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Dual-targeting and pH/redox-responsive multi-layered nanocomplexes for smart co-delivery of doxorubicin and siRNA

Lu Han, Cui Tang, Chunhua Yin*

State Key Laboratory of Genetic Engineering, Department of Pharmaceutical Sciences, School of Life Sciences, Fudan University, Shanghai 200433, China

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ABSTRACT

Multi-layered nanocomplexes (MLNs) were designed here to provide smart co-delivery of doxorubicin (DOX) and vascular endothelial growth factor (VEGF) siRNA. The electrostatically self-assembled MLNs were constructed by TAT peptide modified mesoporous silica nanoparticles (TAT-MSN) as the cationic core for DOX loading, poly(allylamine hydrochloride)-citraconic anhydride (PAH-Cit) as the anionic inner layer, and galactose-modified trimethyl chitosan-cysteine (GTC) conjugate as the cationic outer layer to encapsulate siRNA. Their strong stability at pH 7.4 and 6.5 protected siRNA from degradation in the blood and tumor microenvironment. Galactose ligands on the GTC outer layers effectively facilitated the internalization of MLNs through receptor-mediated endocytosis. Afterwards, the endosomal/lysosomal acidity (pH 5.0) triggered the charge reversal of PAH-Cit, thereby inducing the disassembly of MLNs and their escape to the cytosol. Cytoplasmic glutathione further accelerated siRNA release through cleaving disulfide bonds in GTC layers, leading to high silencing efficiencies. Meanwhile, the exposed DOX-loaded cores were transported into the nuclei by virtue of TAT peptide and exhibited sustained release thereafter. As a result, potent antitumor efficacies of MLNs were noted following intravenous injection at a low dose with no apparent toxicity detected. Therefore, MLNs served as an effective and safe vector to maximize synergistic effect of chemodrugs and therapeutic genes.

1. Introduction

Cancer cells continually secrete diverse cytokines, such as the vascular endothelial growth factor (VEGF), to activate cancerous angiogenesis which in turn augments the supply of nutrients and oxygen for their rapid proliferation [1,2]. Such concomitant progression of angiogenesis and proliferation represents the major hallmark of most cancers [3,4], making them notoriously refractory to treatments aiming at one single target. To solve this problem, the cocktail therapy that combines cytotoxic chemotherapeutics with anti-angiogenesis genes has been adopted to simultaneously kill off cancer cells and block vascularization. A well-known example is the co-administration of doxorubicin (DOX) and VEGF siRNA (siVEGF) [5–7]. However, the instability and non-selectivity of these anticancer entities have seriously restricted their application [8].

The flexibility of nanotechnology provides opportunities to address the above challenges by introducing multiple functional moieties [9]. A myriad of nanocarriers have demonstrated highly efficient performance in terms of single delivery of either chemodrugs or genes [10–12]. Unfortunately, when co-delivering chemodrugs (*e.g.* DOX) with genes (*e.g.* siVEGF), current vectors, although effective, are still far from clinical requirements. The major difficulty underlying their incapability stems from the different mechanisms of action of different drugs [13]. For example, rapid dissociation of siRNA from vectors in the cytosol is a prerequisite for eliciting effective silencing of target mRNA [14], while sustained release of DOX to the nucleus is preferable for provoking long-term interruption against DNA replication [15]. However, previously reported co-delivery systems for DOX and siRNA often underwent similar intracellular kinetics in cancer cells for both drugs, *i.e.* releasing them in the same subcellular compartment at the same time [16–25]. Such delivery behaviors could not coordinate well with the different mechanisms of DOX and siRNA. Their incapability to provide individually on-demand delivery inevitably resulted in limited synergistic effect and thus high consumption of both drugs [16–18,20,22]. To achieve desired antitumor efficacies at a clinically preferable dosage, it is of great challenge and urgent demand to develop a co-delivery system that can precisely deliver DOX and siRNA to the nucleus and cytosol, respectively, and release

* Corresponding author.
E-mail address: chyin@fudan.edu.cn (C. Yin).

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them in an individually on-demand manner thereafter.

To this end, we herein designed and evaluated a multifunctional nanosystem based on layer-by-layer self-assembly for the intelligent co-delivery of DOX and siVEGF. As illustrated in Fig. 1a, these multi-layered nanocomplexes (MLNs) with a positive/negative/positive structure were constructed by TAT peptide modified mesoporous silica nanoparticles (TAT-MSN) as the cationic core for DOX loading, poly(allylamine hydrochloride)-citraconic anhydride (PAH-Cit) as the anionic inner layer, and galactose-modified trimethyl chitosan-cysteine (GTC) conjugate as the cationic outer layer to encapsulate siRNA. Amino-functionalized MSN were adopted here owing to their high surface area and large pore volume for drug loading. Their surface was further decorated with HIV-1 TAT peptide, a nuclear localization signal [26], for active nuclear transport of DOX. PAH-Cit, the inner layer, was a charge-reversible polymer that can be rapidly converted from negative (PAH-Cit) to positive (PAH) *via* side chain hydrolysis at pH < 6.0 [27]. Accordingly, MLNs were expected to be structurally stable in the blood (pH 7.4) and tumor extracellular matrix (pH 6.5) to prevent the premature loss of drugs, while rapidly disassembled in response to the intracellular acidity (pH 5.0) to activate the individually on-demand delivery of DOX and siRNA in cancer cells. Meanwhile, such charge reversal process was normally accompanied by abundant proton influx [28], thus endosomal/lysosomal escape could be achieved *via* the "proton sponge" effect. GTC conjugate was a multifunctional vector we recently developed for siRNA delivery [29], wherein the trimethyl, galactose (Gal), and cysteine groups respectively endowed this polymer with desired binding affinity for siRNA *via* electrostatic interactions, targeted entry into hepato-carcinoma cells by recognizing asialoglycoprotein receptors, and accelerated intracellular release of siRNA through the reductive cleavage of disulfide bonds by cytoplasmic glutathione (GSH).

With the abovementioned designing strategies, MLNs were synthesized here to integrate multiple essential attributes in a smart fashion as illustrated in Fig. 1b, including sufficient extracellular stability, active entry into hepato-carcinoma cells, pH-sensitive intracellular disassembly, effective escape from endosomes/lysosomes, redox-responsive cytoplasmic release of siRNA, and targeted transport of DOX to the nuclei, thereby providing precise delivery of DOX and siRNA to their individual sites of action. In this study, the structural stability and release profiles of MLNs were investigated at various pH values. Cellular uptake, intracellular kinetics, gene silencing efficiencies, and anti-proliferation effects of MLNs were determined in human hepato-carcinoma QGY-7703 cells. *In vivo* antitumor efficacies of MLNs were evaluated in tumor-bearing nude mice following intravenous injections.

2. Materials and methods

2.1. Materials, cell culture, and animals

Cetyltrimethylammonium bromide (CTAB), tetraethylorthosilicate (TEOS), and 3-aminopropyltriethoxysilane (APTES) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Fluorescein isothiocyanate (FITC) labeled TAT (FITC-TAT) and *N*-acetylated TAT (TAT: YGRKKRRQRRR) were purchased from

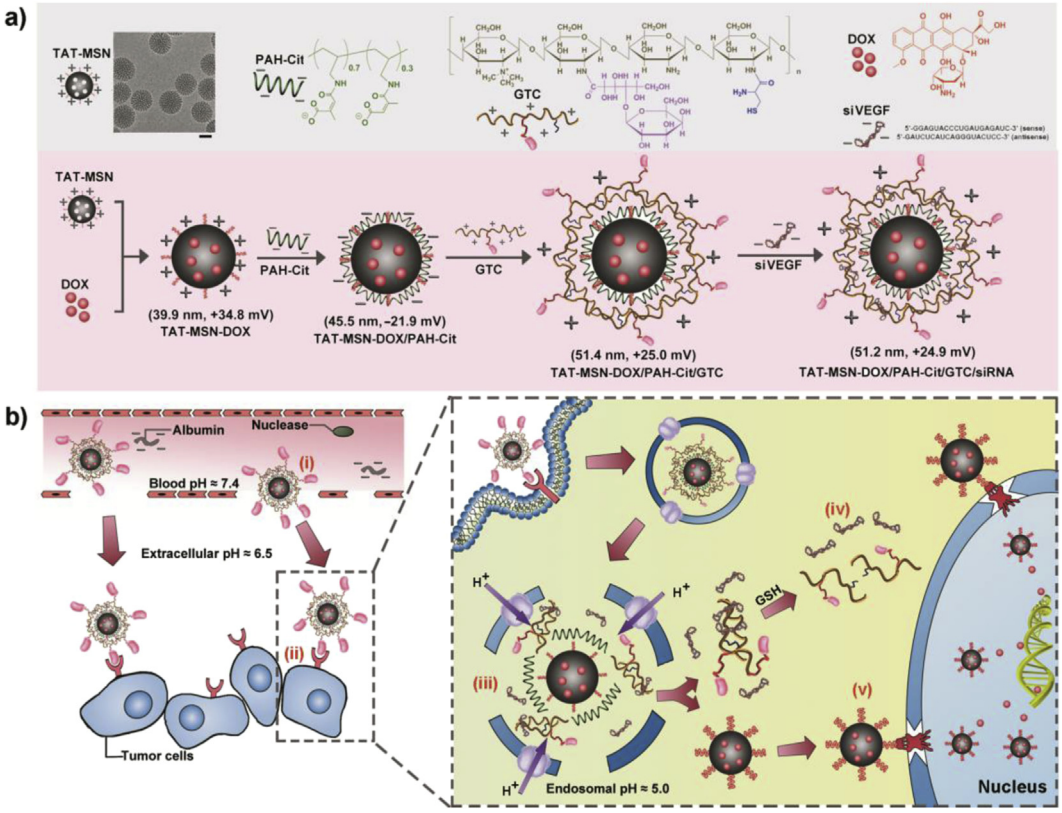

Fig. 1. Schematic illustration showing the formation of MLNs and their delivery kinetics. (a) Schematic structure of MLNs which were constructed *via* layer-by-layer self-assembly driven by the electrostatic coverage of PAH-Cit and GTC onto the TAT-MSN core (morphology shown by the TEM image, bar represents 20 nm). (b) Schematic presentation of MLNs-mediated delivery for DOX and siVEGF. MLNs maintain structural integrity in the blood and tumor milieu (i), actively enter cancer cells *via* galactose receptor-mediated endocytosis (ii), undergo structural disassembly and endosomal escape in response to intracellular acidity (iii), release siVEGF into the cytoplasm upon GSH-triggered disulfide cleavage (iv), and deliver DOX into the nuclei *via* TAT-mediated targeting (v).