pH-Controlled Delivery of Doxorubicin to Cancer Cells, Based on Small Mesoporous Carbon Nanospheres

Jie Zhu, Lei Liao, Xiaojun Bian, Jilie Kong, Pengyuan Yang, and Baohong Liu*

Mesoporous carbon nanospheres (MCNs) with small diameters of $\approx 90$ nm are developed as an efficient transmembrane delivery vehicle of an anticancer drug, doxorubicin (DOX). MCNs exhibit a high loading capacity toward DOX due to hydrophobic interactions and the supramolecular $\pi$ stacking between DOX and the carbonaceous structures, on which the pH-dependent drug release are successfully achieved. Specifically, DOX can be loaded onto MCNs in basic solution and in a physiological pH range, while release occurs in acidic solution in its ionized state. By effective passive and active targeting, MCNs can be readily internalized into HeLa cells, where the carried DOX can be efficiently released in the acidic microenvironment of the tumors for further therapy. The endocytosis and cytotoxicity of DOX@MCNs toward HeLa cells are investigated by confocal microscopy and MTT assay. This smart pH-dependent drug loading and release property of DOX@MCNs makes it possible to reduce the cytotoxicity to normal tissues during circulation in the body since the normal physiological pH is $\approx 7.4$.

1. Introduction

Drug delivery is of importance for cancer therapy as conventional chemotherapeutic agents are distributed nonspecifically in the body, thus affecting both normal and tumoral cells. One of the key challenges of such drug delivery is to have the ability of transporting an effective amount of drug with fewer acute and chronic side effects. Various nanoparticles, such as liposomes, carbon nanotubes, and mesoporous silica nanoparticles, have been explored as drug carriers to enhance the drug loading capacity, effectively transport drugs through the cell membrane, and efficiently release the cargo into cells. Moreover, with regard to the decrease of cytotoxicity of drugs, development of stimuli-responsive intracellular controlled delivery systems is the latest important breakthrough based on functional mesoporous materials. Lin et al. modified mesoporous silica nanoparticles with chemically removable CdS or gold nanoparticle caps; drugs or DNAs in the mesopores can be released by the introduction of uncapping triggers. Fujiwara et al. reported the photocontrolled release of guest molecules from coumarin-modified mesoporous materials, and Stoddart et al. described redox-controlled nanovalves on MCM-41 spheres for the release of luminescent molecules.

Tumors exhibit a lower extracellular pH than normal tissues, as well as in their intracellular lysosomes and endosomes. Based on this property, pH-controlled drug delivery will be a fascinating protocol for cancer therapy while reducing the side effects to the human body. Doxorubicin (DOX) is one of the commonly used chemotherapeutic agents (the $pK_a$ of its amino group is 7.6) which are highly toxic to humans and result in severe suppression of hematopoiesis, and gastrointestinal and cardiac toxicity.

DOI: 10.1002/smll.201200217

J. Zhu, L. Liao, X. Bian, Prof. J. Kong, Prof. P. Yang, Prof. B. Liu
Department of Chemistry
Institute of Biomedical Sciences
and State Key Lab of Molecular Engineering of Polymers, Fudan University
Shanghai 200433, China
E-mail: bhliu@fudan.edu.cn
design of a suitable delivery vehicle for DOX is thus of crucial importance. Carbon nanomaterials are the potential candidates which exhibit specific interactions toward DOX at different pH values based on its nonionized and ionized states. Dai and co-workers first reported the pH-controlled release of DOX from single-walled carbon nanotubes and successfully applied this to in vivo cancer therapy.[5] Ordered mesoporous carbon is another kind of novel carrier for intracellular drug release due to not only its large surface area and pore volume for high drug adsorption, but also its abundant adjustable mesopores that could protect the guest drugs with less premature release before reaching the targets. As far as we are concerned, there are few reports on the use of mesoporous carbon nanospheres (MCNs) as the transmembrane deliverer in human cancer cells; on the other hand, the particle size of MCNs synthesized by hard templates is usually larger than 100 nm.[10] It is accepted that the optimal size of a transmembrane delivery vehicle should be less than 100 nm in diameter and the surface should be hydrophilic to circumvent clearance by macrophages, to maximize circulation times and targeting ability.[11]

To achieve these aims, MCNs with smaller diameters of ~90 nm have been developed to be a transmembrane carrier of the anticancer drug DOX. The prepared MCNs show a large loading capacity for DOX due to hydrophobic interactions and supramolecular π stacking between DOX and MCNs. Excitingly, we successfully observed pH-dependent DOX loading and release properties from MCNs. DOX is easier to load in basic solutions (pH ≥ 7.4) in its nonionized state, whereas it is easier to release in acid solutions (pH 5.5) in its ionized state (shown in Scheme 1). The in situ cytotoxicity of DOX-loaded MCNs (DOX@MCNs) was further studied toward HeLa cancer cell lines. The results indicated that the released DOX can kill HeLa cells with high efficiency after being internalized by MCNs, while no cytotoxicity was observed by MCNs.

2. Results and Discussion

The smaller MCNs were synthesized using triblock copolymer Pluronic F127 as a template.[12] A low-concentration controlled hydrothermal treatment was carried out to create the nanostructure and to confine the particle size. A typical transmission electron microscopy (TEM) image of calcined MCNs is shown in Figure S1a (Supporting Information). The average diameter is ~87 nm, obtained by measuring 100 individual MCNs directly from the TEM images (Figure S1b). The small-angle X-ray scattering (SAXS) pattern of MCNs shows two resolved scattering peaks at q values of 0.73 and 1.27 nm⁻¹, respectively (Figure S1c). The peaks with q ratio of 1.3⁷⁺ can be indexed as the 110 and 211 reflections of a body-centered cubic Im 3 m mesostructure. The calcined MCNs exhibit a specific surface area of 746 m² g⁻¹, a large pore volume of 0.76 cm³ g⁻¹, and a narrow pore size distribution centered at 2.7 nm (Figure S1d), thereby providing an enhanced drug loading capacity.

Different from the conventional MCNs synthesized by using mesoporous silica nanoparticles as hard templates which have been removed by hydrofluoric acid,[10] the MCNs prepared herein are quite hydrophobic. To improve their dispersion property in aqueous solutions for drug loading and release as well as following cell experiments, MCNs were treated with concentrated nitric and sulfuric acids, by which carboxylic and hydroxyl groups were successfully introduced. Figure 1a shows the scanning electron microscopy (SEM) and TEM images of MCNs after treatment in acid. The average diameter measured by TEM is ~90.0 nm (Figure 1b), which is similar to the results for MCNs before oxidation. The SAXS pattern also shows two resolved scattering peaks at q values of 0.73 and 1.27 nm⁻¹, respectively (Figure S2), thus indicating that the mesostructure of MCNs was maintained during the acid treatment. The specific surface area of the MCNs is 738 m² g⁻¹, and the pore volume slightly changes to 0.99 cm³ g⁻¹ after pretreatment (Figure 1d). All of these characterizations illustrated that the morphology of MCNs was maintained well during the covalent oxidation by acid. Dynamic light scattering (DLS) experiments (Figure 1c) were performed to measure the particle size of the MCNs in phosphate-buffered saline (PBS). MCNs exhibited an average diameter of 91.3 nm in PBS, which was slightly larger than the results from TEM. The results revealed that the MCNs after acid treatment were well dispersed in the aqueous solutions due to the introduction of hydroxyl and carboxylic groups onto the MCN surfaces.

Figure 2a compares the Raman spectra of MCNs before and after the acid treatment. The two most intense features are the G peak at ~1585 cm⁻¹ and a band at ~1340 cm⁻¹, historically named the D peak. The G peak is due to the doubly degenerate zone center E₂g mode, while zone-boundary
phonons give rise to the D peak in defect-containing graphite. The Raman spectra show that all the prepared phonons give rise to the D peak in defect-containing graphite.  

MCNs have a structure of graphite with defects. It is reported that aromatic molecules can interact strongly with the basal plane of graphite via π stacking. DOX is an aromatic anticancer drug, and a good model in this work. Apart from the hydrophobic interactions, the plane of graphite existing in MCNs can also interact strongly with the aromatic molecule DOX via supramolecular π stacking, which contributed to the high loading capacity. To completely remove the free DOX, the loaded DOX@MCNs were dialyzed for 2 days. The DOX adsorption factor (defined as DOX/MCN weight ratio) increased with the increasing amounts of DOX at pH 9.0 and 7.4, while the adsorption factor at pH 5.5 did not increase with different concentrations of DOX, which is similar to the case of carbon nanotubes. At higher concentrations, the extremely large adsorption amounts may result from the possible close packing of DOX or clustering between DOX and MCNs. Considering the pH-dependent drug delivery is mainly induced by supramolecular π-stacking interactions, a lower loading of 1.86 was used for the following experiments. The TEM image and the size distribution of DOX@MCNs with a loading factor of 1.86 measured by DLS are shown in Figure S3. As shown in Figure 2b, free DOX exhibited high fluorescence, while the fluorescence decreased significantly after DOX was bound to MCN. This high degree of fluorescence quenching is evidence of π-stacked DOX.

Figure 2. a) Raman spectra of MCNs before (A) and after oxidation (B). b) Fluorescence spectra of free DOX (8 μg mL⁻¹) and DOX@MCNs (12.3 μg mL⁻¹, the loaded DOX was also 8 μg mL⁻¹) in PBS solutions.

Additionally, we found that the amount of DOX bound to MCNs was pH-dependent. The DOX loading capacity decreased from ~1.86 to ~0.25 and ~0.11 as the pH was reduced from 9.0 to 7.4 and 5.5, respectively (Figure 3a). This trend was attributed to the increased hydrophilicity and higher solubility of DOX at lower pH values, caused by increased protonation of NH₂ groups on DOX, thereby reducing the hydrophobic interaction between DOX and MCNs. In terms of release, as shown in Figure 3b, DOX stacked on MCNs remained stable both in a basic medium at pH 9.0 and in a physiological pH range (e.g., pH 7.4). However, in acidic media (e.g., pH 5.5), ~40 and ~46% DOX was released over 24 and 48 h, respectively (the concentration of DOX@MCNs was 67 μg mL⁻¹). The pH-dependent drug release from MCNs was very interesting because the microenvironments of extracellular tissues of tumors and intracellular lysosomes and endosomes are acidic, potentially facilitating active drug release from MCNs.

The acid-assisted drug release and endocytosis property of DOX@MCNs toward HeLa cells was investigated using confocal microscopy. As shown in Figure 4b and c, a red fluorescence was observed in the whole cell, including the nucleus, after being cultured with DOX@MCNs. However, confocal microscopy of HeLa cells cultivated with rhodamine B (RhB)-labeled MCNs (RhB@MCNs; Figure 4e and f) showed no red fluorescence of RhB in the nucleus, which indicated that MCNs cannot be internalized into the nucleus. The above strong red fluorescence in the nucleus is attributed to the unbound DOX molecules, which were released from MCNs in the acidic microenvironment after being internalized into HeLa cells. Since the fluorescence signal of free DOX is much stronger than that of π-stacked DOX (Figure 2b), the unbound DOX shows an enhanced red signal inside the nucleus. It is worth mentioning that DOX is an anticancer drug that interferes with...
the topoisomerase II–DNA complex, thus leading to the formation of double-stranded breaks of DNA. Our MCNs show very high loading capacity of DOX, so that the large amounts of DOX loaded by the MCNs can be released and further internalized into the nucleus, thus interfering with the topoisomerase II–DNA complex there. Meanwhile, DOX@MCNs remain stable in the PBS, which has a similar pH value to blood. Additionally, no fluorescence signal of DOX was observed from HeLa cells cultivated with DOX@MCNs at 4 °C (Figure S5b), which indicated that DOX@MCNs were internalized into HeLa cells through an energy-dependent pathway.[17]

The cytotoxicity of DOX@MCNs toward HeLa cells was further evaluated using the standard MTT assay. As shown in Figure 5a and b, the cytotoxicity of DOX@MCNs was both dose- and time-dependent. Here the cytotoxicity of DOX@MCNs is tested at three different concentrations (shown in Table S1). After 24 h, about 85.5% of cells were killed at the highest concentration tested (e.g., 67 μg mL⁻¹, the loaded DOX was 44 μg mL⁻¹), and the inhibition ratio reached 95.6% after 48 h. The cytotoxicity of DOX stacked on MCNs was slightly lower than that of free DOX. As shown in Figure 5b, the inhibition ratio of DOX reached 99.7% after 48 h at 44 μg mL⁻¹. This phenomenon may be attributed to the partial release of DOX from MCNs, where only ≈46% DOX was released after 48 h for a concentration of DOX@MCNs at 67 μg mL⁻¹. However, considering that free DOX is highly toxic in humans, the use of MCNs as carrier and protector is necessary to reduce premature release before arrival at the target sites. The cytotoxicity effect of MCNs was further studied, and no obvious cytotoxicity was observed toward HeLa cells at the three concentrations tested, which indicated that the cytotoxicity of DOX@MCNs was attributable to DOX being released in cells. There are several advantages of using MCNs as a drug carrier. First, rapid vascularization in fast-growing cancerous tissues results in a leaky, defective architecture and impaired lymphatic drainage. This structure allows the accumulation of smaller nanoparticles at the tumor site, named passive targeting (Scheme 1).[19] Second, MCNs can facilitate the modification of functional groups such as antibodies and realize the target delivery for selective destruction of certain types of cells, thus reducing the toxicity to nontargeted cells. Furthermore, DOX stacked on MCNs is stable in PBS, thus reducing the cytotoxicity to normal cells during circulation in the body.

3. Conclusion

In summary, smaller MCNs with diameters of ≈90 nm have been developed to be a
novel transmembrane delivery vehicle of anticancer drug, and the pH-dependent drug loading and release from MCNs was successfully realized. MCNs show high loading capacity toward DOX due to hydrophobic interactions and supramolecular π stacking between DOX and MCNs in basic solutions or the physiological pH range, and exhibit a lower cytotoxicity; in acidic solutions, DOX is more easily released from MCNs in its ionized state. The MCNs can be readily internalized into HeLa cells, and the carried DOX can be efficiently released inside for chemotherapy, since the microenvironment of tumors is acidic. The potential advantages of using such MCNs as the vehicle of anticancer drugs are that the MCNs exhibit good biocompatibility, high loading and protection of the guest molecules, less premature release, efficient cellular uptake, and effective passive and active targeting. The exciting pH-dependent loading and release properties of DOX@MCNs make their use a promising strategy for enhancing efficient therapy towards tumors, while reducing the cytotoxicity of DOX to humans during body circulation since the pH of blood is ≈7.4.

4. Experimental Section

Materials: All chemicals were used as received without further purification. Triblock copolymer Pluronic F127 was purchased from Sigma–Aldrich. The HeLa cell line was provided by the Institute of Biochemistry and Cell Biology, SIBS, CAS (China). Formaldehyde, phenol, and ethanol were purchased from Shanghai Chemical Corp. Doxorubicin hydrochloride (DOX) was purchased from Shanghai Hualan Chemical Corp. Doxorubicin hydrochloride (DOX) was purchased from Shanghai Hualan Chemical Corp.

Synthesis of MCNs: The MCNs used in this work were synthesized according to a literature report. Briefly, phenol (0.6 g), formalin aqueous solution (2.1 mL, 37 wt%), and NaOH aqueous solution (15 mL, 0.1 M) were mixed and stirred at 70 °C for 0.5 h to obtain low-molecular-weight phenolic resols. After that, triblock copolymer Pluronic F127 (0.96 g) dissolved in H2O (15 mL) was added. Then the mixture was stirred at 66 °C with a stirring speed of 340 ± 40 rpm for 2 h. Water (50 mL) was added to dilute the solution. After 16–18 h, the obtained solution (17.7 mL) was transferred into an autoclave, diluted with H2O (56 mL), and heated at 130 °C for 24 h.

Surface Modification of MCNs: The obtained MCNs were added to an acid mixture (the volume ratio of H2SO4 to HNO3 was 3), and treated by ultrasound for 4 h. After that, the acid-refluxed MCNs were obtained by centrifugation and washed by PBS until the pH value of the spent liquor was neutral.

Loading and Release of DOX: MCNs (10 mg) and DOX (20 mg) were added to buffers with different pH values. The mixture was stirred at room temperature for 24 h. After adsorption, free DOX was removed by dialysis for 2 days. The dialyze (500 mL) was renewed every few hours. The dialyze was collected for quantitative analysis of the loading capacity by UV/Vis spectrophotometry; no UV/Vis peak of DOX was found from the final dialyze. In terms of release, DOX@MCNs were dispersed in buffers with different pH values and shaken at 100 rpm. The final concentration of DOX@MCNs was 67 μg mL−1. The supernatant after centrifugation was collected for quantitative analysis by UV/Vis spectrophotometry.

Characterization: TEM images were obtained with a JEOL 2011 microscope operated at 200 kV. The size distribution and zeta potential of particles were measured at 20 °C by a Nanosizer ZS-90 (Malvern Instruments). Nitrogen sorption isotherms were measured at 77 K with a Micromeritics Tristar 3000 analyzer. SAXS measurements were taken on a Nanostar U SAXS system (Bruker, Germany) using CuKα radiation (40 kV, 35 mA). Laser Raman spectroscopy was carried out with a LabRam-1B spectrometer. UV–visible (UV/Vis) spectra were obtained with a UV/Vis spectrophotometer (Agilent, 8453). The fluorescence spectra were measured with a fluorescence spectrophotometer (Varian, Cary Eclipse).

Subcellular Localization: HeLa cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C and 5% CO2. Cells (5 × 104) were seeded on 14 mm glass coverslips in a chamber and allowed to adhere for 24 h. After 24 h of incubation, the cells were washed with PBS three times. Then, HeLa cells were incubated with DOX@MCNs (6.7 μg mL−1) in a serum-free medium for 3 h at 4 or 37 °C under 5% CO2. After incubation, the cells were fixed with 4% paraformaldehyde, and their nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, 5 μg mL−1) in 10% glycerol. Confocal fluorescence imaging was performed with a Leica confocal laser scanning microscope (SPS) and a 63× oil-immersion objective lens. Excitation at 488 nm was provided by a Multi Ar laser. The images were taken at the same voltage (910.7 V), offset (~3.0%) and pinhole (106.2 μm).

Cytotoxicity: Cell viability was determined by the MTT assay. Briefly, cells were plated in 96-well flat-bottomed plates at 5 × 103 cells per well and allowed to grow overnight prior to exposure to MCNs, DOX, or DOX@MCNs with different concentrations, followed by irradiation with red light. After 24 or 48 h of further incubation, the MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added for 4 h at 37 °C to allow conversion of MTT to a purple formazan product by active mitochondria. Then the formazan product was dissolved in DMSO and quantified by absorption spectrophotometry at 490 nm on an enzyme-labeled instrument (SUNOSTIK SPR-960).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

This research was supported by NSFC (20925517, 21175028) and SKL EAC201101.

References