Fabrication of magnetite hollow porous nanocrystal shells as a drug carrier for paclitaxel

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A facile and simple route is employed to synthesise a new type of magnetite hollow porous nanocrystal shells (HPNSs) as hydrophobic drug delivery vehicles. The morphological evolution of spherical clusters from solid to hollow porous shells is controlled in a straightforward fashion through the reaction time. The obtained magnetite HPNSs possesses high magnetization, well-defined structure, and porous shell, the channels and cavity in HPNSs benefit the drug storage, delivery, and release. The structure of HPNSs was characterized by SEM, TEM and XRD, VSM (vibrating sample magnetometer) data showed that the saturation magnetization values of the Fe3O4 HPNSs are 67.5, 73.2, 79.4, and 88.7 emu/g, corresponding to reaction times of 6, 8, 12, and 16.5 h, respectively. This result clearly proved that the crystallinity could be improved by Ostwald ripening of nanocrystals through a dissolution–recrystallization process, the formation of the hollow porous structure was promoted as well. FT-IR and TGA results showed that the porous shell facilitated paclitaxel diffusion into the cavity of hollow structure, and the drug loading of magnetite HPNSs for paclitaxel is very high (20.2 w%). The antitumor efficacy of the drug-loaded HPNSs measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was clearly enhanced, compared with free drugs.

Introduction

Paclitaxel (TXL) is a promising anticancer therapeutic agent.1,2 Being able to promote tubulin polymerization leading to abnormally stable microtubules and cause cell death by disrupting the dynamics necessary for cell division, TXL is used to treat a variety of malignancies, such as ovarian, breast, non-small cell lung cancers and head and neck carcinomas.3 Despite the applicability and effectiveness that TXL has shown so far, the hydrophobic nature of TXL impedes the use of traditional mode of intravenous administration, thereby limiting its application in the biomedical field. Modification of paclitaxel to a prodrug form, such as polymer-paclitaxel conjugates, could help overcome the problem, although usually the resultant will show a decreased antitumor efficacy, compared with the native TXL.4,5 Consequently, the designed fabrication of novel delivery systems for TXL has been intensively pursued. The use of nanomaterials in biotechnology merges the fields of material science and biology.6,7 Nanoparticles provide a particularly useful platform, demonstrating unique properties with potentially wide-ranging therapeutic applications.8,9 Among the broad diversity of nanoparticles, hollow nanostructures are of great interest owing to their advantageous characteristics.10,11 Their capacity for encapsulating sensitive materials such as therapeutic drugs,12 fluorescent markers,13 and field-responsive agents14 has been exploited for drug delivery12,13 and biomedical imaging.11,14 A key factor determined the applications is convenient access to the hollow interior space. In this regard, facile synthetic approaches for producing hollow carriers with a porous shell which permits easy encapsulation and release of guest species are significant contributions for their application as drug carriers. Meanwhile, magnetic nanoparticles, especially iron oxides, have been subjected to extensive studies in the past few decades owing to their stability in physiological conditions, biocompatibility, ease of surface functionalization, and unique magnetic responsiveness.15–20 These specific characters endow them with great potential in nanomedicine, serving as targeting carriers for drug delivery.21–23 Usually, magnetic drug nanocarriers comprise magnetic nanoparticulate cores with an organic or inorganic shell, and therapeutic agents are encapsulated within the shell structure.22,23 Magnetic core/shell nanocarriers can easily be manipulated by an external magnet, which have become known as the ‘magnetic motor’ for site-specific drug delivery applications.21,22 However, for an antitumor drug with poor water solubility, conjugating it onto magnetic nanocarriers often could not achieve satisfactory therapeutic efficacy due to the limited loading capacity, poor colloidal stability and weakened magnetic response. Therefore, integration of magnetic nanomaterials and hollow structures to form magnetic hollow nanocrystal shells, which possess large cavity, strong magnetic responsiveness and enhanced drug loading capacity, is undoubtedly of significance as versatile carriers for diagnostic and therapeutic applications.

Herein, we report the fabrication of magnetite hollow porous nanocrystal shells (HPNSs) as hydrophobic drug delivery vehicles. The obtained magnetite HPNSs possess high magnetization

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(88.7 emu/g), well-defined structures, and porous shells. The channels and cavity in the HPNSs benefit the drug storage, delivery, and release. We demonstrated that such magnetite HPNSs are ideal as drug delivery vehicles for TXL, the experimental results exhibited an excellent drug loading up to 20.2 wt%. The porous shell facilitates TXL diffusion into the cavity of hollow structure. Furthermore, antitumor efficacy of the drug-loaded HPNSs measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was enhanced, compared with free drugs.

**Experimental**

**Materials**
Iron(III) chloride hexahydrate (FeCl₃ 6H₂O), ammonium acetate (NH₄Ac), ethylene glycol (EG), and anhydrous ethanol were purchased from Shanghai Chemical Reagents Company (China) and used as received. Paclitaxel (TXL) was purchased from Beijing HuaFeng United Technology Co., Ltd. (China) and used as received. Dulbecco’s modified Eagle’s medium (DMEM), RPMI-1640, fetal bovine serum (FBS), penicillin G, streptomycin, and trypsinase were obtained from GIBCO BRL (Grand Island, NY, USA). Dimethyl sulfoxide (DMSO) and [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (MTT) were purchased from Sigma. Deionized water was used in all experiments.

**Synthesis of monodisperse magnetite hollow porous nanocrystal shells**

The magnetite hollow porous nanocrystal shells (HPNSs) were prepared through a modified solvothermal reaction. Typically, 1.350 g of FeCl₃ 6H₂O and 3.854 g of NH₄Ac were dissolved in 70 mL of ethylene glycol to form a homogeneous solution, and then transferred into a Teflon-lined stainless-steel autoclave (100 mL capacity). The autoclave was heated to 200 °C and maintained for 16.5 h, then it was cooled to room temperature. The black magnetite HPNSs were then rinsed several times with ethanol under ultrasonic conditions to effectively remove the solvent. The HPNSs were separated from the supernatant by centrifugation and stored under vacuum for further characterization and application.

**Incorporation of TXL into HPNSs**
TXL was loaded into HPNSs using a nanoprecipitation method. Briefly, 16.2 mg as-made HPNSs were dispersed in 4 mL mixed solution of DMSO and H₂O (1 : 3, v/v) containing 6 mg TXL. The mixture was sonicated for 3 min and then was mechanically stirred for 24 h at room temperature. The solvents were then evaporated to allow penetration of TXL through porous shell channels and deposition into the hollow interiors of HPNSs. The resultant TXL-loaded particles were washed and sonicated with PBS (pH = 7.4), then isolated by an external magnet. The process was repeated for another two times to ensure that weakly adsorbed drugs on the surface were removed, and finally dried under vacuum and stored in a freezer for further characterization and application.

**In vitro drug release study**
10 mg magnetite HPNSs were dispersed into 200 μL PBS saline (pH = 7.4), and ultrasonicated for homogeneity. The dispersion was added into a dialysis tube (cut-off Mn = 14 000) and immersed in 30 mL of PBS at 37 °C and a fixed pH value under stirring. 3 mL of the release medium was extracted at given time intervals and its UV-vis absorbance was measured. It is worth mentioning that the medium was totally replaced with another 30 mL preheated fresh PBS saline after each sampling. The amount of release TXL could be calculated from the corresponding UV-vis absorbance.

**Cell culture**

A human embryonic kidney cell line, HEK 293T cells, was cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (100 U/mL penicillin and 0.1 mg mL⁻¹ streptomycin) at 37 °C in a humidified incubator containing 5% CO₂. A human cervical carcinoma cell line, HeLa cells, was cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (100 U/mL penicillin and 0.1 mg mL⁻¹ streptomycin) at 37 °C in 5% CO₂ humid incubator. The cells were subcultured twice weekly.

**In vitro cytotoxicity and cell viability study**

The in vitro cytotoxicity of native HPNSs was assessed on HEK 293T cells, using the MTT method. Specifically, 100 μL of cells was seeded in a 96-well flat culture plate at a density of 1 × 10⁴ cells per well and were subsequently incubated for 24 h to allow attachment. Then samples with different concentrations were added to each group (three wells) for 24 h. 20 μL MTT solution (5 mg mL⁻¹ in PBS) was added to the wells and incubated for 4 h. MTT internalization was terminated by aspiration of the media, and the cells were lysed with 150 μL DMSO. The absorbance of the suspension was measured at 490 nm on an ELISA reader. Cell viability was calculated by means of the following formula:

\[
\text{Cell viability(\%)} = \frac{\text{OD}_{490(\text{sample})} - \text{OD}_{490(\text{blank})}}{\text{OD}_{490(\text{control})} - \text{OD}_{490(\text{blank})}} \times 100% 
\]

The cell viability of HPNSs, TXL-loaded HPNSs, as well as free TXL against Hela cells was measured by the MTT assay. The experiment was carried out as an in vitro cytotoxicity method. Cell viability graphs were plotted as TXL concentration.

**Characterization**

Transmission electron microscopy (TEM) images were collected by an H-600 (Hitachi, Japan) transmission electron microscope at an accelerating voltage of 75 kV. High-resolution transmission electron microscopy (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.

Scanning electron microscopy (SEM) images were performed using a TS-5136MM (TESCAN, Czech) scanning electron
microscope at an accelerating voltage of 20 kV. Samples dispersed at an appropriate concentration were cast onto a glass sheet at room temperature and sputter-coated with gold.

Powder X-ray diffraction (XRD) patterns were obtained using a X’Pert Pro (Panalytical, Netherlands) diffraction meter with Cu Kα radiation at λ = 0.154 nm operating at 40 kV and 40 mA.

X-Ray photoelectron spectrum (XPS) data were obtained using an RBD upgraded PHI-5000C (Perkin Elmer, USA) ESCA system with Mg Kα radiation (hν = 1253.6 eV) at 250 W and 14.0 kV with a detection angle at 54°.

Magnetic characterization was carried out with a vibrating sample magnetometer on a Model 6000 physical property measurement system (Quantum Design, USA) at 300 K.

Fourier transform infrared (FT-IR) spectra were recorder on a Magna-550 (Nicolet, USA) spectrometer. The samples were dried and mixed with KBr to be compressed to a plate for measurement.

Thermogravimetric (TG) analysis data was obtained with a Pyrisis-1 (Perkin Elmer, USA) thermal analysis system under a flowing nitrogen atmosphere at a heating rate of 20 °C min⁻¹ from 100 to 700 °C.

Ultraviolet-visible (UV-vis) absorption spectra were performed on a UV-3150 (Shimadz, Japan) ultraviolet-visible spectrophotometer.

Results and discussion

Monodisperse magnetite HPNSs were synthesized via a solvothermal reaction with iron(III) chloride hexahydrate as precursor, NH₄Ac as electrostatic stabilization agent, and ethylene glycol (EG, a polyhydric alcohol with boiling point of 196–198 °C) as reducing agent. NH₄Ac played a key role in the synthesis. Firstly, NH₄Ac was selected for electrostatic stabilization to prevent particle agglomeration. Secondly, NH₄Ac functioned as an assistant-reducing agent. The reductive environment provided by EG and NH₄Ac at high temperature, partially transforms Fe(OH)₃ into Fe(OH)₂, and finally Fe₃O₄ nanocrystals are formed through dehydration.²⁸ Control experiments showed that no products were obtained by EG alone under the same reaction conditions. Thirdly, NH₄Ac served as the structure-directing agent in the morphology transformation of spherical aggregates from a solid to hollow structure. NH₄Ac was hydrolyzed into HAc and NH₃, and partial NH₃ was evaporated and formed little gaseous bubbles, which combined with the Ostwald ripening process, resulting in the favored formation of hollow structures.²⁶

The morphology transformation of spherical clusters from solid to hollow porous shells is controlled in a straightforward fashion through the reaction time while keeping all other parameters fixed. Fig. 1a shows that the spherical clusters with 6 h reaction time have a loose spherical nanostructure. The loosely packed nanocrystals in the outer layer serves as the starting growth site for the gradual outward migration of nanocrystals through a dissolution–recrystallization process known as Ostwald ripening.²⁹ This ripening process results in the evolution of the outer layer from loose to compact packed structures, and the rough surface turned smooth, which can be observed in Fig. 1b after 8 h reaction time. With a lengthened Ostwald ripening process, the large nanocrystals result in continued expansion, the small nanocrystals and noncrystal part are dissolved gradually, then the interior space becomes hollow. As shown in Fig. 1c, the intensive contrast between the black margin and the dotted bright center of the clusters with 12 h reaction time confirmed the existence of hollow structures in the resulting microspheres. With a further prolonged reaction time of 16.5 h, the crystallinity of HPNSs could be improved, and

![Fig. 1](representative TEM images of magnetic nanocrystal clusters synthesized with reaction times of (a) 6, (b) 8, (c) 12, and (d) 16.5 h. All scale bars are 200 nm.)
small nanocrystals in the shell were dissolved gradually as well, then the hollow porous shells were formed, as shown in Fig. 1d.

Powder XRD measurements were carried out for the samples obtained with different reaction times, and the results are shown in Fig. 2A. All the diffraction peaks of the powder XRD patterns were indexed to typical XRD patterns of Fe₃O₄ (JCPDS 75-1609). As shown in Fig. 2B, the X-ray photoelectron spectrum (XPS) of HPNSs synthesised with a 16.5 h reaction time exhibits peaks at 711.4 and 724.8 eV, which are the characteristic peaks of Fe 2p3/2 and Fe 2p1/2 oxidation states, respectively. XRD and XPS results confirm that the magnetite HPNSs have been successfully synthesised by this facile and flexible one pot route.

In parallel to analysis of structures, magnetic properties were investigated with a vibrating sample magnetometer at 300 K, as shown in magnetization curves in Fig. 3. The Fe₃O₄ clusters have saturation magnetization values of 67.5, 73.2, 79.4, and 88.7 emu/g, corresponding to HPNSs synthesized with reaction times of 6, 8, 12, and 16.5 h, respectively. This result proved that the crystallinity could be improved by prolonged reaction time and Ostwald ripening, which agreed well with the TEM studies.

Under optimized conditions with the reaction time of 16.5 h, the primary Fe₃O₄ nanocrystals aggregate to form porous shells with the average diameter of about 310 nm and shell thickness of about 70 nm, as shown in the representative TEM image in Fig. 4a. The magnetite HPNSs are uniform both in size and shape, as shown in the representative SEM image in Fig. 4b. Some opened channels in the shell could be also distinctly observed in SEM image further confirming that clusters process channels leading to cavity of hollow structure. The secondary structures of HPNSs can be observed more clearly in Fig. 4c and 4d for representative isolated HPNS. As shown in the TEM image in Fig. 4c, the HPNS is clearly composed of small primary crystals with clear crystal orientation. The HRTEM image showed in Fig. 4d provides more detailed structural information of the HPNSs and the selected area electron diffraction (SAED) pattern taken from individual magnetic HPNSs (inset in Fig. 4d) reveals the single-crystalline nature of the nanocrystals. The fact that primary nanocrystals crystallographically align with adjacent ones is the result of oriented attachment and subsequent high-temperature sintering during synthesis. To examine the colloidal stability of HPNSs, magnetite HPNSs powder (20 mg) was dispersed in doubly distilled water (80 mL) by sonication and the suspension could be well dispersed. Being applied with a permanent magnet (2000 G), the HPNSs in their homogeneous dispersion showed a fast response, agglomerated within 30 s. and redispersed quickly with slight shaking once the magnetic field was removed. All these suggest that the HPNSs possess excellent colloidal stability, redispersibility, and strong magnetic responsiveness, which are advantageous to their biomedical applications.

Successful loading of TXL is confirmed by Fourier transform infrared (FT-IR) spectroscopy as shown in Fig. 5. The peak appearing at 573 cm⁻¹, attributed to the typical band of Fe₂O₃, corresponds to the stretching vibration modes of Fe–O. Spectra of standard TXL show characteristic absorptions at 1718 and 1244 cm⁻¹, which are ascribed to the symmetric or asymmetric stretch of C=O and C–O, respectively. As for HPNSs after
loading with TXL, the new peak located at 1718 cm\(^{-1}\) along with the one at 1244 cm\(^{-1}\) corroborate that TXL has been loaded into the HPNSs.

To determine the amount of TXL storage in the magnetite HPNSs, TG was employed to directly measure the weight loss of as-prepared product as shown in Fig. 6. Compared with the native HPNSs before drug loading, TXL-carrying HPNSs revealed an approximate 20.2% weight loss in the range 100–700 °C, indicating that a considerable amount of TXL was loaded into the carriers through the available pore channels. It is worth mentioning that, while being incorporated into nanoparticles, hydrophobic agents, such as TXL and camptothecin (CPT), traditionally could not achieve such a high loading capacity.\(^{34,35}\) Besides, drugs are often encapsulated within the shell structure of the conventional core/shell magnetic vehicles, which unavoidably results in undesired release of the guest drug during the course of delivery. The magnetite HPNSs, however, could effectively prevent such spontaneous, premature drug release due to the relatively dense, single crystalline Fe\(_3\)O\(_4\) nanoshells.\(^{36}\) This suggested the potential of HPNSs to be efficient drug delivery vehicles, special fat-soluble therapeutic agent.

Fig. 7 shows the release behavior of TXL-loaded HPNSs over a 48 h period in PBS (pH = 7.4) at 37 °C. A burst release was observed at the early stage under pH 7.4, indicative of the released drug adsorbed in the shallow nanochannels. Then the drug release rate decreased dramatically and the released percentage reached a value approximately 36.0% at 48 h, which showed that most of the incorporated drug was kept stable within the vehicles under the protection of dense magnetite nanoshells at pH 7.4. For more acidic conditions, such as pH 5.0,
however, it is noteworthy that the carriers are susceptible to acid etching, followed by subsequent burst release of the entrapped TXL. This is undoubtedly an excellent property for a drug delivery system since the release of the drug was pH-dependent and the carriers could be “degradable”, leaving no harm to other normal cells and tissues. More detailed investigations are underway to further explore this phenomenon and design rational pH-responsive carriers.

In vitro cytotoxicity of non-drug-loaded HPNSs, TXL-loaded HPNSs, and free TXL was then evaluated towards Hela cell line using MTT assay (Fig. 8). It could be found that the unloaded HPNSs did not show any significant cytotoxicity against Hela cells even in high concentrations, revealing the good biocompatibility of the vehicles. And TXL-loaded HPNSs exhibited a dose-dependent cytotoxic effect, which was comparable to or slightly higher than that observed with an equivalent dose of free TXL after 24 h incubation (Fig. 8a). After 48 h incubation, however, TXL-carrying HPNSs exhibited higher growth inhibition effect than free TXL (43.1 ± 7.3% vs. 34.6 ± 5.1% of 100 ng mL⁻¹, Fig. 8b). Such improved cytotoxicity is probably attributed to the sustained release of encapsulated TXL from the hollow interior of HPNSs. Additionally, nanoparticle-based delivery vehicles are known to enhance cellular internalization and provide access for the guest drug molecules through cell membranes to take intracellular pharmacological action, inducing cell apoptosis. In comparison, the cellular uptake of free TXL may unavoidably be hindered due to its hydrophobic nature.

In contrast, assays of as-synthesized HPNSs alone on a human embryonic kidney (HEK) 293T cell line (a normal cell line) clearly demonstrate that no significant cytotoxicity was detected to the proliferation and growth of HEK 293T cells, even at a high dose, exhibiting the biocompatibility of the nanovehicles (Fig. 9). Thus, combined with their inherent magnetic property, the HPNSs will open up a new avenue in in vivo delivery of drugs for killing cancer cells.

Conclusions

In summary, we have successfully prepared the magnetite HPNSs, and used it as drug delivery vehicles. The resulting magnetite HPNSs possess high magnetization, well-defined structure and porous accessible shells, which provided a significant step towards the exploitation for therapeutics. As presented by the preliminary resulted of TXL payload in antitumor efficacy by a sustained release of loaded TXL in Hela cell medium. These unique properties laid groundwork for magnetically guided in vivo diagnoses and therapy, which are in progress. Additionally, we expect that the magnetite HPNSs with useful magnetic
properties and unique microstructure may provide great promise for bioseparation and catalysis.

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