Reduction-responsive drug delivery based on mesoporous silica nanoparticle core with crosslinked poly(acrylic acid) shell

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A novel reduction-responsive drug delivery system was successfully constructed with mesoporous silica nanoparticle (MSN) core as a drug carrier and poly(acrylic acid) (PAA) shell crosslinked by disulfide linkages as a drug release switcher. To keep the pore structure of MSN intact, PAA was covalently attached to the exterior surface of MSN before removing structure-template via radical polymerization. After removing structure-template and loading doxorubicin (DOX), the PAA shell was crosslinked by cystamine dihydrochloride through amidation reaction. The loading content and the entrapment efficiency of DOX could reach up to 40.2% and 80.4%, respectively. Because that the dissociation of disulfide linkage is reduction-responsive, the release behavior of DOX could be controlled by varying the concentration of reductant, and the release rate was 49.4% after 24 h with the existence of 2 mM glutathione (simulated environment of cancer cells), about three times higher than that of without glutathione (corresponding to normal human cells), which was only 16.9%. The in vitro cell assays demonstrated that the disulfide linkages crosslinked MSN–PAA (MSN–PAA-crosslinked) was highly biocompatible and suitable to use as drug carrier, and the DOX loaded MSN–PAA-crosslinked showed remarkable cytotoxicity to HeLa cells (human cancer cells), and relatively lower cytotoxicity to 293 cells (human normal cells). These results imply that the MSN–PAA-crosslinked is a promising platform to construct reduction-responsive controlled drug delivery system for cancer therapy.

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1. Introduction

In the last few decades, a controlled drug delivery system (CDDS) has gained much attention because of its superior performance in chemotherapy, such as reduced toxicity and side effects, reduced frequency of doses, improved drug efficacy, and convenience [1–3]. An ideal CDDS should not only efficiently load anticancer drugs, but also release the loaded drugs at the specific focal sites [4]. To achieve this goal, two important components, drug carrier and drug release switcher, are critical to CDDS [5–7].

Up to date, many materials, for example liposome [8], polymer micelles [9], and organic and inorganic nanoparticles [10,11], have been applied as drug carriers in CDDS. Among them, mesoporous silica nanoparticle (MSN) has been widely investigated, due to its extraordinary chemical and physical properties, e.g. tunable particle and pore size, large specific surface area, high chemical and thermal stability, excellent biocompatibility, and versatile chemistry for further functionalization [12,13]. It is feasible to embed the drug molecule in the porous channel of MSN, which will be favorable not only to improve the drug loading efficiency, but also to achieve the aim of sustained release [14].

A CDDS utilized MSN as drug carriers usually realized the controllable drug release via two strategies. One is relying on adjusting the pore size and morphology of MSN. For instance, Horcajada et al. [15] have studied the influence of pore size of MCM-41 type of MSN on the drug release behavior, and found that the release rate of ibuprofen in synthetic body fluid (SBF) solution decreased with the decrease of the pore size from 3.8 to 2.5 nm. However, this strategy can only alter the release rate of drug to a limited extent, rather than to decide when or where to release drug. The other strategy is through the chemical functionalization of MSN [16]. The exterior and internal surfaces of MSN are rich with silanols, which can be further modified with chemical groups or polymer chains to afford MSN with some new properties [17]. To take advantage of these new properties, the drug release behavior could be controlled by external stimuli, such as pH [18], thermo [19], and light [20]. Fujiwara et al. [21] have functionalized MCM-41 type of MSN with photosensitive coumarin, and found that the system was stable while irradiating with ultraviolet light of wavelength longer than 310 nm, and would release guest molecules at wavelength of 250 nm. You et al. [22] have grafted thermo-sensitive poly(N-isopropylacrylamide) (PNIPAM) onto the exterior
surface of MSN, to give a novel nanodevice with MSN core as the guest molecule carrier and thermo-responsive PNIPAM shell as a smart nanovalve. Our group has prepared pH-sensitive poly(acrylic acid) (PAA) covalently modified MSN, and found that the release rate of doxorubicin increased with the decrease of pH [23]. However, the existing stimuli-responsive CDDSs could not respond sensitively enough to distinguish the tiny difference of pH or thermal between normal cells and cancer cells. In vitro extra stimuli, such as photo-irradiation, light or magnetism induced heating, are normally indispensable to trigger the drug release switcher. Therefore, it is still a challenging work to explore a new type of CDDS, which could utilize the difference between normal cells and cancer cells to trigger the drug release switcher.

Recently, CDDS using disulfide linkage as the drug release switcher has attracted more and more attention [24]. Because glutathione (GSH) is a bond breaking agent to disulfide linkage, and the concentration of GSH in cancer cells (2–8 mM) is 1000 times higher than that in blood and normal cells (1–2 μM), a disulfide linkage would be stable in blood and normal cells, and be scissored in cancer cells [25,26]. For example, Ojima et al. [27] have constructed a CDDS by covalently linking monoclonal antibody with paclitaxel via disulfide linkage, and found that the drug would only be released with the existence of GSH. Mortera et al. [28] have covalently linked cysteine with MSN via a disulfide bond, and the in vitro cell assay demonstrated that the CDDS could kill HeLa cells more efficiently than cysteine non-covalently loaded MSN. In previous work, we have constructed a CDDS based on copolymer covalently linked paclitaxel via a disulfide linkage, and found that the CDDS showed apparent cytotoxicity to OS-RC-2 cells (kidney cancer cells) and low cytotoxicity to macrophage cells (human normal cells) [29]. However, the CDDSs mentioned above loaded drug via covalent bonds, which need multi-step chemical reactions and tedious post-treatment [30]. Lin et al. [31] have prepared a CDDS using cadmium sulfide (CdS) nanoparticles as gatekeeper by covalently linking CdS to MSN via a disulfide linkage, however, it would be inevitably to release CdS at the same time of release drug, which might induce toxic and side effect.

Herein, we reported a novel reduction-responsive CDDS with MSN core as the drug carrier and poly(acrylic acid) (PAA) shell crosslinked via disulfide linkages as the drug release switcher. This CDDS could efficiently load DOX, and the release rate of DOX in simulated environment of cancer cells after 24 h was three times higher than that in simulated environment of normal cells. The in vitro cell assays demonstrated that the MSN–PAA-crosslinked was highly biocompatible and suitable to use as drug carrier, and the DOX loaded MSN–PAA-crosslinked showed remarkable cytotoxicity to HeLa cells, and relatively lower cytotoxicity to 293 cells.

2. Experimental section

2.1. Chemicals

Acrylic acid (AA, 99%), azodisobutyronitrile (AIBN, 98%), cystamine dihydrochloride, methacryloxy propyl trimethoxyl silane (MPS), and glutathione (GSH, 98%) were purchased from Aldrich. Doxorubicin hydrochloride (DOX) was purchased from Beijing Huafeng United Technology Co., Ltd. Tetraethyl orthosilicate (TEOS), cetyltrimethylammonium bromide (CTAB, 99%), acetonitrile, and mesitylene (TMB) were purchased from Shanghai Chemical Reagent Co., Ltd. NaOH (96%), NH4NO3 (99%), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl, >98%) were purchased from Sinopharm Chemical Reagent Co., Ltd. All the reagents were analytical grade and used without further treatment.

2.2. Preparation of poly(acrylic acid) functionalized MSN (MSN–PAA)

MSN was synthesized as our previous work [23]. Typically, 0.5 g of CTAB and 3.5 mL of TMB were successively added into a solution containing 240 mL of deionized water and 1.75 mL of 2 mol·L−1 NaOH (aq). After vigorous stirring at 80 °C for 4 h, 2.5 mL of TEOS was quickly added into the mixture. Then, the reaction was vigorously stirred at 80 °C for another 2 h. The resultant white precipitate (MSN) was separated by filtration, washing with copious ethanol, and drying overnight in a vacuum at 45 °C. Double bond functionalized MSN (MSN–PAA) was obtained by refluxing 1 g of the resultant MSN with 2 mL of MPS in 240 mL of ethanol for 12 h, followed by filtration, washing with copious ethanol, and drying overnight in a vacuum at 45 °C. 50 mg of MSN–PAA, 0.3 mL of AA, and 7.5 mg of AIBN were successively added into 40 mL of acetonitrile. Then, the mixture was refluxed for 2 h. The resultant was separated by filtration and

![Fig. 1. Schematic preparation process of PAA–MSN.](image-url)
washing with copious ethanol. The structure-template CTAB and TMB were removed by refluxing in ethanol solution of ammonium nitrate (NH₄NO₃/C₂H₅OH, 10 mol·L⁻¹) for 6 h. The final product was filtrated and dried in a vacuum at 45 °C for 12 h, to give MSN–PAA as a white powder.

2.3. Drug loading and in vitro release

Typically, MSN–PAA and DOX were dispersed in deionized water to form 2 mg·mL⁻¹ solutions, respectively. Different ratios of the DOX solution and the MSN–PAA solution were mixed together under stirring at room temperature for 24 h to reach the equilibrium state. Then, the PAA shell of DOX loaded MSN–PAA was crosslinked by adding stoichiometric cystamine dihydrochloride and EDC·HCl, and following by reacting at room temperature for 24 h. The shell-crosslinked DOX loaded MSN–PAA (DOX@MSN–PAA) was obtained by filtrating, washing with pH = 7.4 PBS solution for three times, and drying overnight in a vacuum at 40 °C. The amount of DOX in DOX@MSN–PAA was determined by a UV–vis spectrophotometer at 480 nm.

In the in vitro drug release experiment, a certain amount of DOX@MSN–PAA was dispersed into deionized water. The dispersion was transferred into a dialysis bag (cut off molecular weight of 14,000 g·mol⁻¹), and then the bag was immersed into 30 mL of PBS solution with or without 2 mM of GSH. An amount of 1.0 mL of solution was withdrawn at a given time interval, followed by supplying the same volume of fresh PBS solution. The amount of released drug was measured by a UV–vis spectrophotometer at 480 nm.

2.4. Cytotoxicity assay

The cytotoxicity of MSN–PAA against HeLa cells and DOX@MSN–PAA against HeLa and 293 cells was evaluated by MTT assay. The cells were seeded in 96-well plates with a density of 5000 cells per well and cultured in 5% CO₂ at 37 °C for 24 h. Then, the cells were washed with PBS solution, and the medium was changed to fresh PBS mediums containing the indicated concentrations of MSN–PAA and DOX@MSN–PAA, respectively. The concentration of DOX in DOX@MSN–PAA in each group was equal by calculating with prudence. At the end of the incubation (24 or 48 h), the cells were washed with fresh PBS solution to remove the MSN–PAA and DOX@MSN–PAA that were not taken up by the cells. 20 μL of MTT in PBS solution was added to each well, and the cells were incubated for another 4 h at 37 °C in dark. The absorbance of each well was measured at 490 nm with a BioTek Elx800 apparatus. All the tests were performed in triplicate.

2.5. Characterization

Transmission electron microscopy (TEM) images were obtained on a JEOL JEM 2100 F transmission electron microscope, and samples for TEM measurements were made by casting one drop of the sample’s solution on carbon copper grids. The size distribution of the nanoparticles was measured by dynamic light scattering (DLS) using a Malvern autosizer 4700, and the dispersant was deionized water. Fourier transform infrared (FT-IR) spectra were recorded on a Nicolet 6700 FT-IR spectrometer using the KBr pellet method. Thermogravimetric analysis (TGA) was performed on a Perkin-Elmer Pyris 1 TGA instrument at a heating rate of 20 °C·min⁻¹ in a nitrogen flow from 100 to 800 °C. The zeta potentials were measured by a Malvern Nano-HT Zetasizer. The UV–vis absorbance spectra were measured with a Perkin-Elmer Lambda 35 spectrophotometer.

![Fig. 2. TEM images of (A) MSN and (B) MSN–PAA.](image-url)  
![Fig. 3. (A) FTIR spectra and (B) TGA curves of MSN, MSN–MPS and MSN–PAA.](image-url)
3. Results and discussions

3.1. Preparation of MSN–PAA

MSN was synthesized according to the classic route [22,32] using N-cetyltrimethylammonium bromide (CTAB) as structure-template and mesitylene (TMB) as expanding agent, as shown in Fig. 1. To keep the channel structure of MSN intact to efficiently load drug, MSN before removing the structure-template was reacted with methacryloxy propyl trimethoxily silane (MPS) to functionalize the exterior surface with double bonds, followed by distillation–precipitation polymerization [33] with acrylic acid, to obtain PAA covalently grafted MSN (MSN–PAA). Finally, the structure-template CTAB and TMB were removed by refluxing in an ethanol solution of ammonium nitrate (NH₄NO₃/C₂H₅OH, 10 mol·L⁻¹) for 6 h.

As it could been seen in Fig. 2, the synthesized MSNs were uniform spherical nanoparticles with clear porous structure. The periodically well-organized hexagonal array of mesopores represented by the bright dots shown on the TEM micrograph (Fig. 2A, inset) is the characteristic of MCM-41 type MSN. After covalently grafted with PAA, the exterior surface of MSN was uniformly covered by a shell of polymer, and the porous structure could hardly be seen. Due to the covalent functionalization of hydrophilic PAA, the dispersibility of MSN–PAA in PBS solution of pH = 7.4 was obviously enhanced, there was no apparent precipitate even after two weeks’ standing. This is a critical factor in biomedicine applications.

After functionalized with MPS to introduce double bonds to the exterior surface of MSN, a new absorption band at 1710 cm⁻¹ assigned to the stretching vibration of C=O in ester groups was appeared in the FTIR spectrum of MSN–MPS (Fig. 3A), indicating the successfully attachment of MPS. Due to the existence of CTAB in the channels, the characteristic C–H stretching vibrations at 2930 and 2860 cm⁻¹ and C–H deformation vibration around 1470 cm⁻¹ were also observed. After covalently grafted with PAA, a broad band centered at 3400 cm⁻¹ and a strong band at 1720 cm⁻¹ were appeared, which indicated the existence of a large amount of carboxyl groups [34]. To evaluate the graft amount of PAA on the exterior surface of MSN, the thermogravimetric analysis (TGA) experiments were performed on the structure-template removed MSN, MSN–MPS, and thoroughly washed MSN–PAA. As it was seen from Fig. 3B, MSN, MSN–MPS, and MSN–AA showed a weight loss of 6 wt.%, 13 wt.%, and 50 wt.%, respectively. Thus, the graft ratio of PAA could be calculated to be about 37 wt.%. All these results indicated that the PAA chains have been successfully grafted onto the exterior surface of MSN.

3.2. Drug loading and in vitro release of DOX

The drug loading process was conducted by mixing DOX and MSN–PAA aqueous solution together under stirring at room temperature for 24 h to reach the equilibrium state, followed by adding stoichiometric cystamine dihydrochloride, a disulfide crosslinkage, and catalysts to crosslink the PAA shell via amidation reaction. After crosslinking, the characteristic C=O stretching vibration of carboxyl groups at 1720 cm⁻¹ red-shifted to 1640 cm⁻¹, ascribed to the C=O stretching vibration of amide groups (Fig. 4 A), indicating the successful crosslinking of PAA shell. This result was also confirmed by the zeta potential measurement. Because that the amidation reaction would decrease the amount of active carboxyl groups on PAA chains, the zeta potential of crosslinked MSN–PAA was only −1.8 mV in PBS of pH = 7.4, much lower than that of non-crosslinked MSN–PAA,

<table>
<thead>
<tr>
<th>DOX/MSN–PAA</th>
<th>Loading content (%)</th>
<th>Entrapment efficiency (%)</th>
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<tr>
<td>0.1</td>
<td>2.2</td>
<td>25.1</td>
</tr>
<tr>
<td>0.25</td>
<td>11.3</td>
<td>51.2</td>
</tr>
<tr>
<td>0.5</td>
<td>24.5</td>
<td>64.8</td>
</tr>
<tr>
<td>1</td>
<td>40.2</td>
<td>80.4</td>
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Fig. 4. [A] FTIR spectra and [B] DLS results of DOX@MSN–PAA before and after crosslinking.

Fig. 5. The drug release curves of DOX@MSN–PAA (a) with and (b) without adding 2 mM of glutathione in PBS of pH = 7.4.
which was -42.5 mV. Furthermore, compared with non-crosslinked MSN–PAA, the mean diameter of crosslinked MSN–PAA dispersed in deionized water determined by dynamic light scattering (DLS) was decreased from 250 nm to 180 nm (Fig. 4B). This was because that the crosslinkage among PAA chains limited the free spread of PAA. The crosslinking network of PAA would be dissociated with the action of glutathione, leading to the release of DOX. In vitro cell assays demonstrated that MSN–PAA was highly biocompatible and suitable to use as a drug carrier. The disulfide linkage crosslinked DOX@MSN–PAA showed a remarkable inhibition to the proliferation of HeLa cells, and relatively lower cytotoxicity to 293 cells. This novel reduction-responsive CDDS provided a promising platform for chemotherapy.

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