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Intracellular redox-activated anticancer drug delivery by functionalized hollow mesoporous silica nanoreservoirs with tumor specificity

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ABSTRACT

In this study, a type of intracellular redox-triggered hollow mesoporous silica nanoreservoirs (HMSNs) with tumor specificity was developed in order to deliver anticancer drug (*i.e.*, doxorubicin (DOX)) to the target tumor cells with high therapeutic efficiency and reduced side effects. Firstly, adamantanamine was grafted onto the orifices of HMSNs using a redox-cleavable disulfide bond as an intermediate linker. Subsequently, a synthetic functional molecule, lactobionic acid-grafted-β-cyclodextrin (β-CD-LA), was immobilized on the surface of HMSNs through specific complexation with the adamantyl group, where β-CD served as an end-capper to keep the loaded drug within HMSNs. β-CD-LA on HMSNs could also act as a targeting agent towards tumor cells (*i.e.*, HepG2 cells), since the lactose group in β-CD-LA is a specific ligand binding with the asialoglycoprotein receptor (ASGP-R) on HepG2 cells. *In vitro* studies demonstrated that DOX-loaded nanoreservoirs could be selectively endocytosed by HepG2 cells, releasing therapeutic DOX into cytoplasm and efficiently inducing the apoptosis and cell death. *In vivo* investigations further confirmed that DOX-loaded nanoreservoirs could permeate into the tumor sites and actively interact with tumor cells, which inhibited the tumor growth with the minimized side effect. On the whole, this drug delivery system exhibits a great potential as an efficient carrier for targeted tumor therapy *in vitro* and *in vivo*.

1. Introduction

Tumor illness is one of the leading causes of human death and difficult to be cured [1]. It is, therefore, imperative to develop potent treatment methods to cure tumor illness, while with less side effects to patients [2]. Recently, the approach of "nanomedicine" has provided an immense potential to revolutionize tumor treatments by designing nanoscale drug delivery systems for targeted administration in order to achieve the optimal treatments [2,3]. Mesoporous silica nanoparticles (MSNs), as one of representative chemotherapeutic agent delivery vehicles, have been utilized to fabricate controlled drug release systems on account of their unique features including ordered framework, tunable pore size, large specific surface area and very low cytotoxicity [4–6]. Moreover, the abundant original silanol groups (Si–OH) on MSNs further facilitate them for post-functionalization [4–6]. Up to now, various types of MSN-based stimuli-responsive drug delivery systems have been developed [7–28]. For example, inorganic nanoparticles (*e.g.*, Au [7,8], Fe_3O_4 [9,10], CdSe [11], and Zinc [12]), biomacromolecules (*e.g.*, lactose [13,14], antibody

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[15,16], DNA [17,18], liposome [19,20], and collagen [21]) and (supra)molecular machines (*e.g.*, pseudorotaxanes and rotaxanes [4,13,14,22–25]) serving as the end-cappers have already been incorporated onto the orifices of MSNs through cleavable intermediate linkages. However, a crucial issue still remains in these systems, which is how to efficiently "switch off" and "switch on" the mesopores in response to biological signals or external stimuli for controlled drug delivery *in vivo*.

Generally, the approaches for stimuli-triggered "opening" of the end-cappers can be classified into the following categories, *i.e.*, redox reaction [11,21,25–28], pH change [9,10,12,22,24], enzyme-mediated action [7,8,13–15,18], light irradiation [17], temperature change [29], magnetic field [23] and so on. For instance, Stoddart and Zink reported a series of (supra)molecular machine-functionalized MSNs by employing switchable [2] pseudorotaxanes and [2] rotaxanes as the end-cappers to control the cargo loading and release under pH, enzyme and temperature stimuli [13,14,22–25]. These systems showed high drug encapsulation capability and high sensitivity to external stimuli, and were highly modular for specific requirements. We also developed diverse types of redox- [25,27,30], pH- [31,32], enzyme- [33], and light- [34] responsive release systems based on MSNs for controlled and targeted drug delivery. In our previous studies, lactobionic acid (LA) was employed as one of the targeting ligands to HepG2 cells, since the lactose group is a specific ligand binding to the asialoglycoprotein receptor (ASGP-R) on the membrane of HepG2 cells [32,35]. Since most of these studies did not involve the investigation and evaluation of (supra)molecular machine-functionalized MSNs for *in vivo* drug delivery, it is urgent to accumulate the proofs of the interactions between these systems and tumor cells, as well as animal curative effects of these systems *in vivo* for potential clinical applications.

The disulfide bond, one of versatile and bio-cleavable linkages, has been used as an intermediate linker to connect the end-cappers with MSNs for redox-triggered release of loaded cargos, since the amount of reducing agent (*i.e.*, glutathione (GSH)) within tumor cells is nearly 10^3-fold higher than that of GSH in the extracellular matrix [26,28]. Herein, we reported the fabrication of intracellular redox-responsive hollow mesoporous silica nanoreservoirs (HMSNs), in which adamantanamine was grafted onto the orifices of HMSNs through a redox-cleavable disulfide bond linkage followed by end-capping with lactobionic acid-grafted-β-cyclodextrin (β-CD-LA) *via* strong complexation between the adamantane unit and β-CD (Fig. 1). In this work, HMSNs were used as the carriers of anticancer drug doxorubicin (DOX) with enhanced loading capacity as compared with ordinary MSNs. β-CD-LA could act as both an end-capper of HMSNs and a targeting ligand towards HepG2 cells [32,35]. DOX-loaded nanoreservoirs were then employed to treat HepG2 cells and tumor-bearing nude mice in order to investigate their therapeutic effects *in vitro* and *in vivo*, respectively.

2. Materials and methods

2.1. Materials

All chemical reagents were purchased from Sigma–Aldrich and used without further purifications. Cell culture medium and cellular imaging dyes were purchased from Invitrogen Company (USA). All solvents and inorganic reagents were commercially available.

2.2. Synthesis of 2-carboxyethyl 2-pyridyl disulfide

2-Carboxyethyl 2-pyridyl disulfide was synthesized according to our previous report [27], and its preparation was also described in the Supplementary data.

2.3. Synthesis of β-CD-LA

Firstly, β-CD was reacted with *p*-toluenesulfonyl chloride (*p*-TsCl) according to a previous report with some changes [36]. Briefly, β-CD (31.7 mmol) was dispersed into deionized water (300 mL) by using a 500 mL round bottom flask. NaOH (98.4 mmol) in deionized water (8 mL) was added dropwise into the β-CD solution within 5 min. After the solution became clear, *p*-TsCl (31.7 mmol) in acetonitrile (18 mL) was added dropwise into the above mixture solution under an ice bath within 8 min. The solution was continuously stirred at room temperature for another 3 h. Then, the solution was adjusted to neutral condition (pH = 7.0) by using HCl and then placed in a refrigerator under 4 °C overnight. After the filtration, the precipitate was collected and re-dispersed into acetone and ethanol to remove unreacted *p*-TsCl. The precipitate was recrystallized with distilled water for several times, and dried by vacuum freeze-dryer to obtain the final product denoted as tosyl-β-CD.

Secondly, tosyl-β-CD was functionalized with ethylenediamine to obtain amino β-CD according to a previous report with some modifications [36]. Briefly, tosyl-β-CD (3.0 g, 2.33 mmol) was suspended in dry DMF (15 mL) in a 250 mL two-necked round bottom flask under nitrogen protection. Ethylenediamine (3 mL) was then added into the flask *via* a syringe and the mixture was refluxed at 60 °C overnight. After cooling the solution to room temperature, the resulted product was added dropwise to acetone (150 mL) for purifications. The obtained precipitate was filtered and washed extensively with ethanol (50 mL) and acetone (50 mL × 2). After that, the powder was re-dissolved in water (3 mL) followed by the precipitation with acetone (150 mL) for two times. Finally, the precipitate was collected, dialyzed with a cellulose bag filter (MW: 500) for 3 days, and dried at vacuum drying oven for overnight to afford the final product denoted as β-CD-NH_2.

Thirdly, β-CD-NH_2 was reacted with LA to produce β-CD-LA. Briefly, a mixture of LA (0.3 g, 0.85 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC, 0.64 mmol) and *N*-hydroxysuccinimide (NHS, 0.64 mmol) was dissolved into hexamethylenetetramine/HCl buffer solution (20 mL, pH = 5.4) under gentle stirring for 4 h. Then, β-CD-NH_2 (0.5 g, 0.43 mmol) was added into the above solution, which was stirred for overnight. The resultant mixture was added dropwise to acetone (150 mL) with stirring. After the filtration, the precipitate was washed extensively with ethyl ether (50 mL). The obtained powder was re-dissolved in water (5 mL) followed by the precipitation with cold ethyl ether (−40 °C). Finally, the product was dialyzed with a cellulose bag filter (Mw: 500) for 3 days, and dried under vacuum freeze-dryer for overnight.

2.4. Preparation of solid SiO_2 nanoparticles

Solid SiO_2 nanoparticles (~100 nm) were synthesized *via* Stöber method according to previous reports [25,37]. Briefly, ammonium hydroxide (10 mL) and tetraethoxysilane (TEOS, 10 mL) were sequentially dissolved into an ethanol/water (428 mL/60 mL, v/v) mixture, which was stirred at 30 °C for 2 h. After centrifugation (10,000 rpm), the product was rinsed with ethanol and distilled water for several times, respectively. The final nanoparticles were dispersed into distilled water by ultrasonication for further uses.

2.5. Preparation of SiO_2@CTAB-SiO_2 core/shell nanoparticles

The core/shell silica nanoparticles were synthesized according to previous reports with some modifications [25,37]. Briefly, hexadecyltrimethylammonium bromide (CTAB, 150 mg) was dissolved into a mixture solution of water/ethanol (30 mL/30 mL, v/v) containing concentrated ammonia water (0.55 mL). After treatment with ultrasonication for 30 min, the solid SiO_2 nanoparticles (100 mg) in distilled water (20 mL) were added into the above solution, which was stirred at room temperature for 30 min. TEOS (0.25 mL) was subsequently added to the mixture solution, and the reaction was continued for another 6 h. After centrifugation (10,000 rpm), the crude material was sequentially rinsed with ethanol and water for several times, and then re-dispersed into distilled water (15 mL) to afford the final product denoted as SiO_2@CTAB-SiO_2.

2.6. Preparation of HMSNs

HMSNs were prepared by a selective etching method based on previous reports [25,37]. Briefly, the water suspension of the obtained SiO_2@CTAB-SiO_2 was ultrasonicated for 20 min and then vigorously stirred at room temperature for 4 h. Sodium carbonate (Na_2CO_3, 470 mg) in water (5 mL) was subsequently added into the mixture solution, which was remained at 50 °C for 8 h to selectively etch the SiO_2 core. After centrifugation and extensive washing with water (30 mL × 3), the obtained HMSNs were dispersed in distilled water under stirring for overnight to remove the residues.

2.7. Sulfhydration of HMSNs

The obtained HMSNs were dispersed into anhydrous toluene (50 mL) containing 3-mercaptopropyl-trimethoxysilane (MPTS, 0.5 mL), and the suspension was refluxed at 60 °C with gentle stirring for 24 h [25]. After centrifugation, the products were extensively washed with acetone and ethanol to remove excess MPTS. To further extract CTAB from the hollow silica nanoparticles, the samples were dispersed into methanol/hydrochloric acid mixture solution under reflux at 80 °C for 36 h, leading to MPTS-functionalized HMSNs denoted as HMSNs-HS.