Dual-Responsive Mechanized Mesoporous Silica Nanoparticles Based on Sulfonatocalixarene Supramolecular Switches

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Mesoporous silica nanoparticles (MSNs) have been functionalized with supramolecular switches, composed of cleavable disulfide bond-containing alkylammonium stalks encircled by water-soluble sulfonatocalix[4,6]arenes, to result in smart mechanized MSNs. Cargo can be encapsulated tightly in the nanocavities of these mechanized MSNs in their closed state, but are released efficiently either in response to L-glutathione (GSH), by cleaving the disulfide bonds in the stalks, or in response to pH variation, by turning on the calixarene-based supramolecular switches. The higher concentration of GSH in cancer cell cytosol and the relatively lower pH value of cancer cell lysosome can simultaneously activate the mechanized MSNs, enabling them to release the pre-loaded cargo in place. This efficient use of the different environments of cancer cells and normal healthy cells can enhance the targeting effect of delivery vehicles and effectively lower the side effects of delivered anti-cancer drugs. In vitro cytotoxicity tests suggest good biocompatibility and low toxicity of these newly developed drug-delivery systems.

Over the last couple of decades, porous materials with excellent physical and chemical features, including large surface area, robust storage ability, tunable pore volume, and known functionalization strategies, have attracted much attention owing to their potential applications in the fields of sensing, biomedicine, and catalysis. Mesoporous silica nanoparticles (MSNs) have shown to be a promising type of drug carriers in nanomedicine and, and, significantly, the surface-functionalized, end-capped versions of MSNs have been well demonstrated to be ideal nanocarriers for regulating the on-command release of drugs and genes. In this regard, materials grafted on the surfaces of MSNs include polymers, magnetic nanoparticles, gold nanoparticles, pillararenes, crown ethers, cucurbiturils, cyclodextrins, antibodies, and so on. Meanwhile, a range of external triggering motifs including temperature, pH changes, ultrasound, enzyme, redox, competitive binding and light have been employed to activate the mechanized MSNs to release the loaded cargo.

Recently, calixarenes as the third generation of synthetic macrocyclic receptors, especially water-soluble sulfonated calix[n]arenes, have been widely used in various fields such as self-assembly membranes, materials science, and separation technology due to their good water solubility and unique host-guest properties. Sulfonatocalixarenes are excellent hosts for some positively charged organic or inorganic molecules in aqueous solutions and possess many advantages in supramolecular assemblies and molecular recognition. They can also encircle some typical stalks on the surfaces of MSNs by host-guest interactions, acting as gatekeepers in the motif of systemsb ased on biocompatible sulfonatocalix[4,6]arenes, SC[4]A, SC[6]A-alkylammonium supramolecular switches. And their performances of encapsulating and release of cargo molecules in response to GSH or pH change have also been investigated (Scheme 1). In these systems, alkylammonium stalks anchored onto the pore orifices of MSNs via cleavable disulfide bonds were encircled by SC[4]A/SC[6]A as gates to seal the mesopores after cargo loading through host-guest interactions. On one hand, redox disulfide bonds can be cleaved by relatively high concentration of GSH. On the other hand, host-guest interactions can be weakened by changing pH, subsequently allowing the loaded cargo to diffuse out. Thus, the dual-responsive mechanized MSNs can be obtained, which are promising candidates for diagnostics and treatments of cancer diagnostics and treatments, taking advantage of the inherent environments in cancerous cells as stimuli is a more preferable method to regulate drug release. Incorporating disulfide linkages into drug release systems is a promising approach because the cleavable disulfide bonds can be reduced by high concentration of L-glutathione (GSH) in the cytosol. The pH value is another important factor to distinguish normal healthy cells and cancer cells, which can also be used as a stimulus in mechanized MSNs.

Herein, we have fabricated novel supramolecular nanocarrier systems based on biocompatible sulfonatocalix[4,6]arenes (SC[4]A and SC[6]A)-alkylammonium supramolecular switches. And their performances of encapsulating and release of cargo molecules in response to GSH or pH change have also been investigated (Scheme 1). In these systems, alkylammonium stalks anchored onto the pore orifices of MSNs via cleavable disulfide bonds were encircled by SC[4]A/SC[6]A as gates to seal the mesopores after cargo loading through host-guest interactions. On one hand, redox disulfide bonds can be cleaved by relatively high concentration of GSH. On the other hand, host-guest interactions can be weakened by changing pH, subsequently allowing the loaded cargo to diffuse out. Thus, the dual-responsive mechanized MSNs can be obtained, which are promising candidates for diagnostics and treatments of can-

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cers for the following reasons: 1) the disulfide bonds can provide sensitive cleavage sites for cargo delivery in cancer cells by the significantly higher GSH level in cancer cell cytosol (10 mM) compared with that in the bloodstream (2–20 μM);[24] 2) the gate-keepers, that is, SC[4]A/SC[6]A, of the mechanized MSNs would be separated from the stalk components as triggered by acidic pH inside lysosome to achieve controlled intracellular delivery; 3) the dual-responsive nanovalves are able to decrease the undesired pre-cargo delivery before reaching cancerous cells and reduce the detrimental side effects of external stimuli; 4) different loading capacity and release amount of cargo in these mechanized MSNs can be regulated by employing water-soluble sulfonatocalixarenes with different cavity sizes.

Results and Discussion

We firstly prepared the scaffold MSNs by means of the frequently used template-directed sol–gel method according to our published procedure.[10a,13a] Tetraethylorthosilicate (TEOS) was used as silicon source with cetyltrimethylammonium bromide (CTAB) as template to form pore structures under alkalesscent conditions. Then, after removal of CTAB by extracting MSNs with hot acidified methanol, the monodisperse MSN-OH nanoparticles with homogeneous particle sizes were obtained as evidenced by scanning electron microscopy (SEM) (Figure 1a). Next, MSN-OH materials were treated with 3-mercaptopropyl-trimethoxysilane (MPTS) to introduce hydrosulphonyl onto their surfaces to get the reductive MSN-SH, which was further covalently surface-functionalized through reacting with s-(2-aminoethylthio)-2-thiopyridine hydrochloride[26] (M1) to result in MSN-SS.[27] Finally, smart cargo delivery systems were fabricated after loading with rhodamine (Rh6G) dye and capping with negatively charged supramolecular macrocycles, that is, SC[4]A and SC[6]A, which were synthesized (see the Supporting Information, SI) according to a modified procedure on the basis of previous literature reports.[28]

The synthesized mesoporous silica materials were fully characterized by SEM, transmission electron microscopy (TEM), Brunauer–Emmett–Teller (BET) and small-angle powder X-ray diffraction (PXRD). Mono-dispersed and homogeneous MSN-OH and MSN-SH nanoparticles with an average particle size of about 56 nm were observed in the SEM images (Figures 1a and 1b). Ordered 2D hexagonal array of cylindrical nanopores in the original nanoparticles was also evidenced by TEM (Figure 1c). Furthermore, as shown in Figure S5 (see the SI), PXRD patterns of freshly obtained MSN-OH exhibited standard Bragg peaks of (100), (110), (200), reflecting a highly ordered 2D hex-
agonal array. In addition, the characteristic Type IV BET adsorption isotherm (Figure S6 in the SI) also demonstrated the existence of ordered mesopores in the newly prepared MSN-OH. The surface-functionalized nanoparticles (MSN-SH and MSN-SS) still maintained their original sizes and morphologies even though the mesopores were not that clear compared with bare MSN-OH (Figures 1d and 1e). However, a fuzzy pore structure appeared in the SEM image of the Rh6G-loaded, SC[4]A-capped MSN-SS, which can be ascribed to the installation of stalks that covered on the pore outlets of MSNs and the replenished Rh6G in the nanopores.

The successful preparation of functionalized mesoporous silica materials was also further confirmed by Fourier transform infrared (FTIR) spectroscopy. As shown in Figure 2a, the strong absorption signals of 1084 and 935 cm\(^{-1}\) were corresponding to the asymmetric stretching of Si–O–Si bridges and skeletal vibration of C–O bond stretching. The thiol units on the surfaces of MSN-SH can be detected by the thiol absorption band at 2563 cm\(^{-1}\) in its FT-IR spectrum. The disappearance of the thiol absorption and the appearance of additional stretching vibration of \(-\text{NH}_2\) bending (1597 cm\(^{-1}\)) in the spectrum of MSN-SS (Figure 2b) demonstrated that small molecule M1 was coupled with MSN-SH by covalent bonds. The typical absorption peak of aromatic ring (1500 cm\(^{-1}\)) presented in Figure 2c proved that the stalks on MSN-SS have been successfully encircled by calixarene macrocycles. Comparing MSN-SS with MSN-OH, a weight loss of about 4.93% in the thermal gravimetric analysis (TGA) spectra (Figure S8) suggested that 0.47 mmolg\(^{-1}\) of small molecules (M1) were successfully immobilized onto the surfaces of MSNs. Besides, the successful functionalization was also verified by elemental analysis, as the percentages of N and S elements increased remarkably after surface immobilization (Table S3 in the SI).

The release of luminescent dye Rh6G, a probe molecule due to its optical properties, from the dye-loaded MSNs materials with and without the attachment of calixarenes was investigated by tracking the changes of UV absorption intensity along with time by UV/Vis spectroscopy (Figure 3 and Figures S2 and S11). The release capacity and the relative release percentage of the dye-loaded, sulfonatocalixarene-capped MSN-SS were calculated based on competitive binding method (in the SI). Intriguingly, the loading percentages of dye molecules in two dye-loaded, sulfonatocalixarene-capped MSNs, calculated by measuring the UV/Vis absorption of the supernatants (in the SI), were different, resulting from the different binding affinity of the two stalks. The relatively stronger binding endowed SC[4]A with macrocycles (SC[4]A and SC[6]A) towards alkylammonium more prominent ability to prevent the cargo from escaping from the mesopores, thus decreasing the undesired premature cargo release more efficiently. On the other hand, the dye-loaded MSNs without attaching any calixarene caps showed the lowest loading percentage and the most obvious premature leakage (Figure S11), indicating the important role of sulfonatocalixarene-based supramolecular switches in our system that effectively trapped the cargo inside the nanopores and prevented premature leakage of cargo.
To investigate the gating performance of the calixarene nanovalves triggered by GSH, release experiments were conducted in PBS solution (pH 7.4) with and/or without the addition of GSH. As shown in Figure 3, in the PBS solution (pH 7.4) without addition of GSH, that is, a similar GSH environment extracellular fluids or bloodstream in normal tissues, there was no premature release over a long time of 14 h, confirming that SC[4]A (and SC[6]A rings) capped on the surfaces could block the pores and prevent Rh6G within the pores against adverse leaching out. However, when the concentration of GSH was increased to 10 mM, a sudden growth of the release curves of the mechanized MSNs (Figure 3) was detected, indicating that GSH could trigger remarkable cargo release by successfully reducing the cleavable disulfide bonds in the stalks. Besides, comparing the release behaviour of these two systems, the SC[4]A-capped nanovalve showed a slightly lower premature release of dyes in the absence of GSH but larger effective release percentage in the presence of GSH, which can be ascribed to the higher loading capacity of Rh6G resulting from the stronger binding affinity of SC[4]A towards the stalks (see the SI). As is known, electrostatic interactions are the primary driving forces in the complexation of sulfonatocalixarenes and amino acids (or dipeptides). Similarly, the pattern of the complexation between sulfonatocalixarenes and the alkylammonium stalks containing amino groups is that the positively charged alkylammonium electrostatically interacts with the negatively charged sulfonate groups on sulfonatocalixarenes. However, size-fit (or induced size-fit) is another important factor governing host-guest binding in supramolecular chemistry. Compared with SC[4]A, SC[6]A exhibits less efficient binding affinity towards the alkylammonium stalks due to the bigger cavity and flexibility, making SC[6]A relatively loose binding pocket for the alkylammonium stalk.

In acidic pH (5.0), mimicking the lysosomal pH within cancer cells, the amount of cargo released showed a minor increase in the smart SC[4]A-based and SC[6]A-based nanovalve systems compared with that in neutral environment (Figure 3). The release of dyes under acidic conditions can be attributed to the fact that the binding affinities between sulfonatocalixarenes and the alkylammonium stalks anchored on the surfaces of the MSNs were largely influenced by the changes of pH values in external environment. Other studies also suggested the consistent result that the association constant between sulfonatocalixarenes and guest molecules with alkylammonium cations decreases at lower pH.

It is worth mentioning that the fastest release rates of both two smart mechanized MSNs were successfully achieved in the condition that GSH and pH dual-stimuli were utilized at the same time (Figure 3).

As mentioned above, sulfonatocalixarenes could bind to amino acids and dipeptides through electrostatic interactions, and GSH, as a peptide, may also bind to sulfonatocalixarenes. To better understand that GSH cleaves the disulfide bonds of the stalks, leading to subsequent opening of the nanovalves, S-methylglutathione (GSMe)-triggered response of the nanovalves was testified. GSMe was used in the control experiments instead of GSH, because it has almost the same structure as GSH, but no reducing thiol groups. In Figure 3, under the influence of GSMe the mechanized MSNs materials showed just a little higher release amount than that system without addition of GSMe. As compared with GSH activations, GSMe leads to a negligible/insignificant release. This is because GSH or GSMe, being a tri-peptide, has two carboxylate groups and one amino group, showing a net negative charge even at pH 5.0, due to its relatively low isoelectronic point of 2.86, which prevents its binding towards negatively charged sulfonatocalixarenes under our experimental conditions. We can conclude that the release of Rh6G from the mechanized MSNs upon addition of GSH in high concentrations could be the result of the removal of the cap by cleaving the disulfide bond, rather than by binding of the sulfonatocalixarenes to GSH preferably over binding to the stalks.

All these experimental results demonstrated that Rh6G-loaded SC[4,6]A-capped MSN-SS materials are capable of GSH- and pH- cooperatively triggered intracellular cargo delivery. And they could avoid undesired premature release of drugs before being endocytosed by cancer cells.

To detect if our obtained nanomaterials can be used in bio-systems, it is necessary to investigate their cytotoxicity. The cytotoxic effects of MSN-SS and SC[4]A-capped MSN-SS on cells were evaluated with A549 cells by performing an MTT assay. Figure 4 represents a 24 h incubation of A549 cells with two kinds of nanoparticles at different concentrations. The two materials showed negligible cytotoxicity in the lung cancer cells within the examined concentration range. The cellular viabilities were able to remain at high levels (higher than 70 %), and the concentration was as high as 200 μg/mL after incubation for 24 h. From the results of the cytotoxicity study, we could conclude that the newly obtained sulfonatocalixarene-based mechanized MSN materials possess very low cytotoxicity, showing great potential for dual stimuli-responsive targeted drug delivery.
Conclusions
Overall, we have shown that enquiring alkylammonium stalks installed on MSN surfaces via cleavable disulfide bonds with water-soluble sulfonatocalixarenes to form supramolecular switch-based mechanized MSNs is a feasible approach not only to trap cargo in the mesopores against adverse leaking but also to release them in response to GSH and pH variations. The low cytotoxicity, confirmed by MTT assay, allows them to be utilized as smart drug carriers in bio-systems. Their good biocompatibility and dual-stimuli responsiveness make them perfect gated nanoreservoirs that can be used in drug delivery within tumour tissues possessing a high concentration of GSH and an acidic pH. SC(4)A- and SC(6)A-based delivery systems exhibited negligible premature cargo release but different cargo release percentages as a consequence of different host-guest binding affinities. The biocompatible mechanized MSN systems presented herein would be promising candidates as effectively multifunctional smart scaffolds for on-demand drug delivery in cancer therapy.

Experimental Section
Materials
The starting materials and reagents were purchased from Aladdin, J&K Chemicals, and Sigma–Aldrich, and used as received. A range of phosphate buffers (PBS buffers) used in the release experiments were prepared beforehand according to the Appendix XV of the Chinese Pharmacopeia (Second Part, 2010 Edition).

Methods
TEM images were obtained on a JEM-2100F instrument (Japan), with an accelerating voltage of 200 kV. SEM images were recorded on a Hitachi SU8020 electron microscope at 20 kV. Gold coating of the nanoparticles prepared for preferable imaging was performed beforehand by sputtering for about 120 s. PXRD experiments were performed using a Rigaku Smart Lab III powder diffractometer. FT-IR spectra were recorded on a Bruker Vertex 80 V spectrometer. TGA was carried out on a TA Q500 instrument in high-resolution mode with a temperature ramp of 10 °C min⁻¹ to 800 °C under a flow of dry synthetic air in an alumina pan. The radiation source was copper (1.39225 Å). Nitrogen adsorption-desorption isotherms were obtained on a Micromeritics Gemini instrument. Specific surface areas were determined from the adsorption data in the low-pressure range through BET model. Pore sizes were calculated using the Barrett–Joyner–Halenda (BJH) method (see the SI). The controlled-release profiles were collected by means of UV/Vis spectroscopy on a Shimadzu UV-2550 spectrophotometer.

Cell Viability Assay
The in vitro cytotoxicity test was performed using an MTT assay with lung cancer A549 cells. Cells during the logarithmic phase of growth were seeded onto a 96-well at a density of 5 × 10⁴ cells per well. After 24 h of cultivation, these cells were incubated with different concentrations of MSN-SS and SC(4)A-capped MSN-SS materials at 37 °C for 24 h in 5 % CO₂. Then, an MTT solution (10 μL, diluted in culture medium to a concentration of 1 mg mL⁻¹ in PBS) was added to each well (20 mL per well, 5 mg mL⁻¹) and the cells were incubated for another 24 h. The resulting culture medium was discarded and DMSO (150 μL) was added to each well. The absorbance of MTT was confirmed at 490 and 630 nm by using an automated microplate reader (BioTek, ELX808). A culture medium without any addition of nanomaterials was used as the blank control test. The results were expressed as an average percentage of the cell viability over three nominally, as compared with the blank control.

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Keywords: cytotoxicity · disulfide bonds · glutathione · mesoporous silica nanoparticles · sulfonatocalixarenes

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