Drug Delivery

Acetylcholine-Triggered Cargo Release from Supramolecular Nanovalves Based on Different Macrocyclic Receptors

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Parkinson’s disease (PD), a degenerative disorder of the central nervous system, affects millions of people, especially those past the age of 55.[1] The motor symptoms of PD result from the death of dopamine-generating cells in the substantia nigra. So far, the best treatment for PD is dopamine replacement therapy, which can ameliorate dyskinesia and dystonia effectively, but the side-effects of the treatment on the motor system are troublesome and have a significant impact on quality of life.[3] Consequently, an effective therapy that minimizes these side-effects is urgently needed. Acetylcholine (ACh) plays an important role as a neurotransmitter by restoring impaired functions to a normal and healthy operational level. Pathological changes occur to the substantia nigra in the midbrain region of PD patients, that is, damage occurs to dopaminergic nigrostriatal neurons that reduces their ability to synthesize acetylcholinesterase (AChE) inhibitors. Consequently, the concentration of ACh in the synapses of PD patients will increase remarkably compared with normal people.[5] If we can take advantage of these characteristics of PD patients, we may find a new way to treat PD.

Over the past decade, much research on drug delivery systems incorporating mesoporous silica nanoparticles (MSNs) and supramolecular macrocycle-based nanovalves (or nanocaps) has been carried out.[4] MSNs can be a solid support for supramolecular nanovalves, and have been considered to be superior nanocontainers for drug loading and controlled release, due to their good biocompatibility, robust storage ability, tunable pore size, and known functionalization procedures.[4, 5] To reduce premature cargo release before nanovalve activation, a variety of materials have been grafted onto the surface of MSNs and the valve tightness has been fine-tuned. Meanwhile, a series of external stimuli, including pH changes,[4b–d, 6a, 7] enzyme activation,[4a, 8] light,[6b–c, g, 9] temperature,[10] and competitive binding,[6c–g, 7c, 8b] have been employed to operate the nanovalves and release probe molecules from the pores of MSNs. Different kinds of synthetic macrocycles can encircle the guest stalks on MSNs by host–guest interactions to serve as the moving element of gating macrocycles. Although this proof-of-concept study is far from a real-life application, it provides a possible route to treat diseases related to the central nervous system.

**Abstract:** Acetylcholine (ACh), a neurotransmitter located in cholinergic synapses, can trigger cargo release from mesoporous silica nanoparticles equipped with calixarene- or pillararene-based nanovalves by removing macrocycles from the stalk components. The amount and speed of cargo release can be controlled by varying the concentration of ACh in solution or changing the type of gating macrocycle. Although this proof-of-concept study is far from a real-life application, it provides a possible route to treat diseases related to the central nervous system.
rocycles and stalk components (or ACh), demonstrating that not only robust competitive binding can open up the nanovalves but also the relatively weaker competitive binding of ACh towards macrocycles (as compared with the stalks on the MSN surfaces) can realize the release of cargo when ACh reaches a certain excess level. Hence, this methodology is expected to broaden the application of competitive binding methods in triggering nanovalve-based controlled cargo release.

The scaffold MSNs were synthesized according to a modified literature procedure. MCM-41 nanoparticles were prepared using a template-directed sol–gel method and the monodisperse nanoparticles with homogeneous particle sizes were obtained as evidenced by SEM (Figure 2 e). 3-Chloropropyl trimethoxysilane was reacted with MCM-41 nanoparticles to achieve 3-chloropropyl-modified MCM-41 nanoparticles (MSN-Cl). Upon reaction with pyridine in DMF, pyridine-modified MSNs (MSN-Py) were obtained, then the final drug delivery systems were achieved after loading MSN-Py with Rh6G and capping it with macrocycles (the detailed description can be found in the Supporting Information). The morphology study of the MSNs before and after functionalization was done by transmission electron microscopy (TEM). Figure 2 f clearly shows the morphology of MSN-Cl, which is spherical in shape with a particle size around 180 nm, and exhibits an ordered 2D hexagonal porosity of cylindrical nanopores. It should be noted that MSN-Py (Figure 2 g) still maintained their mesoporosity even though the pores were not that clear as compared with the MSNs before pyridine functionalization (the detailed description can be found in the Supporting Information). For MSN-Py, an ordered 2D hexagonal structure still remains, which indicates that the mesoporous scaffolds have not been damaged despite a slight decrease in intensity and a broadening of the (110) and (200) peaks (line ii). This is consistent with the ordered pore structure observed from its TEM image (Figure 2 g). However, after loading with Rh6G and capping with CP[5]A or SC[4]A (Figure S2 in the Supporting Information), the diffraction peaks of MSNs disappeared, except the main peak (100) was still visible at a reduced intensity. Meanwhile, the distance between adjacent pores and the pore diameter calculated by the Bragg equation decreased (Table S2 in the Supporting Information). Furthermore, the surface areas and pore sizes of the materials were confirmed by Brunauer–Emmett–Teller (BET) and Barrett–Joyner–Halenda (BJH) analyses, which clearly revealed the physical properties (Figure 2 a, Figures S3 and S4, Table S3 in the Supporting Information). Figure 2 a shows the characteristic type IV adsorption isotherms with surface areas of 871 (line i) and 628 m$^2$ g$^{-1}$ (line ii), consistent with the presence of MSN cylindrical pores.

FT-IR spectra show the successful modification of MSNs in the grafting steps. Compared with the spectrum of MSN-Cl (Figure 2 b, line i), the new absorption peaks at 1637 and 1490 cm$^{-1}$ of MSN-Py (Figure 2 b, line ii) correspond to the characteristic absorption peaks of aromatic rings, indicating that pyridine units have been successfully added to the surfaces of MSNs by covalent bonds. However, the final material, Rh6G-loaded, SC[4]A-capped MSN-Py (Figure 2 h) exhibits a less clear pore structure because of the loaded probe molecules inside of the MSNs and a layer of macrocyclic receptors installed on MSN surfaces.

To further test the microcrystallinity of our newly synthesized materials, small-angle powder X-ray diffraction (XRD) patterns were obtained. As shown in Figure 2 d, the original nanoparticles (line i) show a clear standard Bragg peaks of (100), (110), (200) and a faint peak of (210), reflecting a highly ordered 2D hexagonal array. In addition, the average interplanar spacing and pore distance of the (100) peak of MSN-Cl were 3.56 and 4.11 nm, respectively (Table S2 in the Supporting Information). For MSN-Py, an ordered 2D hexagonal structure still remains, which indicates that the mesoporous scaffolds have not been damaged despite a slight decrease in intensity and a broadening of the (110) and (200) peaks (line ii). This is consistent with the ordered pore structure observed from its TEM image (Figure 2 g). However, after loading with Rh6G and capping with CP[5]A or SC[4]A (Figure S2 in the Supporting Information), the diffraction peaks of MSNs disappeared, except the main peak (100) was still visible at a reduced intensity. Meanwhile, the distance between adjacent pores and the pore diameter calculated by the Bragg equation decreased (Table S2 in the Supporting Information). Furthermore, the surface areas and pore sizes of the materials were confirmed by Brunauer–Emmett–Teller (BET) and Barrett–Joyner–Halenda (BJH) analyses, which clearly revealed the physical properties (Figure 2 a, Figures S3 and S4, Table S3 in the Supporting Information). Figure 2 a shows the characteristic type IV adsorption isotherms with surface areas of 871 (line i) and 628 m$^2$ g$^{-1}$ (line ii), consistent with the presence of MSN cylindrical pores.

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Figure 2. a) N$_2$ adsorption and desorption (BET), b) FT-IR spectra, c) TGA, d) XRD of (i) MSN-Cl and (ii) MSN-Py. Typical SEM image of e) MCM-41 and TEM images of f) MSN-Cl, g) MSN-Py, h) Rh6G-loaded, SC[4]A-capped MSN-Py.
MSN-Py (Figure S1 in the Supporting Information, line a) and Rh6G-loaded, SC[4]A-capped MSN-Py (Figure S1, line b), which means the original materials have been successfully covered by CP[5]A and SC[4]A. Thermal gravimetric analysis (TGA) (Figure 2c) shows a difference in weight loss of about 5.4% when comparing MSN-Py to MSN-Cl, indicating that 0.677 mmol g\(^{-1}\) pyridine molecules were successfully added to the MSN surfaces. In addition, the elemental contents of the samples further disclosed that the surfaces of MSNs have been functionalized with pyridine units, because the percentages of N and C elements were remarkably increased compared with those before pyridine modification. Meanwhile, the coverage percentage of pyridine units anchored to the outer rims of MSNs was around 30%, which was calculated by the mole contents of C (4.749 mmol g\(^{-1}\)) and N (0.464 mmol g\(^{-1}\)) elements (Table S1 in the Supporting Information). The number of pyridine units found by elemental analysis was less than the value calculated by TGA, which might be because the percentage of nitrogen was very small in comparison with Si, O, etc.

To investigate the influences of concentrations and kinetics on the cargo release systems, we used ACh as an inducing agent, Rh6G as a probe molecule, and SC[4]A/CP[5]A as the blocking agents. Rh6G release was performed in deionized H\(_2\)O in the absence or presence of different concentrations of ACh, and the process was monitored by UV/Vis absorption spectroscopy. The release curves of Rh6G-loaded, macrocycle-capped MSNs are presented in Figure 3a, which indicates that ACh could trigger the systems with a negligible premature release. In addition, the effective release percentage of Rh6G-loaded, SC[4]A-capped MSN-Py was larger than the Rh6G-loaded, CP[5]A-capped system (Figure 3a), which was mainly due to the different binding constants (Figure 4, Table 1). Isothermal titration calorimetry (ITC) experiments gave the complex stability constants (\(K_s\)) and other relative thermodynamic parameters (enthalpy and entropy changes, \(\Delta H^\circ\) and \(\Delta S^\circ\)). In all cases, host–guest inclusion complexes of 1:1 binding stoichiometry were observed and Gibbs free energy changes \(\Delta G^\circ\) < 0 indicated that the inclusion complexation is spontaneous. In addition, it should be mentioned that the thermodynamic parameters are mainly influenced by macrocycle type and the inclusion complexations are mainly driven by favorable enthalpy changes (Table 1). The relative release percentage was calculated based on competitive binding method; Rh6G-loaded, SC[4]A-capped MSN-Py was triggered by ACh (\(K_s\) with SC[4]A is 58 500 M\(^{-1}\)) and Rh6G-loaded, CP[5]A-capped MSN-Py was treated by methyl viologen (\(K_s\) with CP[5]A is 82 000 M\(^{-1}\)) until the absorption value did not increase any more.

As is known, ACh is an important neurotransmitter and exists widely in the synapses of human body, which is not good for drug delivery if the system is hypersensitive to ACh. So, when the concentration of ACh is in a normal range, it should not trigger a significant release, but once the concentration of ACh is up to a certain level, such as in PD patients, a controlled release of drugs could then be achieved with careful design. This is the principle that we used to design our current systems. Rh6G-loaded, SC[4]A-capped MSN-Py was chosen as a model system to investigate the release experiments. ACh-triggered cargo release was monitored under different concentrations of ACh, 1.66 \times 10^{-4} \text{M} (0.58 \text{mmol}), 1.66 \times 10^{-3} \text{M} (5.8 \text{mmol}), and 1.66 \times 10^{-2} \text{M} (58 \text{mmol}), as a function of time. As can be seen in Figure 3b, when the concentration of ACh (in vitro) is compared with the concentration in the human body, only 2.6% of the cargo was released, which is mainly negligible premature release. Furthermore, it also shows that different percentages of Rh6G were released from...
the mesopores of MSNs when the ACh concentration was elevated, which means the drug release rate will be faster and the release will be enhanced when the amount of ACh in the treated region is greater. Moreover, we explored the release kinetics of Rh6G-loaded, CP[5]A-capped MSN-Py. CP[5]A forms a more stable complex (an order of magnitude greater) with 1-(4-(2,6-diisopropylphenoxy)butyl)pyridin-1-iumbromide (M-1, see the Supporting Information) than ACh (Figure 4, Table 1), according to the existing knowledge. Initially, the cargo was hardly released from the system, but as time went by, more ACh was added into the cuvette and the release rate of Rh6G rapidly increased (Figure 3 c) when the amount of ACh was much more than that of pyridine units, so that it could remove macrocycles from the stalks and thus open the nanovalves. The release curve shows the absolute absorption over a long time period and the inset is the relative absorption, which is determined by subtracting the premature release value from the absolute absorption. These results indicated that not only the stronger binding agents can activate the nanovalves but also the weaker players. Consequently, this competitive binding method can broaden the scope of stimuli-responsive drug delivery systems.

In conclusion, a novel proof-of-concept therapeutic method was demonstrated by release experiments. This system has the potential to ameliorate dystonic, dyskinetic, and choreiform behaviors, through ACh acting as a competitive agent to operate calixarene or pillarene-based supramolecular nanovalves. Functionalization of MSNs by organic components and supramolecular nanovalves was confirmed by a series of experimental characterizations. Different amounts of cargo can be released from the mesopores of the final materials when the concentration of competitive agents is changed or the gating macrocycle is changed. In other words, smart nanocontainers have been successfully designed and synthesized in this work. Both calixarene and pillarene-based systems presented a negligible premature release and exhibited different percentages of released from the mesopores of the final materials when the concentration change of external ACh, and, although ACh is a weaker competitor in comparison with pyridine units for CP[5]A, the release of Rh6G can be achieved by increasing the concentration of ACh in the Rh6G-loaded, CP[5]A-capped MSN-Py system to compete with and conquer pyridine stalks. These functionalized MSN-based drug delivery systems are promising for in vivo applications of site-specific drug release for the treatment of diseases, especially PD.

**Experimental Section**

**Materials and methods**

All the commercially available chemicals were purchased from Aldrich and Aladdin and were used as received. Reactions were carried out under nitrogen atmosphere using dry solvents, unless otherwise noted. The controlled release experiments were collected with UV/Vis spectroscopy on a Shimadzu UV-1800 spectrophotometer. Fourier transform infrared (FT-IR) spectra were carried out using a Bruker Vertex 80 V spectrometer. Scanning electron microscope (SEM) images were recorded on a JEOL JSM 6700F instrument. Gold coating of the materials used for imaging was carried out by sputtering for 2 min. Transmission electron microscopy (TEM) images were measured on a Hitachi H-800 instrument with an accelerating voltage of 200 kV. Powder X-ray diffraction (XRD) measurements were carried out using a Rigaku SmartLab III powder diffractometer. The radiation source was Cu Kα (1.39225 Å).

**Table 1.** Complex stability constants (K), standard enthalpy (ΔH°), entropy changes (ΔS°) and Gibbs free energy (ΔG°) for the 1:1 complexation of hosts and guests at 25 °C in water.

<table>
<thead>
<tr>
<th>Host</th>
<th>Guest</th>
<th>K (M⁻¹)</th>
<th>ΔH° (kJ mol⁻¹)</th>
<th>ΔS° (kJ mol⁻¹ K⁻¹)</th>
<th>ΔG° (kJ mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC[4]A</td>
<td>M-1</td>
<td>95800 ± 3677</td>
<td>35.10 ± 2.52</td>
<td>-0.56 ± 0.23</td>
<td>34.54 ± 2.30</td>
</tr>
<tr>
<td>SC[4]A</td>
<td>ACh</td>
<td>58500 ± 2687</td>
<td>31.12 ± 0.10</td>
<td>-0.33 ± 0.01</td>
<td>30.79 ± 0.10</td>
</tr>
<tr>
<td>CP[5]A</td>
<td>M-1</td>
<td>221000 ± 849</td>
<td>18.95 ± 0.49</td>
<td>0.49 ± 0.05</td>
<td>19.45 ± 0.45</td>
</tr>
<tr>
<td>CP[5]A</td>
<td>ACh</td>
<td>996 ± 40</td>
<td>6.11 ± 1.68</td>
<td>0.93 ± 0.13</td>
<td>7.04 ± 1.55</td>
</tr>
</tbody>
</table>
N₂ Adsorption and desorption isotherms (BET and BJH) were measured on using a Micromeritics Gemini instrument. Elemental analysis was done on an Elementar vario micro 45 cube to test the content of C, H, N, and S. Thermal gravimetric analysis (TGA) was carried out on a Q500 instrument from 100 to 900 °C at a heating rate of 10 °C min⁻¹ under nitrogen flow. ITC experiments were carried out on a thermostated and fully computer-operated isothermal titration calorimetry (Microcal ITC200) instrument, purchased from General Electric. In the ITC experiments, the host solutions were placed in the reaction cell and the guest solutions were then added. The binding isotherm data were fitted by "one set of binding sites" model.

Cargo loading and macrocycle capping

Rh6G-loaded, macrocycle-capped MSNs were prepared as follows: MSN-Py (0.5 g, see the Supporting Information for the detailed synthesis) was suspended in an aqueous solution of Rh6G (0.5 mm, 60 mL), followed by sonication for 5 min and stirring for 12 h at room temperature under darkness. An excess of the macrocycle (CPS5A or SC4JA) was added into the mixture and stirred for another 2 days. Finally, to remove excessive Rh6G and macrocycles, the solids were centrifuged and washed with deionized H₂O twice to result in red products after drying in high vacuum oven overnight.

Controlled release experiments

The Rh6G-loaded, macrocycle-capped MSNs (1.2 mg) were suspended in deionized H₂O (0.5 mL) and put into dialysis bag, which was then immersed into the cuvette loading with deionized H₂O (3 mL) or the corresponding ACh solution under gentle stirring. To monitor the release of Rh6G, UV/Vis absorption spectra of these solutions were recorded at predetermined times. The amount of released Rh6G was quantified by plotting the absorption curve with Rh6G solution of different concentrations at a fixed time.

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Keywords: acetylcholine · drug delivery · macrocyclic chemistry · mesoporous materials · silica nanoparticles

A new term, that is, pillarene, has been introduced into this field and is suggested to replace the use of pillararene/pillar[n]arene, and hopefully will benefit the pillarene research community. See the first example and detailed explanation: L.-L. Tan, Y. Zhang, B. Li, K. Wang, S. X.-A. Zhang, Y. Tao, Y.-W. Yang, New J. Chem. 2014, 38, 845 – 851.