Mesoporous magnetic colloidal nanocrystal clusters (MCNCs) are featured with high magnetization, adequate surface area, excellent colloidal stability, good biocompatibility, and acid degradability. It is thus highly anticipated that MCNCs can serve as vehicles for target drug delivery. Herein, the mesoporous MCNCs stabilized by poly(γ-glutamic acid) (PGA) were fabricated by the modified solvothermal route, showing a high specific surface area (126.4 m²/g), strong magnetic response (63 emu/g) and appropriate mesoporosity including a large pore volume (0.27 cm³/g) and accessible pore size (8.1 nm). Docetaxel (DOC) was then loaded in the resultant MCNCs using the nanoprecipitation method, and a high drug loading capacity was achieved up to 24 wt%. The chemotherapeutic effect and mechanism of DOC-MCNC conjugates in bladder cancer was evaluated in vitro. A series of analyses for cell uptake, cell viability, cell cycle, cell apoptosis and some cell proteins were performed by transmission electron microscopy, MTT assay, flow cytometry, cell nuclei staining, Annexin V staining assay, western blot assay and caspase-3 activity assay, respectively. The results demonstrated that DOC-MCNC conjugates enhanced the inhibitory effect by hampering mitoschisis and increased the apoptotic effect by changing the expression of apoptosis-related proteins in T24 cells, substantially proving their remarkable efficiency in treatment of bladder cancer.

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imaging, and has improved the treatment of cancer.12 A wide range of nanoparticles, such as liposomes, stealth liposomes, polymeric nano/microspheres with equivalent stealth properties, polymeric nanocapsules, solid lipid nanoparticles, and inorganic nanoparticles (including mesoporous silica nanoparticles and magnetic nanoparticles) have been designed and constructed for drug delivery systems (DDS) by encapsulating or conjugating therapeutic agents. These drug delivery carriers not only allow controlled and sustained drug release, but also improve the efficacy and safety of cancer chemotherapy.23 Recently, hydrophobically hyperbranched polyglycerols (HPG-C8/10-PEG-NH₂) were used to load DOC, and showed promising efficacy and safety in bladder cancer.14,15

Of these nanomaterials, magnetic nanoparticles with distinctive magnetic susceptibility and the desired biocompatibility have attracted much attention in different biomedical fields, including DDS, biosensing, hyperthermia and magnetic resonance imaging.16–21 To optimize the performance of DDS, the magnetic nanoparticles require adequate surface areas to maximize guest loading content, as well as a sensitive magnetic response for fast enrichment and separation. However, it is very difficult to explore such magnetic materials as these two vital factors contradict each other. In this context, mesoporous magnetic colloidal nanocrystal clusters (MCNCs) stabilized by poly(γ-glutamic acid) (PGA) with high magnetization, adequate surface area, excellent colloidal stability, good biocompatibility, and acid degradability were synthesized by our team.22,23 PGA can improve water-dispersibility, manipulate the secondary structure and flexibly tune both the surface areas and pore sizes of MCNCs. The MCNCs were used as a hydrophobic DDS for loading paclitaxel, which showed significantly enhanced anticancer efficacy.22 Furthermore, DOC-loaded MCNCs (DOC-MCNCs) were synthesized and used in the treatment of prostate cancer. The results indicated that MCNCs with superior biocompatibility were a promising DDS for the treatment of prostate cancer.23 In the present study, the chemotherapy effect and mechanism of DOC-MCNCs in bladder cancer were evaluated in vitro.

MATERIALS AND METHODS

Materials and Apparatus

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. Poly(γ-glutamic acid) (Mₚ = 1000 kDa) was purchased from Dingshuxin Biotechnology Company (China). Docetaxel (DOC) was purchased from Knowshine Pharmachemicals Inc. (China). RPMI-1640, fetal bovine serum (FBS), penicillin G, streptomycin, and trypsinase were obtained from Beyotime Institute of Biotechnology (China). Dimethyl sulfoxide (DMSO) and [3-(4,5-dimethyl-1H-tiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) were purchased from the Sigma Company (USA).

Transmission electron microscopy (TEM) images were obtained using an H-600 (Hitachi, Japan) transmission electron microscope at an accelerating voltage of 75 kV. High-resolution transmission electron microscopy (HRTEM) images were obtained using a JEM-2010 (JEOL, Japan) transmission electron microscope at an accelerating voltage of 200 kV. Scanning electron microscopy (SEM) images were obtained using a TS-5136MM (TESCAN, Czech Republic) scanning electron microscope at an accelerating voltage of 20 kV. Powder X-ray diffraction (XRD) patterns were collected on a X’Pert Pro (Panalytical, Netherlands) diffraction meter with Cu Kα radiation at λ = 0.154 nm operating at 40 kV and 40 mA. Nitrogen adsorption–desorption measurements were performed on an ASAP2020 (Micromeritics, USA) accelerated surface area analyzer at 77 K. Before the measurements were obtained, the samples were degassed in a vacuum at 200 °C for at least 6 h. The Brunauer–Emmett–Teller (BET) method was used to calculate the specific surface areas. By using the Barrett–Joyner–Halenda (BJH) model, the pore size distributions were derived from the desorption branches of the isotherms, and the total pore volumes were estimated from the adsorbed volume at a relative pressure of 0.971. Magnetic characterization was carried out with a vibrating sample magnetometer on a Model 6000 physical property measurement system (Quantum Design, USA) at 300 K. 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 from 100 to 800 °C.

Synthesis of MCNCs

The solvothermal reaction was used to fabricate the MCNCs as previously reported.22,23 First, 1.35 g of FeCl₃·6H₂O and 3.85 g of NH₄Ac were dissolved in 70 mL of ethylene glycol to form a yellow solution. The mixture was then transferred into a three-necked flask (100 mL capacity), stirred vigorously and heated to 165 °C. Next, 0.8 g of PGA was added to the above mixture and the reaction was allowed to proceed for 1 h, during which the mixture gradually changed from yellow to dark brownish. The dispersion was instantly transferred into a 50 mL Teflon-lined stainless-steel autoclave and kept at 200 °C for 24 h. The product was separated by a magnet and rinsed several times with deionized water to remove the residue.

Preparation of DOC-Loaded MCNCs (DOC-MCNCs)

DOC was loaded into MCNCs using the nanoprecipitation method.23 Typically, 15 mg of MCNCs were dispersed in 5 mL ethanol solution and mechanically stirred for 2 h. Then 3 mL of the ethanol solution of DOC (3 mg/mL)
was added dropwise into the above solution. Next, the mixture was stirred for another 6 h until the solvent was fully evaporated to allow the DOC to penetrate into the pore channels of MCNCs. The DOC-loaded MCNCs were rinsed repeatedly with PBS (pH = 7.4) to remove surface-adsorbed DOC.

Cell Culture
Bladder cancer T24 cell lines were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai) and maintained in a 5% CO₂ incubator at 37 °C with a concentration of 50 mg/mL in RPMI 1640 containing 10% of fetal bovine serum and 100 μg/mL of penicillin. The cells were sub-cultured twice weekly.

Cell Uptake Study
T24 cells were incubated with MCNCs for 24 h at 37 °C. The cells were then washed with PBS, and fixed with 2.5% glutaraldehyde in cacodylate buffer at 4 °C. The washed cells were post-fixed with 1% osmium tetroxide at 4 °C for 1 h, and then dehydrated using a graded series of ethanol and placed in propylene oxide. After embedding and polymerization of the cells in epoxy resin at 60 °C for 48 h, ultrathin sections of the cells were cut using an ultramicrotome. The sections were then stained with lead citrate and observed by TEM at 120 kV.

Cell Viability Assay
Cell viability was measured using the MTT method. 200 μL of cells were seeded in a 96-well plate at a density of 4 × 10⁴ cells per well, and subsequently incubated for 24 h to allow attachment. Free-MCNCs, DOC, DOC-MCNCs were added to the culture medium. After incubation for 24 h, 48 h and 72 h, 20 μL MTT solution (5 mg/mL in PBS) were 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 570 nm on an ELISA reader.

Cell Cycle Assay
Cell cycle phases were analyzed by flow cytometry. T24 cells were plated at a density of 1 × 10⁶ cells per well for 24 h. After treatment, the cells were collected and fixed with ice-cold 70% ethanol overnight at −20 °C. Thereafter, the cell pellets were treated with 4 μg/mL propidium iodide solution containing 1% Triton X-100 and 100 μg/mL RNase for 30 min. To avoid cell agglomeration, the cell solutions were filtered through a nylon mesh membrane. Finally, the samples were analyzed using a flow cytometer (FACSCalibur, Becton-Dickinson, San Jose, CA, USA). At least 10000 cells were analyzed for DNA content. The percentage of cell cycle phases was quantified by ModFit LT software (Ver. 2.0, Becton-Dickinson).

Cell Nuclei Staining by Hoechst 33258
The morphology of cell nuclei in apoptotic cells was observed by Hoechst 33258 staining. Following treatment with Free-MCNCs, DOC and DOC-MCNCs, respectively, for 24 h, the cells were carefully and gently washed with PBS and fixed with 4% paraformaldehyde solution in PBS for 1 h at 37 °C. The nuclei were stained with 2.5 μg/mL Hoechst 33258 for 30 min. The nuclei of apoptotic cells were then observed under a fluorescence microscope (Leica TCS SP2).

Annexin V Staining Assay
Cell apoptosis rates were further evaluated using an AnnexinV-FITC detection kit (Beyotime, China). After treatment with Free-MCNCs, DOC and DOC-MCNCs, respectively, for 24 h, the cells were harvested with 0.25% trypsin and re-suspended in 100 μL PBS to achieve a concentration of 1 × 10⁶/mL. Then, following the manufacturer’s protocol, 5 μL Annexin V-FITC and 10 μL propidium iodide (PI) (20 μg/mL) were added and incubated in the dark for 15 min at room temperature. Finally, 400 μL of the binding buffer was added to each reaction tube before the cells were analyzed by FACScan flow cytometry (Becton-Dickinson, USA). The data were analyzed by Cellquest software (Becton-Dickinson, USA).

Western Blot Assay
After treatment with Free-MCNCs, DOC and DOC-MCNCs for 24 h, respectively, the cells were washed with PBS and harvested. Cell lysates were isolated using the protein extraction buffer (containing 150 mmol/L NaCl, 10 mmol/L Tris (pH 7.2), 5 mmol/L EDTA, 0.1% Triton X-100, 5% glycerol, and 2% SDS), and then incubated at 4 °C for 30 min. After centrifugation at 12,000 rpm for 30 min, the protein concentration in cell lysates was determined using the Bradford assay. Proteins were denatured in sample buffer containing 2-mercaptoethanol and bromophenol blue for 10 min at 95 °C. Equal amounts of proteins (50 μg) were fractionated using 8 or 12% SDS-PAGE and transferred to PVDF membranes. After blocking with 5% non-fat milk, the membranes were incubated overnight at 4 °C with the primary antibodies (bcl-2, bax, β-tubulin), the membranes were washed three times with PBS, and then incubated in secondary antibodies at room temperature. The intensity of target proteins was detected using the enhanced chemiluminescence detection system.

Caspase-3 Activity Assay
Caspase-3 activity was detected using the caspase-3 assay kit (Beyotime, China), according to the manufacturer’s protocol. Briefly, the cells were seeded into 96-well plates at 10⁵ cells/well. After being exposed to the sample for 24 h, the cells were washed with PBS. The cell lysates were isolated using the protein extraction buffer (containing 150 mmol/L NaCl, 10 mmol/L Tris (pH 7.2), 5 mM...
EDTA, 0.1% Triton X-100, 5% glycerol, and 2% SDS), and then incubated at 4 °C for 30 min. After centrifugation at 12,000 rpm for 30 min at 4 °C, the supernatants were used to measure the activities of caspase-3. 10 μL Ac-DEVD-pNA (2 mmol/L) and 90 μL caspase buffer were mixed to obtain a homogeneous caspase-3 reagent. 10 μL supernatants were diluted to 100 μL using homogeneous caspase-3 reagent and added to each well. The absorbance of pNA was measured at 570 nm on an ELISA reader. The activity-unit of caspase-3 was obtained by calculating the amounts of pNA.

RESULTS

Characterization of PGA-Stabilized MCNCs

The size and morphology of MCNCs were investigated by TEM and SEM (Figs. 1(a), (b)). MCNCs were spherical, approximately 148 nm in size and had a narrow size distribution in the polydispersity index (PDI) of 0.12. The cluster was composed of small nanocrystals approximately 5–8 nm in size. The powder X-ray diffraction (XRD) patterns revealed the crystallographic structure of the Fe₃O₄ clusters, showing diffraction peaks at 220, 311, 400, 422, 511 and 440, which were assigned to the typical peaks of the cubic structure of Fe₃O₄ (JCPDS 75-1610, Fig. 1(c)). The strengthened and sharp peaks revealed the high crystallinity of the products. Moreover, specific surface area and pore sizes were investigated by nitrogen adsorption–desorption isotherms (Fig. 1(d)), and the isotherms were identified as having mesopore characteristics (type IV) and displayed the H3 hysteresis loop. Brunauer–Emmett–Teller (BET) surface area and pore volume were calculated to be around 126.4 m²/g and 0.27 cm³/g, respectively. Furthermore, MCNCs exhibited a sharp distribution of open and accessible mesopores around 8.1 nm, advantageous for the penetration and storage of guest drugs. The ζ-potential value of MCNCs was measured up to −23.1 mV, which was capable of improving the colloidal stability by electrostatic repulsion effects. In addition to structure analysis, magnetic curves revealed a high magnetization value of 63 emu/g at 300 K.

Characterization of DOC-MCNCs

TG was used to determine the loading content of DOC in MCNCs (Fig. 2). After subtracting the weight loss of Free-MCNCs, a high drug loading capacity up to 24 wt% was achieved, indicating that the desired amount of drug was encapsulated and stored in the pore channels for further drug delivery.

The Effect of DOC-MCNCs on Cell Viability

Following treatment with 1 μmol/L DOC-MCNCs for 24 h, MCNCs were internalized by T24 cells and located in the perinuclear region (Fig. 3). The cell viability of T24 cells following treatment with Free-MCNCs, DOC, and DOC-MCNCs was analyzed by MTT assay. From Figure 4 it can be seen that treatment with 0.1–100 μmol/L Free-MCNCs for 48 h resulted in good biocompatibility and did

Figure 1. (a) TEM image, (b) SEM image, (c) powder XRD pattern, and (d) nitrogen adsorption–desorption isotherms of the MCNCs. Inset is the pore diameter distribution.
not induce significant cytotoxicity in T24 cells. In contrast, DOC and DOC-MCNCs (0.1–50 μmol/L for 48 h) significantly reduced cell viability in a concentration-dependent manner. However, when the concentration of DOC was more than 50 μmol/L, nearly 90% of T24 cells lost viability. We also measured the cell viability of T24 cells treated with Free-MCNCs, DOC and DOC-MCNCs for 24 h, 48 h, and 72 h, respectively (Fig. 5). The results indicated that these agents significantly reduced cell viability in a time-dependent manner. DOC-MCNCs enhanced this inhibitory effect in T24 cells compared with DOC alone. In T24 cells, the IC_{50} (50% cellular growth inhibition) value of DOC-MCNCs (about 0.5 μmol/L) was lower than that of DOC (more than 1 μmol/L).

The Effect of DOC-MCNCs on the Cell Cycle
To investigate the effect of DOC-MCNCs on the cell cycle, T24 cells were treated with Free-MCNCs, DOC and DOC-MCNCs for 24 h, respectively, and then analyzed by flow cytometry. Compared with untreated samples, Free-MCNCs had no effect on the cell cycle of T24 cells. Both DOC and DOC-MCNCs markedly decreased the G1 fractions and increased the G2 fractions in T24 cells. The average percentages of the G2 fractions were elevated to 50.1% after treatment with DOC-MCNCs compared with 17.6% before treatment. Compared with DOC alone, the average percentages of the G2 fractions were increased by 12.2% after treatment with DOC-MCNCs (Fig. 6).

The Effect of DOC-MCNCs on Cell Apoptosis
The morphology of cell nuclei in apoptotic cells was observed by Hoechst 33258 staining. From Figure 7 it can be seen that chromosome derangement was caused by DOC and DOC-MCNCs. However, Free-MCNCs did not induce aberrant chromosomes in T24 cells. The rates of cell apoptosis were further evaluated by flow cytometry.
DOC-MCNCs increased the rate of apoptosis of T24 cells. The flow cytometry results showed that Free-MCNCs did not induce apoptosis of T24 cells. An apoptosis rate of 20.6% was observed with DOC alone, while an apoptosis rate of 35.5% was observed when T24 cells were treated with DOC-MCNCs. The difference in apoptosis between these two groups was statistically significant (Fig. 8).

**DISCUSSION**

MCNCs were synthesized via a solvothermal reaction, by employing iron(III) chloride hexahydrate as the iron precursor, NH₄Ac as an electrostatic stabilization agent, ethylene glycol as a reductant and solvent, and PGA as a structure-directing agent. PGA served as the structure-driving tool to manipulate the morphological transformation of magnetic clusters from solid to mesoporous prototype. Moreover, PGA has been proved to be nontoxic and biocompatible towards cells, resulting in good biocompatibility of the MCNCs. DOC is a cytotoxic chemotherapeutic agent derived from the needles of *Taxus baccata*. It exerts its chemotherapeutic effect by stabilizing microtubules
against depolymerization, resulting in G2/M-phase cell cycle arrest and cell death.\textsuperscript{24} DOC is usually used in the treatment of prostate cancer and breast cancer, however, recent studies indicated the chemotherapeutic value of DOC in bladder cancer treatment.\textsuperscript{10, 11} Mugabe et al. reported that DOC-loaded HPG-C8/10-MePEG-NH\textsubscript{2} potently inhibited bladder cancer proliferation \textit{in vitro} and was the most effective formulation for inhibiting cancer growth in an orthotopic model of bladder cancer \textit{in vivo}.\textsuperscript{14, 15, 25} In this study, DOC-MCNCs were constructed and transferred into bladder cancer cells by endocytosis, and significantly induced cell death. More importantly, DOC-MCNCs exerted anticancer activity by inducing mitotic arrest and cell apoptosis. However, MCNCs alone did not induce damaging effects in bladder cancer cells. The same anticancer effect of DOC-MCNCs has been confirmed in prostate cancer.\textsuperscript{23} DOC-MCNCs provided stabilization, which prevented drug dissociation during the delivery process. MCNCs are potential nanomaterials in DDS for cancer drug delivery and therapy.

The toxicity of nanoparticles is a critical issue of concern in clinical applications.\textsuperscript{26} Haafeli et al. reported that the main toxicity factor is the polymer coating and not the magnetite core. Their findings indicated that short-chain copolymers were cytotoxic and much more so than long-chain copolymers.\textsuperscript{27} Our previous study indicated that non-drug-loaded MCNCs did not show any clear cytotoxicity against normal cells (HEK 293T cells), and the good biocompatibility of PGA-stabilized MCNCs was greater than MCNCs without PGA.\textsuperscript{22} Furthermore, non-drug-loaded MCNCs did not show any clear cytotoxicity against cancer cells. However, DOC-MCNCs significantly increased the chemotherapeutic effect of DOC. The effect of DOC-MCNCs on cell viability was time-dependent and concentration-dependent. In addition, the IC\textsubscript{50} value was significantly reduced with DOC-MCNCs compared with DOC alone. This indicated that MCNCs as the DDS could not only reduce the dose of DOC but also the side effects of DOC.

By analyzing the cell cycle, we found that the average percentage of the G2 fractions was increased by 12.2% after treatment with DOC-MCNCs compared with DOC alone. In addition, the results of western-blotting indicated that the expression of the tubulin subunit, \( \beta \)-tubulin, was significantly lower in cells treated with DOC-MCNCs compared with DOC alone. This suggested that following treatment with DOC-MCNCs, the tubulins would assemble rapidly into microtubules and the microtubules did not disassemble into tubulin subunits, resulting in G2/M-phase cell cycle arrest and the cells did not proceed to mitosis to inhibit cell growth. Our results were similar to those described by Hua and Liu et al.\textsuperscript{28, 29}

Based on the results of flow cytometry, Free-MCNCs did not induce apoptosis of T24 cells. The apoptosis rates of T24 cells after treatment with DOC and DOC-MCNCs were 20.6% and 35.5%, respectively, at 24 h. DOC-MCNCs increased the apoptotic effect of DOC in T24 cells. Apoptosis may occur through specific apoptosis signaling pathways such as death receptors and mitochondria which are regulated by the Bcl-2 protein family.\textsuperscript{30} It is well known that caspase-3 plays a central role in the initiation of apoptosis.\textsuperscript{31} Previous studies indicated that chemotherapeutic agent-loaded magnetic nanoparticles increased the apoptotic effect by changing the expression of the Bcl-2 protein family and increasing caspase-3 activity.\textsuperscript{32} Our study showed that DOC-MCNCs decreased the expression of Bcl-2 and increased the expression of Bax and caspase-3 in T24 cells. Our findings clearly indicate that MCNCs as a DDS can facilitate DOC accumulation in T24 cells and enhance apoptosis in these cells.

**CONCLUSION**

In conclusion, we have synthesized monodispersed PGA-stabilized MCNCs with adequate surface area and pore volume, strong magnetic response, and excellent biocompatibility. MCNCs served as a DDS to load a large amount of DOC (24 wt%), and exhibited enhanced anticancer efficacy against T24 cells \textit{in vitro} compared with DOC alone. Moreover, this study indicated that DOC-MCNCs enhanced anticancer efficacy by significantly hampering mitosis and inducing apoptosis of T24 cells. Based on these results, DOC-MCNCs are a new and effective treatment strategy for bladder cancer, and the evaluation of DOC-MCNCs in bladder cancer \textit{in vivo} is ongoing.

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