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Controllable release of nitric oxide and doxorubicin from engineered nanospheres for synergistic tumor therapy

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ABSTRACT

$NaYF_4$:Yb,Er upconversion nanoparticles (UCNPs) capped with long-chain carboxylic acid were synthesized and then conjugated with chitosan (CS) in the aid of *N*-hydroxysuccinimide. The resultant nanocompound was integrated with doxorubicin (DOX) and Roussin's black salt (RBS), a photosensitive nitric oxide (NO) donor to produce stimuli-responsive UCNPs(DOX)@CS-RBS nanospheres as nanocarriers for controllable drug delivery. On the one hand, the encapsulated UCNPs can efficiently absorb NIR photons and convert them into visible photons to trigger NO release. On the other hand, the entrapped DOX can be released at lowered pH from the swollen nanospheres caused by stretched oleoyl-CS chains under acidic conditions. The UCNPs(DOX)@CS-RBS nanospheres exhibit great therapeutic efficacy, which is attributable to the combination of NO and DOX releases based on NO dose-dependent mechanisms. This study highlights the controllable release of NO and DOX from the same nanocarriers and the synergistic therapeutic effect on tumors, which could give new insights into improving cancer nanotherapeutics.

Statement of Significance

In this paper, core-shell structured UCNPs(DOX)@CS-RBS nanospheres have been designed and synthesized via a step-by-step procedure. The stimuli-responsive UCNPs(DOX)@CS-RBS nanospheres act as nanocarriers for controllable drug delivery towards cancer therapy. The encapsulated UCNPs can efficiently absorb NIR photons and convert them into visible light to trigger NO release. Meanwhile, the entrapped DOX can be released from the swollen nanospheres caused by stretched oleoyl-CS chains at lowered pH typical of intracellular environment. Synergistic cancer therapy will be achieved through the combination of NO and DOX releases based on NO dose-dependent mechanisms. This study provides new drug nanocarriers with high antitumor efficacy for synergistic cancer therapy.

1. Introduction

Nitric oxide (NO) is an endogenously generated short-lived free radical that acts as a bioregulatory molecule in the body [1]. It plays a pivotal role in a variety of physiological and pathological processes [2]. NO has been reported as being able to modulate various cancer-related events such as cell cycle, apoptosis, angiogenesis, metastasis and invasion [3]. It has been suggested that NO has tumoricidal effects at appropriate concentrations and timing [4–6]. The likely mechanisms proposed for the anticancer properties of NO include suppression of DNA synthesis, proapoptotic modulator by activating caspase family proteases, expression alteration of apoptosis-associated proteins, etc. [7–9]. Moreover, it has been found that the resistance of cancer cells to chemotherapeutics may be reversed by NO by means of reducing P-glycoprotein (P-gp) expression levels [10,11]. Considering the potential of NO as a cancer therapeutic agent, developing controllable NO donors for targeted NO delivery attracts extensive interest. Photoexcited NO delivery has unique advantages in that it allows precise temporal and spatial control of NO release with few influences from the surroundings [12,13]. By tuning the photoexcitation signal, dose-controllable NO release to specific physiological targets could be achieved. Compared with visible light and

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ultraviolet (UV), near-infrared (NIR) light is preferable for photogeneration of NO as it has great penetration depth in and negligible damage to tissues [14]. Researchers have devoted their efforts to the development of NIR-triggered NO delivery systems [15–17]. In their NO-generating nanovehicles, upconversion nanoparticles (UCNPs) were used as photoactive centers that harvest NIR photons and upconvert them into visible light. The incorporated Roussin's black salt (RBS) absorbs the visible photons and releases NO via photochemical reaction [18].

As a cationic polysaccharide, chitosan (CS) has been extensively used for various biomedical applications [19–21]. The pK_a of CS is 6.0–6.5 in aqueous media [22], and the charged state and physiochemical properties of CS are significantly influenced by the ambient environmental pH [23]. CS has been found to form dissociated precipitates in aqueous phase at physiological pH of 7.4 due to rapid local aggregation of CS polymeric chains [24]. Sung's group fabricated a comblike associating polyelectrolyte by conjugating a hydrophobic palmitoyl group onto the free amine groups of CS [23,25]. Through balancing charge repulsion and hydrophobic interaction, the chain conformation of the associating polyelectrolyte can be controlled simply by adjusting the environmental pH within a narrow range. Inspired by their work, we have intended to design a pH-sensitive nanostructure composed of a UCNP core and a CS shell for drug delivery purposes. This core-shell nanostructure could be realized by conjugating the organic ligands capping the UCNP with the amine groups of CS.

In the current work, we report preparation of CS-encapsulating $NaYF_4$:Yb,Er UCNPs with entrapped doxorubicin (DOX) and attached RBS for synergistic cancer therapy. Oleic acid-capping $NaYF_4$:Yb,Er UCNPs were synthesized, reacted with N-hydroxysuccinimide and conjugated with CS at some of the amino sites. Coexisting with DOX in aqueous solution, the hydrophobic oleoyl groups tended to form local aggregates and entrap DOX by hydrophobic interaction, producing UCNPs(DOX)@CS nanospheres spontaneously. The core-shell structured nanospheres were further conjugated with the RBS $[NH_4][Fe_4S_3(NO)_7]$ via electrostatic interaction (Fig. 1a). In the resultant UCNPs(DOX)@CS-RBS nanospheres, the UCNPs act as antennae to receive NIR photons and convert them into visible photons, which sensitize the attached RBS to trigger NO release. On the other hand, the oleoyl-CS chains are sensitive to environmental pH changes. At pH $\geq$ 7.0, the DOX is entrapped in the nanospheres owing to the strong hydrophobic interaction between the oleoyl groups. At a low pH, the protonated amine groups increase the charge repulsion between the oleoyl-CS chains. As a result, the entrapped DOX is released as the chains expanded (Fig. 1b). The developed UCNPs(DOX)@CS-RBS nanospheres in this work are aimed at synergistic tumor therapy through dose-controllable NO generation in combination with pH-responsive antitumor drug release.

2. Experimental

2.1. Materials

NaOH (>98%), NH_4F (99%), $YCl_3{\cdot}6H_2O$ (99.99%), $YbCl_3$ (99.99%), $ErCl_3$ (99.99%), 1-octadecene (98%), oleic acid (99%), *N*-hydroxysuccinimide (NHS, 99%), fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), doxorubicin hydrochloride (DOX·HCl, >98%) and *N,N′*-dicyclohexylcarbodiimide (DCC, 98%) were purchased from Sigma-Aldrich. Chitosan (CS, M_w = 35000, degree of deacetylation = 88.7%, pKa = 6.09) was purchased from Sinopharm Chemical Reagent Co., Ltd, China, and used without further purification. Roussin's Black Salt (RBS, $[NH_4][Fe_4S_3(NO)_7]$) was prepared following the procedure reported by Seyferth et al. [26]. The RBS was stored in the dark and inert atmosphere. Thiolated oleic acid was prepared in our own lab. Millipore water was used in all experiments. All other chemicals and solvents were provided by Sinopharm Chemical Reagent Co., Ltd and used as received without purification (analytically pure).

2.2. Synthesis of $NaYF_4$:Yb,Er UCNPs

$NaYF_4$:Yb,Er UCNPs were synthesized according to the following procedure: 1.56 mmol of anhydrous YCl_3, 0.40 mmol of $YbCl_3$ and 0.04 mmol of $ErCl_3$ were added to 12 mL of oleic acid and 15 mL of 1-octadecene under stirring. The mixture was heated to 150 °C and held for 30 min under vacuum to remove oxygen and water before cooling down to room temperature. Then 5 mmol of NaOH and 8 mmol of NH_4F were added and the resultant solution was stirred at room temperature for 30 min. Thereafter, the solution was transferred to an autoclave and heated to 300 °C in argon atmosphere for 1 h and cooled down naturally to room temperature. Precipitates were obtained by adding excessive ethanol and centrifuging at 6830*g* for 15 min. Subsequently, a mixture of as-synthesized $NaYF_4$:Yb,Er UCNPs, 15 mL of thiolated oleic acid, 20 mL of 1-octadecene and 20 mmol of ethanol were stirred at room temperature for 32 h for ligand exchange. The oleic acid-capping $NaYF_4$:Yb,Er UCNPs were obtained by centrifugation at 20,490*g* for 20 min, washing with ethanol, and dialysis against water.

2.3. Preparation of UCNPs(DOX)@CS-RBS nanospheres

The as-synthesized UCNPs were mixed with 35 mmol of NHS in 20 mL of anhydrous dimethyl formamide (DMF), to which 70 mmol of DCC was added slowly and the mixture was allowed to react under stirring for 24 h at room temperature in nitrogen atmosphere. Subsequently, the mixture was filtered, washed by ethyl ether thoroughly and rotation-evaporated to obtain UCNPs capped by oleic acid *N*-hydroxysuccinimide ester. The modified UCNPs were dispersed in ethanol, which was then added dropwise to 20 mL of a CS (0.2 g)/aqueous acetic acid (1 wt%) solution at 95 °C to react for 36 h under stirring. The resultant mixture was cooled down to room temperature and precipitated by adding acetone and adjusting pH to 9.0. The precipitates were filtered, washed with acetone for three times, air-dried and redispersed in aqueous acetic acid. The degree of substitution on CS was 11.1 ± 0.2% (n = 5) based on ninhydrin assay [24]. Thereafter, DOX·HCl was added into the above aqueous acetic acid solution of UCNPs@CS at room temperature. The preset weight ratio of DOX to UCNPs@CS was 1:10. The resultant solution was stirred in the open air for 10 min, whose pH was then adjusted to 7.4. After stirring for another 30 min, the solution was dialyzed against water (pH 7.4) to remove free DOX.

At room temperature, 50 mg of the obtained UCNPs(DOX)@CS was redispersed in 20 mL of aqueous acetic acid in a brown vial, where 20 mL of RBS aqueous solution (0.5 mg/mL) was added and the mixture was stirred for 10 min in the dark under nitrogen protection. Then the pH of the mixture was adjusted to 7.4, and stirring was continued for another 5 h. The resultant UCNPs (DOX)@CS-RBS nanospheres were obtained by centrifugation at 6830*g* for 10 min, washed thoroughly with ethanol for three times, and dried under vacuum for 3 h.

Loading content (LC) and loading efficiency (LE) of DOX were calculated using the equations as follows:

$$LC_{DOX} = (\text{weight of loaded DOX}/\text{weight of nanospheres}) \times 100\%$$

$$LE_{DOX} = (\text{weight of loaded DOX}/\text{weight of DOX in feed}) \times 100\%$$

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