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Cancer therapy and fluorescence imaging using the active release of doxorubicin from MSPs/Ni-LDH folate targeting nanoparticles

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ABSTRACT

Hierarchical structured nanomaterials with diverse functionality, such as magnetic susceptibility, stimuli-responsiveness, environmental sensing and biocompatibility, are highly sought after for biomedicine and biodetection alike. In this study, we designed and fabricated a new kind of multi-functional core/shell nanospheres as biodegradable targeted drug carriers, the controlled drug release progress and therapeutic effect were monitored in-situ by the fluorescent state of the cells. Firstly, the core/shell nanospheres with biodegradability were synthesized using magnetic supraparticles (MSPs) as core and the layered double hydroxide (LDH) as shell via a hydrothermal route, the reaction parameters were well investigated to obtain the desired structure of the LDH shell. The anti-cancer drug doxorubicin was modified with carboxyl group (DOX-COOH) and loaded in the shell of MSPs/LDH nanospheres via an anion-exchange intercalation. To endow the nanospheres with tumor-targeting capability, IDA (imino-diacetic acid)-modified folate was successfully immobilized onto the surface of LDH shell using chelating interaction. These nanospheres behaved as multifunctional carriers for targeted delivery of anti-cancer drug, doxorubicin (DOX), within Hela cells and thus, these nano-drugs exhibited clear cytotoxicity and inhibition toward Hela cells as compared to normal cell-lines of HEK 293T cells. Interestingly, after the internalization of these nano-drugs, there was a sharp contrast in illumination between the tumorous Hela cells and the normal HEK 293T cells, the acidic cytoplasm of Hela cell stimulated DOX-COOH in LDH shell quickly degraded into positive-charged DOX, and then rapidly escaped from the positive-charged intercalation of LDH shell by strong repulsive interaction, the released DOX rapidly lit up the whole tumor cells in a short time, but only very weak light was found in HEK 293T cells.

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1. Introduction

Magnetic nano-composites with well-defined core/shell structures have attracted much attentions in recent years [1–5], owing to their unique properties and potential applications in biomedical area, such as magnetically enhanced rapid separation-enrichment [6,7], bio-imaging [8], targeted drug delivery [9,10] and gene transfection [11]. The inherent property, which allows their manipulation through the use of an external magnetic field to be performed in an environment of bio-system, is achieved by its magnetic core [12–14]. Furthermore, the shell can also be made 'tunable', meaning that researchers could tailor the functional shells, from functional polymers [15,16], inorganic oxides [17,18], or even a combination of these to ultimately enhance the key issues surrounding controlled release efficacy and biocompatibility. Meanwhile, the selective degradation in an intracellular environment also demands equal attention and there is a certain urgency in exploring functional materials, which could greatly reduce the cytotoxicity and accumulation of particles within the human body [19–21].

Layered double hydroxide (LDH) is a hydrotalcite-like material with exchangeable anions in the positive brucite-like interlayer, and it has been extensively investigated in the field of biomedicine due to its biocompatibility and colloidal stability [22–24]. Since the unique host–guest super-molecular structure, many therapeutic drugs, biomolecules and functional dyes have been shown to remain intercalated within the LDH structure via ion-exchange to form smart LDH nanohybrids [25–29]. Similarly, magnetic/LDH

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nano-composites have also been reported by Zhang group [30–32], prepared through *in-situ* co-precipitation, where the LDH shell wreathed the magnetic nanoparticle cores, and exhibited a pulsating drug release behavior under an ON/OFF alternate magnetic field [30,31]. Recently, they reported improved magnetic responsiveness by switching the magnetic nanoparticle cores with magnetic clusters [32]. Additionally, the LDH is easily degraded in acidic environment, demonstrating its biodegradable nature [33]. Therefore, magnetic LDH nano-composites stand-out as promising drug vehicles using magnetic manipulation for targeted drug delivery.

Compared with normal magnetic nanoparticles, the magnetic supraparticles (MSPs), consisting of magnetic nanocrystal aggregates, have attracted much attention due to their good colloidal stability and high magnetic responsiveness [34–38]. In these reports, poly(acrylic acid) and sodium citrate were usually used as the stabilizer to construct hydrophilic MSPs and improve the biocompatibility of MSPs [35,36]. Our previous work reported two types of MSPs that can be degraded in an acidic environment [37,38], these biodegradable magnetic supraparticles could supply as an ideal block for biodegradable magnetic nano-carriers after coated with organic or inorganic shell [6,39,40].

Herein, we combined these two types of acid-degradable nanomaterials to form MSPs/Ni-LDH core/shell magnetic nanospheres with MSPs as the magnetic core and Ni-LDH as the functional shell. The carboxylated anti-cancer drug doxorubicin (DOX-COOH) was loaded in the LDH shell via an anion-exchange intercalation. Through chelating interaction, IDA (iminodiacetic acid)-modified folate was immobilized on the surface of LDH shell and enables the MSPs/LDH core/shell magnetic nanospheres to demonstrate an ability to target Hela cells.

2. Materials and methods

2.1. Materials

Iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), nickel(II) chloride hexahydrate ($NiCl_2 \cdot 6H_2O$), aluminum nitrate nonahydrate ($Al(NO_3)_3 \cdot 9H_2O$), sodium hydroxide (NaOH), hydrochloric acid (HCl), ammonium acetate (NH_4Ac), ethylene glycol (EG), ethylene diamine ($NH_2(CH_2)_2NH_2$), folate (FA), anhydrous dimethyl sulfoxide and anhydrous ethanol were purchased from Shanghai Chemical Reagents Company (Shanghai, China) and used as received. Fluorescein isothiocyanate (FITC), glutathione (GSH), 2,3-dimethylmaleic anhydride, *N*,*N*-dicyclohexylcarbodiimide (DCC) and nitrilotriacetic acid (NTA) were purchased from Aladdin (Shanghai, China). *N*-Hydroxysuccinimide (NHS) and citraconic anhydride were purchased from Aldrich (Shanghai, China). Agarose was purchased from GENE TECH (Shanghai, China) Company. Doxorubicin (DOX) hydrochloride was purchased from Beijing Hua Feng United Technology CO., Ltd. (Beijing, China) and used as received. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin G, streptomycin, and trypsinase were obtained from GIBCO BRL (Grand Island, NY). Deionized water was used in all experiments. All other chemicals were commercially available and used without further purifications.

2.2. Preparation of magnetic supraparticles (MSPs)

The magnetic supraparticles (MSPs) were prepared through a facile solvothermal reaction [38]. Typically, 1.08 g $FeCl_3 \cdot 6H_2O$ was ultrasonic dissolved in 35 mL EG in a 50 mL three-necked bottle. The resulting yellow solution was mixed with 0.50 g agarose and 1.85 g NH_4Ac respectively by vigorous stirring at 160 °C for 1.5 h and then transferred into a Teflon lined stainless-steel autoclave (50 mL capacity). The autoclave was heated to 200 °C and maintained for 16 h. Then it was cooled to room temperature, the black MSPs were rinsed several times with ethanol and water under ultrasonic conditions to effectively remove the surplus agarose by magnetic separation, followed by drying in vacuum for 24 h.

2.3. Preparation of Ni/Al layered double hydroxide (Ni-LDH) nanospheres combining MSPs cores (MSPs/Ni-LDH)

MSPs/Ni-LDH core/shell nanospheres were synthesized by a hydrothermal method. Typically, 0.43 g $NiCl_2 \cdot 6H_2O$ and 0.23 g $Al(NO_3)_3 \cdot 9H_2O$ were dissolved in 28 mL water with vigorously stirring, followed by adjusting the solution pH to 10 by adding approximately 2 mL of 10% NaOH. 20 mg of as-prepared MSPs were dispersed with 10 mL of DI water in a 100 mL three-necked bottle; the green hydroxide colloidal solution was stepwise dropped, keeping the pH at 10 by NaOH solution, and then the suspension was ultrasonicated for 5 min to achieve homogeneous dispersion. Finally, 40 mL of the stable suspension was transferred into a Teflon lined stainless-steel autoclave (100 mL capacity) in 140 °C for 24 h. After the completion of the reaction, the autoclave was cooled to room temperature, and the orange product was rinsed with DI water to remove any free LDH and metal ions by magnetic separation. The resultant product was vacuum-dried over night for further use.

2.4. Preparation of folate modified MSPs/Ni-LDH (MSPs/Ni-LDH-folate)

The folate-chelated MSPs/Ni-LDH nanospheres were prepared via IDA chelated with Ni(II) [41]. The IDA modified folate was synthesized by reacting nitrilotriacetic acid (NTA) and aminated folate [42]. Firstly, 200 mg of NTA and an equi-molar dosage of NHS were completely dissolved in 55 mL of anhydrous DMSO. Meanwhile, 1.2 equiv DCC in 5 mL DMSO was prestored in dropping funnel, following by addition of the DCC solution dropwise within 30 min at 50 °C. After reacting for an additional 24 h, 1 equiv aminated folate and 1 mL pyridine were added and reacted for another 24 h. The *N*,*N*′-dicyclohexylurea (DCU) was removed by filtration, and the crude product was then purified through a repeat precipitation process using excess acetonitrile. The folate-modified IDA was collected after drying in vacuum overnight. After that, 1 mg IDA-FA was dissolved in 50 mL deionized water, and the solution pH was adjusted to 9–10 by the addition of 10% NaOH solution, and this was followed by dispersing 20 mg of MSPs/Ni-LDH nanospheres. This mixture was allowed to react for 24 h under stirring and under room temperature. The resultant was rinsed several times using DI water and dried in vacuum overnight for further use.

2.5. Fabrication of DOX or FITC loaded MSPs/Ni-LDH-folate

The carboxyl doxorubicin was firstly prepared according to the report of Matsumura et al. [43], where we altered the reactant from succinic anhydride to 2,3-dimethylmaleic anhydride. After this process, 2 mg carboxyl doxorubicin and 8 mg MSPs/Ni-LDH-folate were dispersed in 20 mL deionized water, where the pH was kept close to 10 by addition of NaOH solution. Then 20 mL suspension was transferred into a 50 mL Teflon lined stainless-steel autoclave for hydrothermal treatment at 110 °C for 24 h. After it was cooled to room temperature, the DOX@MSPs/Ni-LDH-folate was magnetically separated and was washed by DI water until the supernatant became transparent and colorless. The product was freeze-dried for 3 days. FITC-tagged MSPs/Ni-LDH (FITC@MSPs/Ni-LDH) and FITC-tagged MSPs/Ni-LDH-folate (FITC@MSPs/Ni-LDH-folate) were prepared using the same procedure, where the DOX was placed by the FITC molecule.

2.6. Acid-degradable experiment of MSPs/Ni-LDH nanospheres

30 mg MSPs/Ni-LDH nanospheres were dispersed in 150 mL 0.1 M Na_3Cit/H_3Cit buffer (pH = 5.0), and then was divided into fifteen equal parts in glass bottle by shaking in table concentrator at 160 rpm. The sample was taken out one by one in an interval of about 12 h; after magnetic separation by an external magnetic field, 8 mL supernatant was extracted and the concentration of the components in solution was detected by UV–vis and inductively coupled plasma (ICP) spectroscopy.

2.7. In vitro cell assay

In vitro cytotoxicities of MSPs/Ni-LDH-folate, DOX@MSPs/Ni-LDH-folate and free DOX were assessed on HEK 293T cells and Hela cells using the CCK8 method. Specifically, 100 μL of cells was 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 (0.01, 0.1, 1.0, 10 μg/mL) were added to each group (three wells) for 24 h. After removing previous nutrient solution, the cell was incubated in different 110 μL of DMEM containing 10 μL CCK-8 solution for 1 h. The absorbance of the suspension was measured at 450 nm on an ELISA reader. Cell viability was calculated by means of the following formula:

$$\text{Cell viability} = \frac{OD_{450(\text{sample})} - OD_{450(\text{blank})}}{OD_{450(\text{control})} - OD_{450(\text{blank})}} * 100\%$$

The cellular uptake behaviors of the MSPs/Ni-LDH-folate were confirmed by fluorescence microscopy (Olympus IX71, Tokyo, Japan). The Hela cells were incubated in a 24-well cell culture chamber with the MSPs/Ni-LDH-folate (0.1 mg/ml), FITC (0.01 mg/ml), FITC@MSPs/Ni-LDH (0.1 mg/ml) and FITC@MSPs/Ni-LDH-folate (0.1 mg/mL). At predetermined intervals (3, 6 and 24 h), the cells were washed with DMEM and PBS three times prior to fluorescence observation. FITC showed green fluorescence excitated at 488 nm.

The Hela cells and HEK 293T cells were incubated in a 24-well cell culture chamber with the free DOX (0.01 mg/ml), DOX@MSPs/Ni-LDH-folate (0.1 mg/mL) for 24 h. The cells were washed with DMEM and PBS three times prior to fluorescence observation. DOX showed red fluorescence excitated at 520 nm.

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