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Intracellularly monitoring/imaging the release of doxorubicin from pH-responsive nanoparticles using Förster resonance energy transfer

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ABSTRACT

Stimuli-responsive nanoparticles (NPs) have been receiving much attention as a drug-delivery vehicle for therapeutic applications; once internalized into cells, the intracellular fate of NPs and their drug release behavior in response to local stimuli must be understood for efficient delivery of therapeutics. In this study, we prepared pH-responsive doxorubicin (DOX)-loaded NPs, made of *N*-palmitoyl chitosan bearing a Cy5 moiety (Cy5–NPCS), as an anticancer delivery device. The results of our molecular dynamic simulations showed that the ability of Cy5–NPCS to self-associate offered the close proximity between the donor (DOX) and the acceptor (Cy5) required for Förster resonance energy transfer (FRET), while the pH-driven structure transition prescribed the on-to-off switch of the energy transfer. The caveolae-mediated pathway played a major role in the internalization of NPCS NPs. Using the concept of FRET, we found that the DOX fluorescence in the cytosol was first seen when NPCS NPs were present in the slightly acidic early endosomes. Following NPCS NPs trafficking into a more acidic organelle (late endosomes/lysosomes), a more evident release of DOX into the cytosol was observed; the released DOX was then gradually accumulated in the cell nuclei, leading to a significant cytotoxicity. Understanding the fate of NPs with respect to their intracellular localization and drug release behavior is crucial for the rational design of drug carriers.

1. Introduction

Chitosan (CS), a cationic polysaccharide, has been used extensively for various biomedical applications [1]. It is known that the p*K*a of CS is approximately 6.5 [1,2], and the charged state and physiochemical properties of CS are substantially influenced by its environmental pH. In aqueous media at pH 7.4, CS forms dissociated precipitates because the aggregation of CS polymers occurs too rapidly and locally [3]. To enhance the intermolecular contact of CS molecules while retaining their pH-sensitivity, we conjugated a hydrophobic palmitoyl group onto the free amine groups of CS, *N*-palmitoyl CS (NPCS), in a previous study [4]. NPCS is a comblike associating polyelectrolyte characterized by the presence of alternating charges (protonated amine groups) and hydrophobic side chains (palmitoyl groups, Fig. 1). Through a proper balance between charge repulsion and hydrophobic interaction, this associating polyelectrolyte can undergo a rapid hydrogelation triggered simply by its environmental pH within a narrow range.

In dilute aqueous media, we found that NPCS polymers were able to self-assemble into nanoparticles (NPs), due to the hydrophobic interaction between their conjugated palmitoyl groups. NPs made from hydrophobically-modified polymers have been used as a drug-delivery vehicle [5–7]; previous studies have shown that they could accumulate passively in the tumor tissue for therapeutic applications [6,7].

A variety of forms of endocytosis have been demonstrated to be involved in the cellular uptake of polyplexes [8,9]. Current evidences suggest that endocytosis is the main mode of CS-based NPs entering into the cells [10]. In this study, we prepared pH-responsive NPCS NPs encapsulated with doxorubicin (DOX) as an anticancer delivery device; their drug release mechanism intracellularly was studied. The drug release mechanisms from delivery carriers were mostly conducted *in vitro* in simulating release media. Using polymeric micelles loaded with DOX, the intracellular drug release behaviors were reported by a few groups [11,12]. However, they were not able to locate the micelles intracellularly after endocytosis, and when/where the drug was released. Understanding the fate of test particles with respect to their intracellular localization and drug release mechanism is crucial for the rational design of drug carriers [13].

The prepared NPs were characterized using dynamic light scattering (DLS) and their cytotoxicity was evaluated by the MTT

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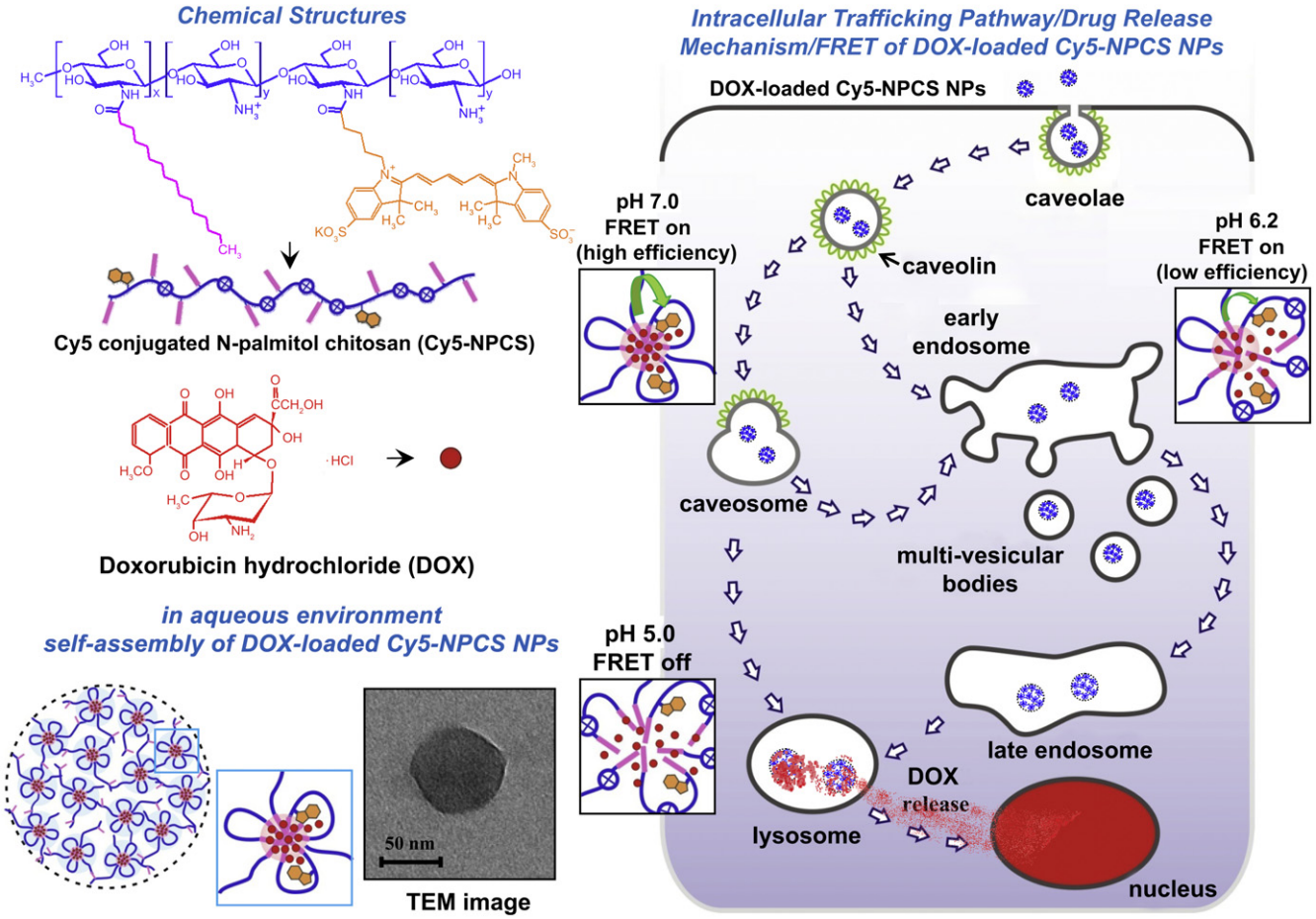

Fig. 1. Schematic illustrations of the concept of the study. pH-responsive doxorubicin (DOX)-loaded nanoparticles (NPs), made of *N*-palmitoyl chitosan bearing a Cy5 moiety (Cy5–NPCS), were prepared as an anticancer delivery device. Using the technique of Förster resonance energy transfer (FRET), the drug release behavior of DOX-loaded Cy5–NPCS NPs can be monitored/imaged intracellularly.

(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. The conformation transition of NPCS NPs in response to the environmental pH was modeled by molecular dynamic (MD) simulations. The emission spectra and dual-emission images of fluorescent DOX-loaded Cy5–NPCS NP suspensions at distinct pH values were determined by a fluorescence spectrometer and an *In Vivo* Imaging System (IVIS), respectively. The *in vitro* release profile of DOX from Cy5–NPCS NPs was examined in different release media; their intracellular drug release mechanism was monitored/imaged using Förster resonance energy transfer (FRET).

2. Materials and methods

2.1. Materials

CS (viscosity 5 mPa s, 0.5% in 0.5% acetic acid at 20 °C, MW 50 kDa) with a degree of deacetylation of approximately 85% was purchased from Koyo Chemical Co. Ltd. (Tokyo, Japan). Palmitic acid *N*-hydroxysuccinimide (NHS) ester and DOX hydrochloride were obtained from Sigma–Aldrich (St. Louis, MO, USA). NHS-functionalized cyanine 5 (Cy5–NHS) and fluorescein (fluorescein–NHS) were acquired from Amersham Biosciences (Piscataway, NJ, USA) and Thermo Scientific (Chicago, IL, USA), respectively. All other chemicals and reagents used were of analytical grade.

2.2. Synthesis of NPCS and preparation of fluorescent NPCS

The procedure used for the synthesis of NPCS with different degrees of substitution (DS; NPCS-5%, NPCS-10%, NPCS-15% and NPCS-20%) was described in details in our previous report [4]. The synthesized NPCS was analyzed by the proton nuclear magnetic resonance and Fourier transformed infrared spectroscopy, and the DS on NPCS was determined by the ninhydrin assay; the results were presented previously [4].

Cy5-labeled NPCS (Cy5–NPCS) and fluorescein-labeled NPCS (fluorescein–NPCS) were synthesized as per the methods described in the literature [14].Briefly, a solution of Cy5–NHS or fluorescein–NHS in dimethyl sulfoxide (DMSO, 1 mg/mL) was prepared and added gradually into an aqueous NPCS (2 mg/mL) while stirring; the weight ratio of fluorescent dye to NPCS was kept at 1:50 (w/w). The reaction was maintained at pH 5.5 and stirred continuously for 12 h in the dark. To remove the unconjugated fluorescent dyes, the synthesized Cy5–NPCS and fluorescein–NPCS were dialyzed in the dark against deionized (DI) water and replaced on a daily basis until no fluorescence (Cy5 or fluorescein) was detected in the supernatant. The resultant Cy5–NPCS and fluorescein–NPCS were then lyophilized in a freeze dryer.

2.3. Preparation and characterization of Cy5–NPCS NPs

The synthesized Cy5–NPCS polymers with different DS were individually dissolved in 1% aqueous acetic acid and its pH value was adjusted to 4.0 by adding a few drops of 1 N NaOH under magnetic stirring to form test NPs. The size distributions and zeta potential values of the prepared NPs at predetermined pH values (adjusted by phosphate buffer) were investigated using DLS (Zetasizer 3000HS, Malvern Instruments Ltd., Worcestershire, UK).

2.4. Preparation and characterization of DOX-loaded Cy5–NPCS NPs

DOX was added into a Cy5–NPCS solution (aqueous acetic acid); the weight ratio of DOX to Cy5–NPCS was 1:5. After stirring in the dark for 10 min, the pH of the mixture was adjusted to 7.4, and the mixture was stirred in the dark for another 20 min to form NPs. Subsequently, the mixture was dialyzed in the dark against DI water (pH 7.4) to remove free DOX in the solution. The morphology of the prepared NPs was examined by transmission electron microscopy (TEM, JEOL 2010F, Tokyo, Japan). The loading efficiency of DOX in NPs was determined by assaying the amount