Hollow-Core Magnetic Colloidal Nanocrystal Clusters with Ligand-Exchanged Surface Modification as Delivery Vehicles for Targeted and Stimuli-Responsive Drug Release


Abstract: The fabrication of hierarchical magnetic nanomaterials with well-defined structure, high magnetic response, excellent colloidal stability, and biocompatibility is highly sought after for drug-delivery systems. Herein, a new kind of hollow-core magnetic colloidal nanocrystal cluster (HMCNC) with porous shell and tunable hollow chamber is synthesized by a one-pot solvothermal process. Its novelty lies in the “tunability” of the hollow chamber and of the pore structure within the shell through controlled feeding of sodium citrate and water, respectively. Furthermore, by using the ligand-exchange method, folate-modified poly(acrylic acid) was immobilized on the surface of HMCNCs to create folate-targeted HMCNCs (folate-HMCNCs), which endowed them with excellent colloidal stability, pH sensitivity, and, more importantly, folate receptor-targeting ability. These assemblages exhibited excellent colloidal stability in plasma solution. Doxorubicin (DOX), as a model anticancer agent, was loaded within the hollow core of these folate-HMCNCs (folate-HMCNCs-DOX), and drug-release experiments proved that the folate-HMCNCs-DOX demonstrated pH-dependent release behavior. The folate-HMCNCs-DOX assemblages also exhibited higher potent cytotoxicity to HeLa cells than free doxorubicin. Moreover, folate-
HMCNCs-DOX showed rapid cell uptake apart from the enhanced cytotoxicity to HeLa cells. Experimental results confirmed that the synthesized folate-HMCNCs are smart nanovehicles as a result of their improved folate receptor-targeting abilities and also because of their combined pH- and magnetic-stimuli response for applications in drug delivery.

Keywords: drug delivery · hollow core · ligand exchange · nanostructures · receptors

Introduction

Nanosized hollow structures are a new class of functional nanomaterials that have gained rapid scientific interest due to their hollow cores, and they show considerable flexibility to load sensitive molecules such as proteins, drugs, enzymes, and genes for delayed and targeted delivery with the added protection of a shell in biomedical applications. Given the undesirable side effects of the majority of anticancer drugs, such targeted delivery could effectively increase the efficacy of the drugs and lead to cheaper formulations and minimization of unwanted side effects.

As smart drug carriers, including any hollow nanomaterials, high loading efficiency, controlled release, low cytotoxicity, good biocompatibility, and specific targeting are ideally expected as key properties. To meet this diverse range of properties, the shell of the hollow materials can consist of various materials, such as silica,[6,7] carbon,[8] metal,[9,10] metal oxides,[11,12] and polymers.[13,14] Generally, the shape and size of the hollow space can be controlled by using inorganic or organic template-based methods, and the shell composition and thickness could also be controlled.[15-17] Furthermore, the structure can be functionalized to address a range of objectives, as demonstrated in recent reports including the “rattle-type” structure with movable magnetic core[18,19] or the magnetic hybrid shell,[20,21] which endows the hollow nanomaterials with a magnetic response. Moreover, a metallic template-based method is another route to synthesize hollow spheres with metal oxide shell, in which the metal core could be removed by in situ oxidation.[22,23] For example, a magnetically responsive shell containing varying amounts of Fe 3O 4 nanoparticles was synthesized by thermal decomposition of an Fe/Fe 3O 4 precursor at high temperatures.[24,25]
On the one hand, a template-based method can be used to prepare nanomaterials with both magnetic susceptibility and a hollow core, but the technique has complex synthetic steps and further, the removal of the template by either thermal or chemical means is complicated and energy-intensive.\cite{29} On the other hand, the Ostwald ripening process lends itself as a “template-free” method for the fabrication of hollow microspheres,\cite{27–29} in which the ripening time is controlled leading to a tunable hollow core.\cite{28} By using this method, a new kind of hollow nanoparticle with Fe3O4 shell was recently prepared, which demonstrated both magnetic stimulus response and fluorescence characteristics due to the magnetic shell and the ripening of FeS spheres in the presence of zinc, which endowed the hollow magnetite particles with the ability to fluoresce.\cite{30}

Furthermore, solvothermal reactions were known to be powerful in directing the formation of sub-micrometer-sized magnetic nanoparticles with the aim of improving their magnetic field responsiveness.\cite{31–35} Recently, the solvothermal method was also employed to prepare hollow-core magnetic colloidal nanocrystal clusters (HMCNCs) containing a large amount of iron oxide single crystals by a template-free method.\cite{36–40} Moreover, the fabrication process was interesting because the material was formed without solid templates or surfactants, and the formation process was considered to be controlled by a template of NH3 gas bubbles.\cite{38,39} Although a high magnetic response from the HMCNCs is obtained, the dispersion of the bare iron oxide shell (without hydrophilic stabilizers) was difficult in water. Also, the large size of the HMCNCs (300–500 nm) did not match the range of the EPR effect,\cite{41,42} which became a key factor that limited application in drug-carrier systems.

Herein, we report a new type of HMCNC with small size, controllable hollow chamber, and functional characteristics that overcome the above-discussed constraints. These HMCNCs are approximately 120 nm in diameter, have controllable porous shell and hollow chamber, and are further stabilized by sodium citrate (Na3Cit). The tunable characteristics of the hollow chamber and porous shell were obtained by varying the concentration of Na3Cit and the feed ratio of ethylene glycol (EG)/H2O (v/v). Further, the versatile ligand-exchange method, which can be used to carry out a range of shell modifications to better target drug carriers, was utilized to anchor folate-modified poly(acrylic acid) (PAA-FA) onto the surface of the HMCNCs. The purpose of the ligand-exchange process was to target cancer cells that overexpressed folate receptors, since the folate medication would enhance the endocytosis capability of such cancer cells. Moreover, we investigated the antitumor efficacy of these novel drug-loaded HMCNCs relative to that of the free drug, doxorubicin (DOX).

**Results and Discussion**

**Effect of sodium citrate and water on the structure of HMCNCs:** According to the report by Zhao et al.,\cite{19} solid magnetic colloidal nanocrystal clusters (MCNCs) could be successfully prepared (Figure S1, Supporting Information). Interestingly, when we doubled the concentration of FeCl3 and kept the concentration of Na3Cit constant, hollow structures were obtained (Figure 1). A TEM image at higher magnification indicated that the overall structure was composed of many small-sized nanocrystals and the aggregate size was about 120 nm (Figure 1b). A high-resolution TEM (HRTEM) image (Figure 1c) revealed that the Fe3O4 shell structure incorporated numerous channels. The selected-area electron diffraction (SAED) pattern with polycrystalline-like diffraction indicated that the shell consisted of many primary magnetite nanocrystals (Figure 1d). The hollow-core structure was found to be relatively stable against large increases in the FeCl3 concentration (about three or four times; Figure S2, Supporting Information). However, the hollow-core structure collapsed and broke into fragments of nanocrystals when the FeCl3 concentration was further increased (Figure S3, Supporting Information). Solid magnetic nanoparticles were obtained by keeping the concentration of FeCl3 constant (0.4 mmol L\(^{-1}\)) in the absence of Na3Cit (Figure 2a), and this was supported through the studies conducted by Li’s group.\cite{20} However, in the presence of Na3Cit the hollow-core structure appeared only when the concentration of Na3Cit reached a certain level (0.17 mmol L\(^{-1}\); Figure 2b), and then the structure gradually contracted until it disappeared at higher levels of Na3Cit (Figure 2c and 2d). Finally, the solid magnetic clusters were regenerated at 0.68 mmol L\(^{-1}\) Na3Cit concentration (Figure 2e). Based on our preparation procedure and previous reports,\cite{29,30} we consider that the formation of the hollow-core structure is based on the so-called bubble template mechanism. Under the solvothermal condition of about 200 °C, gas bubbles of H2O (trace amount in solvent)
form in the reaction system and these bubbles are stabilized by Na$_3$Cit. The stabilized bubbles then act as templates for the subsequent formation of the magnetic nanocrystals, which immobilize and stack on the surface of the gas bubble. With the elapse of time, under a continuous reaction scenario, MCNCs with hollow structure would be formed. However, if the Na$_3$Cit concentration increased in the system, the bubble size would tend to decrease gradually, and thus the hollow chamber would diminish in size. This process would continue until the Na$_3$Cit concentration reached a certain value that caused the size of bubbles to approach the size of primary magnetic nanocrystals, and then the hollow structure would completely disappear.

The influence of water on the morphology of HMCNCs was also studied. Keeping the concentration of both FeCl$_3$ (0.4 mmol L$^{-1}$) and Na$_3$Cit (0.34 mmol L$^{-1}$) constant, the feed amount of water was increased from 0 to 2 mL, and the resulting HMCNCs are shown in Figure 3. High-resolution scanning electron microscopy (HRSEM) images showed that the shell structure was compact without H$_2$O addition (Figure 3a), whereas large gaps appeared in the Fe$_3$O$_4$ shell with the increase of added H$_2$O (Figure 3b and 3c), which indicated that the H$_2$O molecule was a significant factor in controlling the shell structure. The HRTEM image also demonstrated that the Fe$_3$O$_4$ primary nanocrystals assembled into the magnetic shell, and their stacking became loose with an increase in the feed amount of H$_2$O (Figure 3e–3g). It was also found that the whole structure eventually broke down after excess water was added, as shown in Figure 3d and 3h. Nitrogen adsorption–desorption isotherms and the corresponding pore size distributions obtained with the Barrett–Joyner–Halenda (BJH) model were employed to evaluate the pore characteristics of HMCNCs with different amounts of H$_2$O. The isotherm can be attributed to type IV with a clear hysteresis loop in all curves (see Supporting Information, Figure S4a), which indicates the mesoporous characteristic of the HMCNCs. The pore size distributions showed that the major pore size ranged from 2 to 10 nm, which is within the mesoporous scale (2-50 nm; see Supporting Information, Figure S4b). The pore parameters of HMCNCs, including Brunauer–Emmett–Teller (BET) surface area, total pore volume, and major pore dimension, are displayed in Table 1. It was found that the surface area and the total pore volume increased with an increase in the H$_2$O feed amount.
Powder X-ray diffraction (XRD) measurements were carried out for evaluating the as-prepared samples (Figure 4a and 4c). All samples showed the typical XRD patterns of magnetite (JCPDS 75-1609). Without stabilizer, the magnetic nanoparticles possessed strong and sharp diffraction peaks. With the increase of Na$_3$Cit or H$_2$O concentration, the diffraction peaks became weaker and broadened, thus implying that the presence of Na$_3$Cit can significantly suppress the nanocrystal grains.[33] Further, the magnetic property of HMCNCs was investigated with a vibrating sample magnetometer (VSM) at 300 K (Figure 4b and 4d). The results demonstrated that the HMCNCs were prepared with high saturation magnetization, which was ascribed to both the high Fe$_3$O$_4$ content and enhanced crystalline characteristic. The saturation magnetization showed a slight decline from 93.2 to 85.4 emu g$^{-1}$ with a Na$_3$Cit concentration increase from 0 to 0.68 mmol L$^{-1}$, which may relate to the increase of Na$_3$Cit in HMCNCs. Once water was added to the mixture, the saturation magnetization was reduced, but there was no change when the H$_2$O amount was further increased. From the above discussion, we concluded that this method successfully delivered size-controllable and chamber-tunable HMCNCs stabilized by Na$_3$Cit with good crystallization and high saturation magnetization.

Folate modification of HMCNCs and the loading/release characteristics of DOX: As outlined earlier, the HMCNCs could be surface modified through receptor-specific ligand exchange to target those human cancer cells[43,44] that are known to overexpress the folate receptor. This ligand was chosen because folate can be conjugated by facile chemistry.[45,46] To endow the HMCNCs with cancer cell targeting ability, the receptor-specific ligand folate (FA) was employed to graft on the HMCNC surface. This process was achieved through replacing the Na$_3$Cit with PAA-FA on the surface of the HMCNCs and was confirmed by FTIR spec-

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<th>Added water volume [mL]</th>
<th>Surface area [m$^2$ g$^{-1}$]$^a$</th>
<th>Total pore volume [cm$^3$ g$^{-1}$]</th>
<th>Pore size [nm]$^b$</th>
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[a] Calculated by the BET method. [b] Calculated from the desorption branch of the N$_2$ isotherm by the BJH model.

Figure 4. XRD patterns (a,c) and VSM curves (b,d) of HMCNCs prepared with varying Na$_3$Cit concentrations and varying amounts of water, respectively.
troscopy and thermogravimetric analysis (TGA; Figure 5). In Figure 5a, the peaks at 586 and 448 cm\(^{-1}\) appeared in all characteristic curves, which were attributed to Fe\(_3\)O\(_4\) and corresponded to the stretching and vibration modes of Fe–O. The peaks at 1640 (\(v_{\text{C=O}}\)) and 1410 cm\(^{-1}\) (\(v_{\text{as(C=O)}}\)) for HMCNCs (Figure 5a, curve I) are also shown, which are attributed to the carboxyl group interaction with Fe\(_3\)O\(_4\), and these peaks confirm that the Na\(_3\)Cit successfully anchored the magnetite nanoparticles. After the Na\(_3\)Cit was exchanged by PAA-FA, the peaks (Figure 5a, curve II) at 2930, 2850 (\(v_{\text{CH2}}\) and \(v_{\text{as(CH2)}}\)), and 1710 cm\(^{-1}\) (\(v_{\text{C=O)}}\)) highlighted the presence of PAA-FA located on the surface of HMCNCs through the stretching of carbonyl and methyl groups. Additionally, the characteristic vibration of the benzene ring also appeared as peaks at 1600, 1540, and 1460 cm\(^{-1}\), which confirmed the presence of folate and the modification of HMCNCs with PAA-FA. We also measured the fluorescence spectra to support the above conclusions (Figure S5, Supporting Information). The characteristic fluorescence emission peak of folate at 465 nm can be detected in folate-HMCNCs, but these characteristic peaks were absent in HMCNCs and HMCNCs-PAA. Finally, with the DOX encapsulated in folate-HMCNCs, the intensification of the ring skeleton vibration and the appearance of numerous peaks from 1000 to 1300 cm\(^{-1}\) can be observed in the folate-HMCNCs-DOX spectrum (Figure 5a, curve III), which are characteristics of the DOX molecule (Figure 5a, curve IV), and so these FTIR results confirm that the DOX molecules were successfully deposited in the folate-HMCNCs.

TGA curves measured in a N\(_2\) atmosphere are shown in Figure 5b, and the weight loss results summarize the points. For example, relative to pure DOX, the DOX embedded within the hollow chamber of folate-HMCNCs-DOX showed an increased decomposition temperature and its weight loss curve was less “steep”; together, these results highlight the increased stability of the encapsulated drug. As expected, the molecules of Na\(_3\)Cit tended to stabilize the HMCNCs, thus resulting in a reduced decomposition of the HMCNCs. Importantly, the total weight loss observed for folate-HMCNCs (Figure 5b, curve II) was approximately 10 wt%, ascribed to the immobilized PAA-FA, and similarly the weight loss behavior of folate-HMCNCs-DOX enabled the calculation of the DOX in the nanoparticles as approximately 24 wt%.

The dynamic light scattering (DLS) technique was employed to investigate the stability of folate-HMCNCs in 50% serum solution (Figure S6, Supporting Information). Aggregation and precipitation were not observed in 0.01 mgmL\(^{-1}\) folate-HMCNCs, and only a single peak was observed with low polydispersity index (PDI) value (0.04), which implies that there was not a strong interaction between the serum (blood environment) and folate-HMCNCs. Moreover, after standing for 2 h, the dispersion maintained a stable state; meanwhile, the DLS peak and PDI did not alter. In addition, the folate-HMCNCs can be separated rapidly (around 25 s) from solution with a hand-held magnet (2000 G) and easily redispersed by a slight shake of the solution (without magnetic field). This result conclusively proved that folate-HMCNCs had good colloidal stability and enhanced capability of magnetic manipulation, and were appropriate for drug delivery.

To account for the varying pH of plasma and the extracellular and intracellular environments, and to understand the release behavior of DOX in these environments, the drug-release experiment was conducted by subjecting folate-HMCNCs-DOX (0.36 cm\(^3\) g\(^{-1}\) shell-pore volume) to different buffer solutions with pH values of 7.4, 6.0, and 5.0 (Figure 6). After 72 h, 81.6, 30.2, and 10.0 wt% DOX was released from folate-HMCNCs-DOX in media with pH values of 5.0, 6.0, and 7.4, respectively. This result was attributed to pH-induced behavior, achieved by collapsing and stretching of the PAA in environments with varying pH, as shown in Scheme 1. At higher pH (7.4), the PAA molecules anchored on the HMCNCs stretched, while the COOH group combined with the DOX tightly,\(^{[47]}\) which prevented the DOX in the chamber from being released by jamming the shell pores. Meanwhile, there was a stronger bound hydration shell in the swollen state, and the hydration water acted like ice not allowing permeation. Another issue might be that the water formed a barrier for hydrophobic molecules of DOX, and then the DOX could not be released.
under high pH conditions. However, as the pH decreased, the PAA was protonated and tended to collapse step by step,[48] thus allowing the shell pores to open and the DOX in the chamber to escape through the pores. Further to this, the investigation also focused on the impact of shell porosity on the extent of drug release through the HMCNCs. After a period of 48 h and at pH 5 in Na3Cit/H3Cit buffer solution, 69.1, 76.4, and 79.8 wt% DOX was released from folate-HMCNCs-DOX with 0.20, 0.29, and 0.36 cm³/g shell-pore volume, respectively (Supporting Information, Figure S7). The magnetic and pH-responsive shell with suitable porous structure would release large amounts of DOX into tumor cells with a low pH (4.5–5.5) after internalization, and thus enhance “targeted cytotoxicity”, whereas the slow release behavior at higher pH would minimize damage to normal cells in the blood plasma environment.

**In vitro cytotoxicity experiments**: To investigate the inhibition of cell growth, samples of folate-HMCNCs, HMCNCs-DOX, folate-HMCNCs-DOX, and free DOX were incubated with the human embryonic kidney (HEK) 293T cell line (normal cells) and the HeLa cell line (tumor cells). Without DOX loading, folate-HMCNCs did not exhibit clear cytotoxicity against normal cells (HEK 293T) in a broad concentration range (1–1000 µM/g) for 24 and 48 h, clearly revealing that the carrier anchored by PAA-FA had good biocompatibility (Figure 7a). Significantly, the folate-HMCNCs-DOX exhibited a much higher inhibitory effect on HeLa cell growth and proliferation, exceeding the effect of free DOX in equal dose, whereas the folate-HMCNCs showed insignificant cytotoxicity for HeLa cells (Figure 7b and 7c). Whether with folate grafting or not, the DOX-loaded HMCNCs were superior to free DOX, thus illustrating that the novel HMCNC carriers with incorporated DOX
molecules significantly enhanced the therapeutic effect of DOX. Notably, the cell inhibitory effect was enhanced with the folate-modified HMCNCs-DOX. Due to the overexpressed folate receptors in the HeLa cell line, folate-HMCNCs-DOX have improved affinity for entering HeLa cells as the HeLa surface receptor interacts with the folate-HMCNCs-DOX. Together, this provided a targeted delivery approach and improved therapeutic effect due to the pH-modulated release of DOX. Further, as $p < 0.05$ for the trends shown in Figure 7, the results were statistically significant. The folate-HMCNCs-DOX are thus “bidirectional motors”; they demonstrate independent driving and targeting forces (magnetic and folate) such that they can be driven through external magnetic stimuli and be developed to target folate. Therefore, they have great potential as a smart drug carrier for cancer treatment. At the same time, the HMCNCs were also stable at diverse pH values (7.4, 6.0, and 5.0), and so they can be further used in magnetic resonance imaging (MRI).

**Conclusion**

We have successfully synthesized a new kind of biocompatible and nanosized assembly to act as a smart drug-delivery system, which is designed with dual-targeting properties (folate targeting and magnetic response) so that it can be driven by a magnetic field and targeted by folate to cells with overexpressed folate receptors. HMCNCs with controllable hollow chamber and shell porosity could be regulated by the feed amount of Na$_2$Cit and H$_2$O by a one-pot solvothermal method. Through the ligand-exchange method of replacing Na$_2$Cit with PAA-FA, HMCNCs were endowed with good colloidal stability, biocompatibility, pH stimulus, and folate-receptor targeting. Due to the pH-stimulated deformation of PAA chains, the folate-HMCNCs-DOX released greater quantities of DOX in acidic medium than in neutral solution. Compared with free DOX, folate-HMCNCs-DOX exhibited an enhanced targeted approach and comparable cytotoxicity towards HeLa cells. As a result, the dual-targeting mechanism plus the pH-responsive release of DOX from the folate-HMCNCs-DOX could greatly enhance the drug therapeutic efficiency, which is translated into stronger potential as a drug-delivery system. PAA-anchoring HMCNCs also enhanced the chemical stability and biocompatibility of HMCNCs in varying cell environments, which was expected to be utilized in MRI. In addition, the ligand-exchange method allowed HMCNCs to be conveniently modified with a special surface ligand: this study focused on the folate molecule as a model functional ligand, which was grafted on PAA and subsequently used to modify the HMCNCs. The investigation opens avenues in utilizing chemical modification, such as amide reaction, click reaction, and even polymerization, to functionalize HMCNCs and further develop targeted surface modifications of inorganic nanoparticles in the field of drug-carrier systems.

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**Experimental Section**

**Materials:** Iron(III) chloride hydrate (FeCl$_3$·6H$_2$O), sodium acetate (NaAc), ethylene glycol (EG), folate (FA), ethylene diamine (NH$_2$-\(\text{CH}_2\)-NH$_2$), sodium citrate monohydrate (Na$_2$Cit·H$_2$O), and anhydrous ethanol were purchased from Shanghai Chemical Reagents Company (China) and used as received. Polyacrylic acid (PAA, $M_n = 18000$), N,N-diacyloxylicarboxibodiimide (DCC), and N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC·HCl) were purchased from Aladdin (China). N-Hydroxysuccinimide (NHS) was purchased from Sigma–Alrich. Doxorubicin (DOX) hydrochloride was purchased from Beijing Hua-Feng United Technology Co., Ltd. (China) and used as received.

**Synthesis of hollow-core magnetic colloidal nanocrystal clusters (HMCNCs):** HMCNCs stabilized by Na$_2$Cit were prepared through a solvothermal reaction. Typically, FeCl$_3$·6H$_2$O (2.163 g, 8.0 mmol) and a certain amount of Na$_2$Cit were ultrasonically dissolved in EG (20 mL) in a 50 mL beaker. After a certain volume of water was added to the beaker, the orange solution was mixed with NaOAc (2.4 g) under vigorous ultrasonic conditions at room temperature for 0.5 h and then transferred into a Teflon-lined stainless-steel autoclave (50 mL capacity). The autoclave was heated to 200°C, which was maintained for 20 h, then it was cooled to room temperature. The black HMCNCs were rinsed three times with ethanol and three times with water under ultrasonic conditions, and the surplus inorganic molecules were removed by the application of an external magnetic field, followed by drying in vacuum at 40°C for 24 h.

**Modification of HMCNCs with folate-grafted PAA by a ligand-exchange method (folate-HMCNCs):** Typically, PAA (400 mg, $M_n = 18000$ g mol$^{-1}$) was dissolved in deionized water (20 mL) by vigorous stirring, followed by addition of fixed amounts of EDC and NHS and activation for 1 h. Then aminated folate acid (20 mg; the preparation method is reported in ref.[49]) was added to the above solution for 24 h, and the pH was adjusted to 8.9 by 0.1 m NaOH. HMCNCs (200 mg) were added and dispersed ultrasonically, and then the pH was readjusted to 3–4 by 0.1 m HCl, followed by heating to 80°C for 24 h. The resultant brown precipitate was washed with deionized water to remove the unreacted PAA and folate until the supernatant was colorless. The folate-HMCNCs were dried in vacuum at ambient temperature for 24 h. Additionally, HMCNCs without folate anchoring were prepared by the same method but the aminated folate acid, EDC, and NHS were absent in the synthesis route.

**Preparation of doxorubicin-loaded folate-HMCNCs (folate-HMCNCs-DOX) and drug-release experiments:** Folate-HMCNCs (25 mg) were dispersed ultrasonically in DOX solution (10 mL, 1 mg mL$^{-1}$), followed by mechanical stirring for 4 h. After that, the pH of the solution was adjusted to 8.0 and the solution was stirred for 24 h, which enabled gradual migration of the drug into the hollow chambers of the HMCNCs by a slow reduction in the solubility of DOX in water. The resultant particles were separated by a magnet, washed with phosphate-buffered saline (PBS, pH 7.4) until the supernatant was colorless, and the product was freeze-dried for 3 days. The drug-release behavior was studied in Na$_2$Cit/H$_3$Cit buffer of pH 5.0, 6.0, and 7.4. Typically, DOX-loaded folate-HMCNCs (3 mg) were dispersed in Na$_2$Cit/H$_2$O buffer (2 mL, pH 5.0, 6.0, or 7.4) and transferred into a dialysis tube (cutoff $M_w = 14000$). Then the tube was rapidly immersed in 10 mL of the same buffer at 37.5°C with stirring at 200 rpm. In the next step, the supernatant medium (3 mL) was extracted at given time intervals and the absorbance of the solution was measured by UV/Vis spectroscopy. The supernatant medium was replaced with preheated fresh buffer (3 mL) after each sampling. The amount of released DOX was calculated from the corresponding UV/Vis absorbance (wavelength for measurement was 482 nm).

**In vitro cytotoxicity and cell viability study:** The in vitro cytotoxicities of folate-HMCNCs, HMCNCs-DOX, folate-HMCNCs-DOX, and free DOX were assessed on HEK 293T and HeLa cell lines by using the MTT method. Specifically, cells (100 µL) were seeded in a 96-well flat culture plate at a density of 1×10$^4$ cells per well and were subsequently incubated for 24 h to allow attachment. Then samples with different concentrations (0.5, 1.0, 2.5, 5.0 µg mL$^{-1}$) were added to each group (three wells) for 24 or 48 h. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bro-

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mide (MTT) solution (20 µL, 5 mg·mL⁻¹ in PBS) was added to the wells and incubated for 4 h. MTT internalization was terminated by aspiration of the medium, and the cells were lysed with DMSO (150 µL). The absorbance of the suspension was measured at 490 nm on an enzyme-linked immunosorbsent assay (ELISA) reader. Cell viability was calculated by means of Equation (1):  

\[
\text{Cell viability} = \frac{OD_{OD490\text{control}} - OD_{OD490\text{test}}}{OD_{OD490\text{control}}} \times 100 \%
\]

in which OD is optical density.

**Characterization:** TEM images were collected on an H-600 (Hitachi, Japan) transmission electron microscope at an accelerating voltage of 75 kV. HRTEM images were taken on a JEM-2010 (JEOL, Japan) transmission electron microscope at an accelerating voltage of 200 kV. Samples dispersed at an appropriate concentration were cast onto a carbon-coated copper grid. Magnetic characterization was carried out on a Model 6000 physical property measurement system (Quantum Design, USA) at 300 K. Powder XRD patterns were obtained by using an X-ray diffractometer (XRD) (Philips, Netherlands) with CuKα radiation at λ = 0.154 nm operating at 40 kV and 40 mA. X-ray photoelectron spectroscopy data were obtained on an RBD upgraded PHI-5000C (PerkinElmer, USA) ESCA system with MgKα radiation (hν = 1253.6 eV) at 250 W and 14.0 kV with a detection angle at 54°. FTIR spectra were recorded on a Magna-550 (Nicolet, USA) spectrometer, with the samples dried and mixed with KBr to be compressed into a plate for measurement. TGA data were obtained on a Pyris-1 (PerkinElmer, USA) thermal analysis system under a flowing nitrogen atmosphere at a heating rate of 20°C·min⁻¹ from 100 to 800°C. UV/Vis absorption spectra were obtained on a UV-3150 (Shimadzu, Japan) spectrophotometer.

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