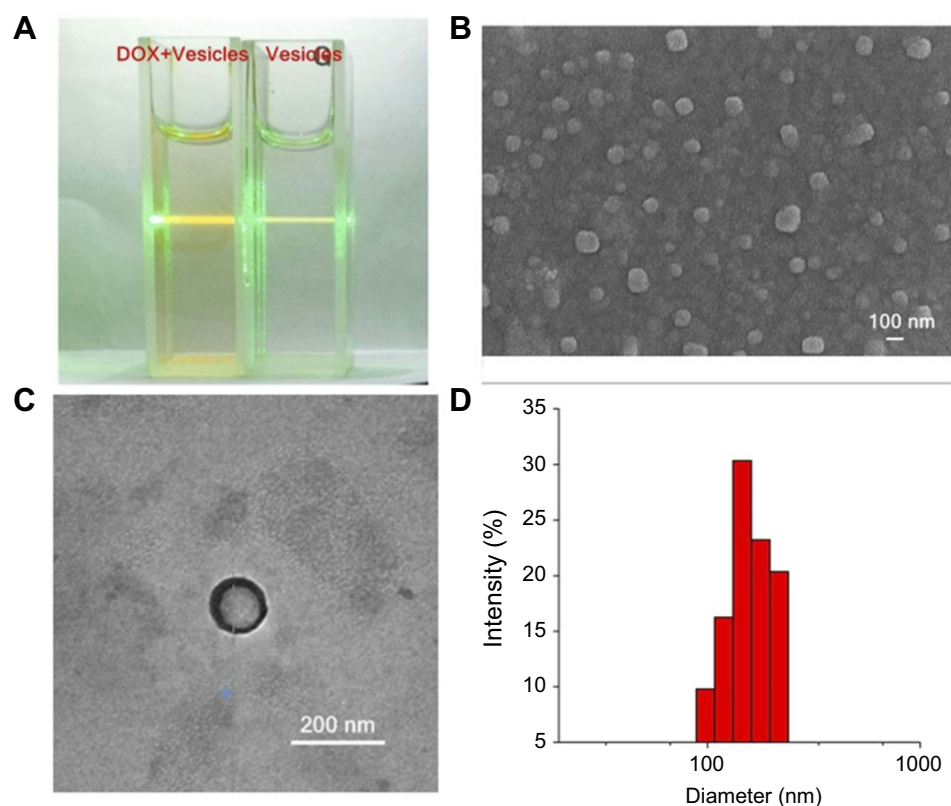

Figure 1 (A) Tyndall effect of **CAAP5G** and DOX-loaded **CAAP5G**; **(B)** SEM image of **CAAP5G** vesicles; **(C)** TEM image of **CAAP5G** vesicles; **(D)** DLS histogram of **CAAP5G** vesicles.
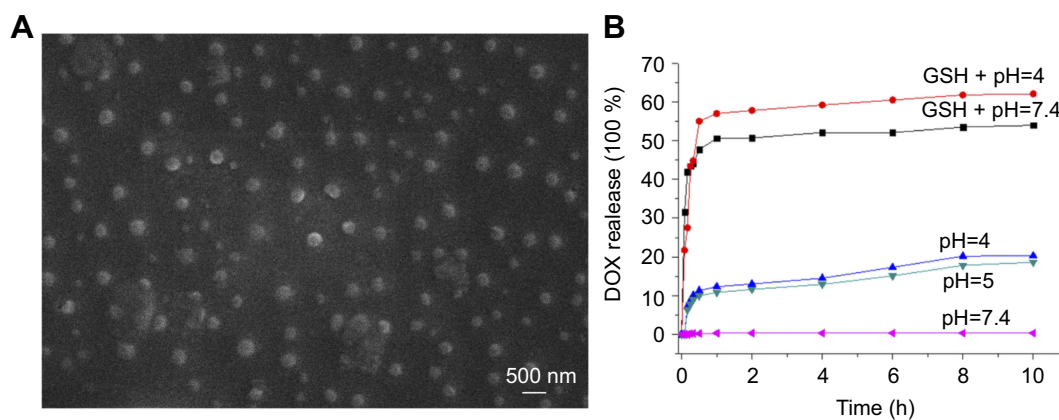
Figure 2 (A) SEM image of DOX-loaded **CAAP5G** vesicles; (**B**) DOX release profiles from DOX-loaded **CAAP5G** vesicles in PBS with GSH (10mM) + pH (4.0), GSH (10 mM)+ pH (7.4), pH (4.0), pH (5.0) and pH (7.4) (n=3).

(10 mM) in solution (pH 7.0) and acidic solution (pH 4.0), respectively, mimicking the concentration of GSH in cancer cells (1–11 mM). As indicated in Figure 2B, 20% and 54% of DOX were released under acidic conditions (pH 4.0) and 10 mM GSH, respectively. In the acidic buffer solutions, **CAAP5** functional groups -NH$_2$ were ionized to -NH$_3^+$, leading to a greater quantity of positive charges, which changed the **CAAP5G** hydrophilic/hydrophobic ratio and further lead to the rupture of vesicles to release drugs. As a result, the solution in the presence of GSH had a quicker release rate. By comparison, the amount of DOX release reached a maximum of 62% when cultured with

GSH (10 mM) in solution (pH4.0), indicating that GSH and pH dual-responsiveness mediated efficient disassembly of DOX-loaded vesicles. These results show that DOX-loaded **CAAP5G** vesicles are relative stable under physiological condition and can be used for drug controllable release.

The cellular uptake of DOX-loaded vesicles was investigated by CLSM. Red fluorescence could be observed clearly in the nuclei of HepG2 cells after 4 h incubation with DOX-loaded **CAPP5G** vesicles, indicating that DOX loaded by **CAPP5G** vesicles could enter the cancer cells (Figure 3A). To evaluate the targeting effect of **CAAP5G** vesicles, HepG2
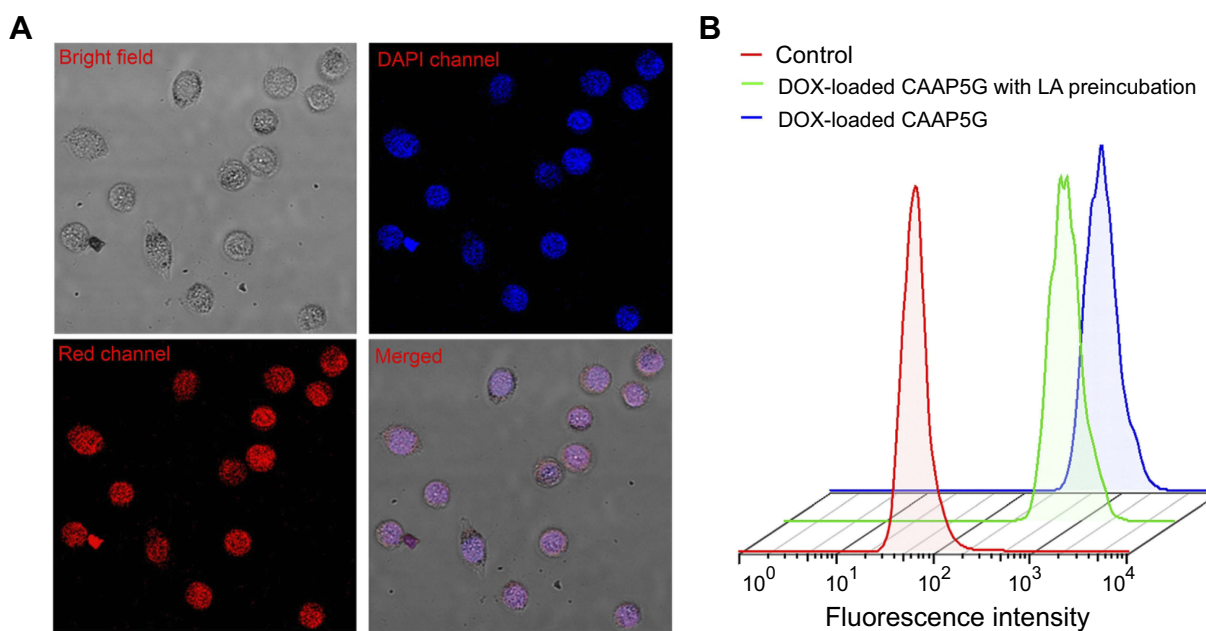

Figure 3 (A) CLSM images of HepG2 cells after being treated with DOX loaded **CAAP5G** vesicles for 4 h. The final concentration of DOX in the medium was 5 μM. (**B**) Fluorescence intensity of HepG2 cells treated with free DOX (red line), DOX-loaded **CAAP5G** vesicles (blue line), DOX-loaded **CAAP5G** after pre-incubation with lactose for 4 h (green line) by flow cytometry analysis, respectively.

cells were incubated with DOX-loaded **CAAP5G** vesicles and free DOX at 5 μM for 4 h, respectively, where the efficiency of cellular uptake was analyzed by flow cytometry. HepG2 cells pre-cultured with lactose (2 mg/mL) for 4 h, were used to culture with DOX-loaded **CAAP5G** vesicles for comparison. As showed in Figure 3B, the fluorescence intensity of HepG2 cells cultured with DOX-loaded **CAAP5G** vesicles (blue curve) was higher than that of DOX-loaded **CAAP5G** with lactose pre-incubation (green curve), indicating a higher uptake of DOX-loaded **CAAP5G** vesicles. The results imply that **CAAP5G** displays hepatoma-targeting ability. In addition, the cell toxicity of unloaded vesicles to 293T cells are evaluated by the MTT cell survival assay. As indicated in Figure S15 (ESI†), the relative cell viability of 293T cells cultured with unloaded vesicles was over 82% in the concentrations of 50 μM **CAAP5G** after 24 h, showing that the vesicles had low cell toxicity to 293T cells, which was essential for the design of drug nano-carriers.

The anticancer efficiency of DOX-loaded **CAAP5G** vesicles was evaluated using MTT cell survival assay, where HpeG2 cells and 293T cells were incubated with free DOX and DOX-loaded **CAAP5G** vesicles for 24, 48, and 72 h, respectively. As indicated in Figure 4A, in all three tested periods, the relative viabilities of HepG2 cells after cultured with DOX-loaded **CAAP5G** vesicles were visibly lower than those with free DOX. By comparison, 293T cells showed higher relative viabilities than those with free DOX (Figure 4A). The results indicated that the encapsulation of DOX by **CAAP5G** vesicles could enhance the drug anticancer efficiency for HepG2 cells while effectively reduce the side effects to 293T cells.

In the presence of GSH, CAAP5G vesicles can produce the cysteamine, an adjuvant chemotherapy drug, that enhances the cytotoxicity of DOX.[42] To study the adjuvant chemotherapy efficiency of **CAAP5G** vesicles, HepG2-ADR cells were cultured with free DOX and DOX-loaded **CAAP5G** vesicles for 24, 48, and 72 h, respectively (Figure 5). The results showed that the relative viabilities of HepG2-ADR cells after incubation with DOX-loaded **CAAP5G** vesicles at all three tested time periods were visibly lower than those with free DOX. It indicated DOX-loaded **CAAP5G** vesicles could enhance anticancer efficiency of HepG2-ADR cells comparison with free DOX, suggesting **CAAP5G** as a multifunctional supramolecular vesicle can be used in adjuvant chemotherapy.

Conclusion

In conclusion, novel multifunctional supramolecular vesicles (**CAAP5G**) based on the host-guest complexation of **CAAP5** and **G** have been successfully constructed and the good drug-loading capability was demonstrated by drug loading experiments. Furthermore, DOX-loaded **CAAP5G** not only exhibited excellent dual stimuli responsiveness and rapid release of DOX in cancer cells but also possesses targeting ability to ASGP-R overexpressing HepG2 cells. Most notably, the vesicles under high concentration of GSH would release cysteamine, which could enhance the anticancer efficiency of DOX and reduce the drug resistance of cancer cells. This work provides a new idea for rational design in the construction of targeted and stimuli-responsive nanocarriers for drug delivery, which have great applications for adjuvant chemotherapy.
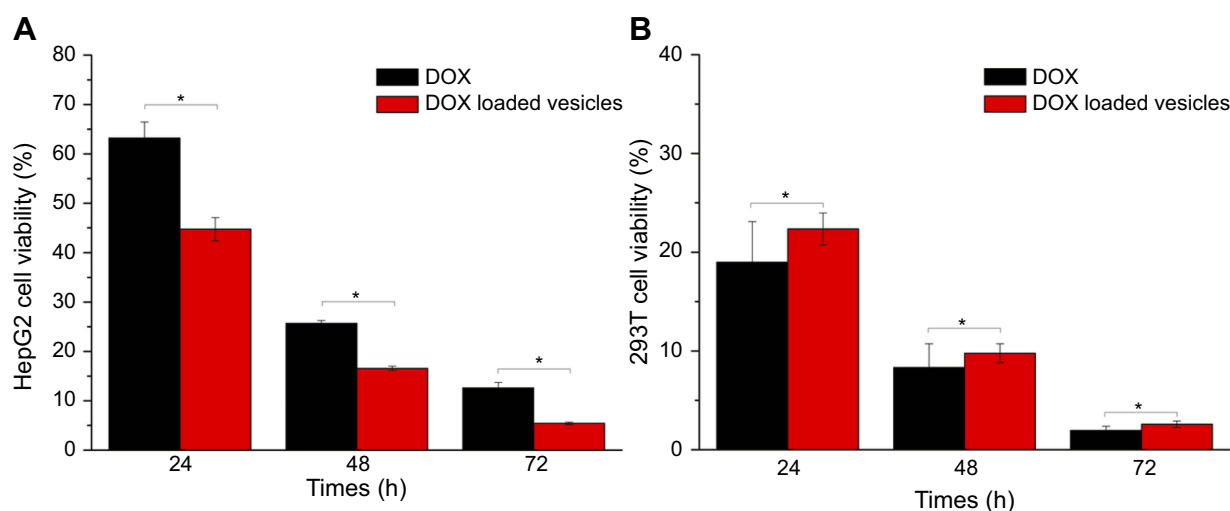

Figure 4 Comparison of DOX-loaded **CAAP5G** vesicles and DOX on viabilities of HepG2 cells (**A**) and 293T cells (**B**) at different time periods; the concentration of DOX was 5 μM. Statistically significant differences were observed (*p<0.05).
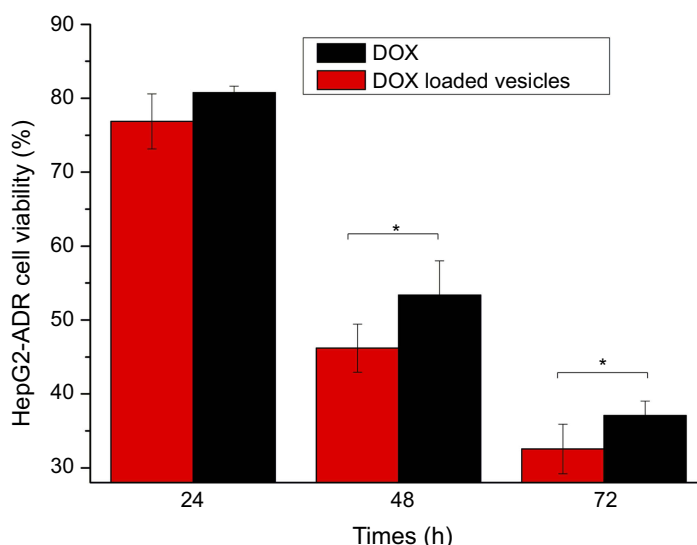
Figure 5 Comparison of DOX-loaded **CAAP5G** vesicles and DOX on viabilities of HepG2-ADR cells at different time periods; the concentration of DOX was 5 µM. Statistically significant differences were observed (*p<0.05).

Acknowledgments

Disclosure

The authors report no conflicts of interest in this work.

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