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Fabrication of doxorubicin and heparin co-loaded microcapsules for synergistic cancer therapy

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ABSTRACT

In this study, a layer-by-layer (LbL) assembly $(HEP/CHI)_5$ microcapsule with doxorubicin hydrochloride (DOX) encapsulating inside was fabricated *via* alternatively depositing heparin (HEP) and chitosan (CHI) onto DOX-loaded $CaCO_3$ templates. The microcapsules were of stable architecture and had good dispersity in aqueous medium. Fluorescence observation showed that DOX distributed both in the wall and in the cavity of microcapsules, while HEP presented in the capsule wall. The release rate of DOX increased at acidic pH as compared with that at basic pH, suggesting a pH-responsive drug release behavior. The microcapsules with positively charged CHI lying on the outer layer could protect HEP from heparanase degradation and achieve intracellular co-delivery of both DOX and HEP. Thus, the DOX-loaded microcapsules could have improved inhibition activity against A549 cells by combining pharmacological actions of DOX and HEP.

1. Introduction

Layer-by-layer (LbL) assembly microcapsules are promising drug vehicles with many advantages [1–3]. A commonly used strategy of constructing multilayered capsule is the alternate deposition of oppositely charged polymers onto colloidal substrates (*e.g.*, calcium carbonate and silica particles) [4,5]. Therefore, to meet practical requirements, the morphological features of microcapsules, like size and shape, can be simply regulated by using suitable templates for the deposition of polymers [6–8]. As a large number of water-soluble polyelectrolytes can be used to create the capsule wall, the assembly can be entirely performed in aqueous solution [9]. Thus, the assembly process is beneficial for biomolecules such as proteins, nucleic acids and polysaccharides, which have limited solubility in organic medium and are susceptible to deterioration [10,11].

Generally, these polymeric capsules can load different types of drugs, *i.e.*, small molecules or biomolecules, and facilitate the circulation of these therapeutic agents in bloodstream [12–14]. Some recent reports demonstrate that polymeric capsules can protect normal tissue from side effects of toxic chemotherapy drugs, like doxorubicin (DOX) [15], or protect DNA and protein drugs from enzymatic degradation during the blood circulation [16]. Moreover, microcapsules can load drugs in different regions. For example, small molecules can be encapsulated into the cavity; biomolecules with charged property can be used as a polyelectrolyte and loaded into the multilayered wall of microcapsules [17–19]. Thus, it inspires to incorporate different drugs with different mechanisms of action into the same carrier and deliver these drugs into a single target to achieve synergistic effect [20].

After years of research, it is found that heparin (HEP), a commonly used antithrombotic agent, can interfere with the action of transcription factor, and thus lead to cell apoptosis when HEP is transported into tumor cell [21]. As HEP is a highly sulfated glycosaminoglycan, the negatively charged nature makes it hard to undergo cellular uptake [22]. Nevertheless, recent reports also demonstrate that positively charged vehicles are able to overcome this barrier and transport HEP across cell membranes [23,24]. Herein, chitosan (CHI), a natural polysaccharide, was employed as the positively charged polyelectrolyte to form the $(HEP/CHI)_5$ multilayered capsule with HEP. It is expected that CHI locates in the outer layer of microcapsules can protect HEP from heparanase, which exists in the extracellular milieu of tumor tissue, and facilitates the intracellular delivery of HEP. Besides, anticancer drug DOX was also encapsulated for the combination therapy (Scheme 1). Since calcium carbonate ($CaCO_3$) can easily be removed in a mild condition of ethylenediaminetetraacetic acid (EDTA),

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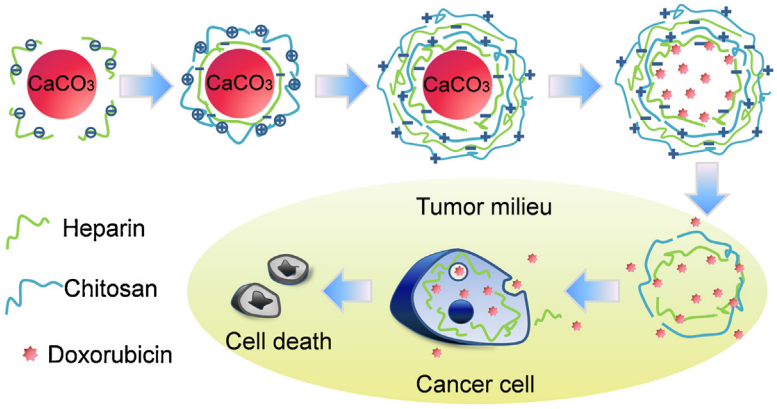

Scheme 1. Fabrication of DOX and HEP co-loaded microcapsules for cancer therapy.

$CaCO_3$ particles were used as the sacrificial template for fabricating the microcapsule and preloading DOX inside [17]. Because of the semipermeable nature of multilayered wall, DOX is anticipated to show a controlled release behavior.

2. Materials and methods

2.1. Materials

Heparin (Mw: 15 kDa), fluorescein isothiocyanate (FITC), was provided by Shanghai Sangon Bio-engineering Co. (China). Chitosan (Mw: 50 kDa, deacetylation degree: 91%) was obtained from Zhejiang Aoxing Biotechnology Co. (China). Heparanase (5 U/ml) was stored in the laboratory. K_2CO_3, $CaCl_2$, DMSO and ethylenediaminetetraacetic acid (EDTA) were purchased from Shanghai Reagent Chemical Co. (China) and used as-received. Doxorubicin hydrochloride (DOX) was purchased from Zhejiang Hisun Pharmaceutical Co. (China). RPMI-1640 medium, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT), fetal bovine serum (FBS), penicillin, streptomycin, and trypsin were purchased from Invitrogen Co. All other reagents and solvents were of analytical grade and used directly.

2.2. Preparation of $CaCO_3$ particles

Briefly, 5 mL of K_2CO_3 aqueous solution (0.33 M) was rapidly poured into 5 mL of $CaCl_2$ aqueous solution (0.33 M) with intense agitation for 30 s. Thereafter, the precipitate was stirred for 2 min and then filtered off. After washing with DI water and acetone in succession, desired particles were dried in air and reserved at 4 °C. The preloading of DOX was achieved by adding 2 mg of DOX into K_2CO_3 solution beforehand. The morphology of $CaCO_3$ particles was observed by a Hitachi S4800 scanning electronic microscope (SEM; Japan).

2.3. Preparation of $(HEP/CHI)_5$ microcapsules

The LbL assembly of $(HEP/CHI)_5$ microcapsules was performed by alternatively depositing HEP and CHI onto the $CaCO_3$ colloidal template. Briefly, 150 mg of $CaCO_3$ particles and 1 mL of HEP solution (2 mg/mL) were mixed and gently shaken for 15 min to establish the HEP layer. After centrifugation (10,000 rpm, 1 min), the particles were separated, followed by washing with DI water for three times. The rinsed precipitate was then dispersed in 1 mL of CHI solution (2 mg/mL) to reach the equilibrium of adsorption. The particles were separated by centrifugation and were washed with DI water three times. The adsorptions of HEP and CHI were then repeated to form the designed $(HEP/CHI)_5$ microcapsule. Subsequently, precipitates were allowed to suspend in EDTA solution (pH 7.4, 0.4 M) for 30 min to remove $CaCO_3$ templates. The microcapsules were isolated by centrifugation (10,000 rpm, 1 min) and washed with DI water carefully. The DOX-loaded microcapsules were obtained by using DOX-loaded $CaCO_3$ particles as the template. FITC-labeled HEP was used instead of HEP for further fluorescent observation and marked as HEP as well. Note that the entire process avoids light irradiation as far as possible. Briefly, the FITC-labeled HEP was prepared by dissolving HEP and FITC into water and stirring for 24 h, and the product was then obtained by dialyzing against water and lyophilization.

2.4. Morphology characterization

The morphology of microcapsules was observed on a JEM-2100 transmission electron microscope (TEM; JEOL, Japan) and a C1-si confocal laser scanning microscope (CLSM; Nikon, Japan). For TEM observation, the sample suspension was dropped onto a copper grid and stained with 0.2% (w/v) sodium phosphotungstate solution. The ζ-potential of each layer was determined on a Zetasizer Nano ZS apparatus (Malvern Instruments, UK) during the LbL assembly process at room temperature.

2.5. Stability measurement

Heparanase was used as the model enzyme to evaluate the stability of $(HEP/CHI)_5$ microcapsules. Lyophilized microcapsules (25 mg) were, respectively, dispersed into 1 mL of heparanase solution (5 U/mL, pH 7.4) and PBS (pH 7.4) followed by incubation at 37 °C. At the assigned time interval, the morphology of microcapsules was viewed under a DMIL LED-inverted fluorescence microscope (Leica, Germany). $(CHI/HEP)_5$ microcapsules were used as the control to estimate whether the designed microcapsule could protect HEP from heparanase degradation.

2.6. In vitro drug release assay

For testing DOX release behavior, 450 mg of DOX-loaded $CaCO_3$ particles coated with $(HEP/CHI)_5$ multilayered films were suspended in 12 mL of EDTA solution (pH 7.4, 0.4 M) to remove the $CaCO_3$ core. After isolation, the microcapsules were re-dispersed in 3 mL of DI water, and then equally divided into three parts and transferred into three dialysis tubes (MWCO 3500 Da), respectively. The dialysis tubes were then immersed into 10 mL of buffer solutions with different pH of 5.0, 7.4, and 10.0 at 37 °C. At the assigned time interval, sample solutions were withdrawn and 10 mL of fresh