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Enhanced antitumor efficacies of multifunctional nanocomplexes through knocking down the barriers for siRNA delivery

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ABSTRACT

Multifunctional nanocomplexes (NCs) consisting of urocanic acid-modified galactosylated trimethyl chitosan (UA-GT) conjugates as polymeric vectors, poly(allylamine hydrochloride)-citraconic anhydride (PAH-Cit) as charge-reversible crosslinkers, and vascular endothelial growth factor (VEGF) siRNA as therapeutic genes, were rationally designed to simultaneously overcome the extracellular, cellular, and intracellular barriers for siRNA delivery. The strong physical stability of UA-GT/PAH-Cit/siRNA NCs (UA-GT NCs) at pH 7.4 and 6.5 endowed protection from massive dilution, competitive ions, and ubiquitous nucleases in the blood and tumorous microenvironment. Their internalization into hepato-carcinoma cells was facilitated through the recognition of galactose receptors, followed by effective escape from endosomes/lysosomes owing to the strong buffering capacity of imidazole residues. At the meantime, the endosomal/lysosomal acidity triggered the charge reversal of PAH-Cit in UA-GT NCs, thus evoking their structural disassembly and subsequently accelerated release of siRNA in the cytosol. As a result, robust *in vivo* performance in terms of both gene silencing and tumor inhibition was achieved by UA-GT NCs at a low siRNA dose. Moreover, neither histological nor hematological toxicity was detected following repeated intravenous administration. Therefore, UA-GT NCs potentially served as an efficient and safe candidate in the treatment of hepatocellular carcinoma through knocking down the overall barriers for siRNA delivery.

1. Introduction

The discovery of RNA interference (RNAi) has pioneered the use of small interfering RNA (siRNA) as a promising modality in cancer therapy [1,2]. However, direct application of naked siRNA has demonstrated minimal *in vivo* success owing to its vulnerability towards nucleases and poor penetration into cells [2,3]. Polymeric vectors provide potential opportunities to address these shortcomings through forming nanocomplexes (NCs) with siRNA to resist nuclease access and facilitate endocytic uptake [4–6]. Unfortunately, the delivery performance of polymeric vectors can be largely compromised by multiple physiological and biological barriers, which would seriously restrict their applications in a clinical setting [7]. Disassembly in ionic body fluids and attack by endogenous nucleases stand as the major extracellular challenges facing polymeric NCs, which normally result in premature release and loss of siRNA cargoes [8,9]. The following obstacle against effective gene silencing stems from the essential demands for active recognition of target cells, otherwise extensive entry into no-specific cells can be frequently seen [10,11]. Once inside target cells, NCs must find their way to escape the endosomes/lysosomes and efficiently release siRNA cargoes in the cytoplasm thereafter to initiate sequence-specific gene silencing [12,13]. Worse still, each of these barriers has the possibility to become the Achilles' heel in siRNA delivery, which would significantly weaken or even totally eliminate the overall therapeutic efficacies of NCs.

Tremendous efforts have been made in the rational design of polymeric vectors to overcome the existing barriers for siRNA delivery. With regard to the extracellular barriers, manipulating the proportion of cationic polymers in NCs and incorporating additional crosslinking agents into NCs are two typical strategies to improve their stability and protective capacity [14,15]. To actively recognize target cells, a variety of specific ligands or antibodies have been conjugated to polymeric vectors to allow for receptor-mediated endocytosis [10]. In attempt to promote the escape of NCs from endosomes/lysosomes, imidazole-containing moieties

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Abbreviations

ANOVA	analysis of variance
Cav-1	caveolin-1
CHC-1	clathrin heavy chain-1
CLSM	confocal laser scanning microscopy
Cit	citraconic anhydride
CTB	cholera toxin B subunit
EDAC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
FBS	fetal bovine serum
GT	galactosylated trimethyl chitosan
H&E	hematoxylin and eosin
LA	lactobionic acid
LPF2000	Lipofectamine™ 2000
LPFMAX	Lipofectamine® RNAiMAX
MTT	methyl tetrazolium
NCs	nanocomplexes
NHS	N-hydroxysuccinimide
PAH	poly(allylamine hydrochloride)
PSS	poly(styrenesulfonate)
RNAi	RNA interference
SEM	scanning electron microscopy
siRNA	small interfering RNA
TC	trimethyl chitosan
UA	urocanic acid
UA-GT	urocanic acid-modified galactosylated trimethyl chitosan
VEGF	vascular endothelial growth factor
siVEGF	VEGF siRNA
siScr	scrambled VEGF siRNA

are extensively employed to elevate osmotic pressure in endosomes/lysosomes and then eventually burst these compartments [16,17]. To facilitate the intracellular release of siRNA, stimulus-sensitive linkages that can be readily cleaved in response to intracellular acidity or redox are introduced into NCs to promote their intracellular disassembly [18,19]. Notwithstanding the individual success of these strategies in vanquishing certain barrier, it's synthetically challenging to integrate them in a single system to address the entire obstacles for siRNA delivery. To our knowledge, siRNA delivery systems that can overcome the overall extracellular, cellular, and intracellular barriers are rarely reported so far.

In this study, we described an easy-to-fabricate multifunctional system consisting of urocanic acid-modified galactosylated trimethyl chitosan (UA-GT) as the polymeric vector, poly(allylamine hydrochloride)-citraconic anhydride (PAH-Cit) as a charge-reversible crosslinker, and vascular endothelial growth factor (VEGF) siRNA (siVEGF) as the therapeutic gene, to pursue robust antitumor efficacy through conquering the successive delivery barriers. Trimethyl, galactose, and urocanic residues were conjugated to chitosan to afford potent capabilities in encapsulating siRNA, targeting hepato-carcinoma cells, and buffering endosomes/lysosomes, respectively. PAH-Cit, an anionic polymer that can be rapidly hydrolyzed to cationic poly(allylamine) at endosomal/lysosomal pH [20], can initiate intelligent transformation of UA-GT/PAH-Cit/siRNA NCs (UA-GT NCs) from extracellularly stable state to intracellularly disassembled one. The structural stability, protective capability, buffering capacity, and *in vitro* release profiles of NCs were investigated. Cellular uptake, endosomal/lysosomal escape, and gene silencing efficiencies were determined in human hepato-carcinoma QGY-7703 cells. *In vivo* antitumor efficacies of NCs were evaluated in tumor-bearing nude mice. *In vitro* and *in vivo* toxicities of NCs were also assessed.

2. Materials and methods

2.1. Materials, cell culture, and animals

Chitosan (deacetylation degree of 85% and Mw of 200 kDa) was purchased from Golden-Shell Biochemical Co., Ltd. (Zhejiang, China). Urocanic acid (UA), poly(-allylamine hydrochloride) (PAH, Mw of 58 kDa), citraconic anhydride (Cit), poly(-styrenesulfonate) (PSS, Mw of 70 kDa), lactobionic acid (LA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), N-hydroxysuccinimide (NHS), galactose, FITC labeled dextran (FITC-dextran, Mw of 70 kDa), and Hoechst 33258 were from Sigma (St. Louis, MO, USA). Lysotracker® Green, cholera toxin B subunit (CTB)-Alexa Fluor 488, transferrin-Alexa Fluor 488, Lipofectamine™ 2000 (LPF2000), and Lipofectamine® RNAiMAX (LPFMAX) were purchased from Invitrogen (Carlsbad, CA, USA). siVEGF (sense: 5′-GGAGUACCCUGAUGAGAUC-3′), its scrambled control (siScr, sense: 5′-AUCGGAGCAGUCGUAAUGC-3′), clathrin heavy chain-1 (CHC-1) siRNA (sense: 5′-GCAGAAGAAUCAACGUUAU-3′), caveolin-1 (Cav-1) siRNA (sense: 5′-GCAUCAACUUGCAGAAAGA-3′), Rac1 siRNA (sense: 5′-GUGAUUU-CAUAGCGAGUUU-3′), and TAMRA labeled siRNA (TAMRA-siRNA) were supplied by GenePharma Co., Ltd. (Shanghai, China). All other reagents were of analytic grade.

Human hepato-carcinoma QGY-7703 cells and human cervical carcinoma HeLa cells were provided by Chinese Academy of Sciences (Shanghai, China) and American Type Culture Collection (ATCC, Rockville, MD, USA), respectively. Both cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS).

Female Balb/c nude mice (16–20 g) were obtained from Slaccas Experimental Animal Co., Ltd. (Shanghai, China) and kept under standard conditions. Animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee, Fudan University.

2.2. Preparation and characterization of UA-GT conjugates

Chitosan (200 mg) was reacted with CH_3I (1.2 mL) in 40 mL of NaOH/*N*-methyl-2-pyrrolidone solution (2%, w/v) at 65 °C for 2 h to yield trimethyl chitosan (TC) conjugates, followed by ion-exchange, dialysis (MWCO 3500 Da) against water, and lyophilization. Next, TC conjugates (100 mg) were reacted with LA (100 mg) in 50 mL of TEMED/HCl buffer (10 mM, pH 5.0) at room temperature for 72 h as catalyzed by EDAC/NHS (1:1, 200 mM). After ultrafiltration (MWCO 10 kDa) and lyophilization, the obtained galactosylated TC (GT) conjugates (100 mg) were reacted with UA (40 mg) for 24 h as mediated by EDAC/NHS (1:1, 200 mM) in 10 mL of 2-morpholino ethanesulfonic acid buffer (25 mM, pH 6.0). The resultant UA-GT conjugates were dialyzed (MWCO 3500 kDa) against HCl solution (pH 5.0), lyophilized, and stored at −20 °C. The substitution degrees of trimethyl, galactose, and urocanic residues were calculated by ^{1}H NMR in D_2O with an AVANCE DMX 500 spectrometer (Bruker, Germany). The viscosity-average molecular weight of UA-GT was measured with an IVS300 viscometer (ZonWon, China).

2.3. Preparation and characterization of PAH-Cit

PAH (200 mg) was dissolved in 1 M NaOH solution (6 mL), followed by the dropwise addition of Cit (0.8 mL). The mixture was stirred at room temperature for 24 h, during which 2 M NaOH solution was added to maintain pH value above 8. The mixture was then dialyzed (MWCO 3500 Da) against NaOH solution (pH 8.0), lyophilized, and stored at −20 °C. The structure of PAH-Cit was characterized by ^{1}H NMR in D_2O. The viscosity-average molecular weight of PAH-Cit was measured with an IVS300 viscometer.

2.4. Preparation and characterization of UA-GT NCs

UA-GT conjugates, PAH-Cit, and siRNA were dissolved in DEPC-treated water at 2.0, 1.0, and 0.2 mg/mL, respectively. PAH-Cit solution was first mixed with siRNA solution at 15:1 (w/w), into which the UA-GT solution was added at various UA-GT/PAH-Cit weight ratios (2.5, 5, 7.5, 10, and 15) under stirring. The resultant UA-GT NCs were incubated for 30 min at 37 °C before use. Particle sizes and Zeta potentials of UA-GT NCs were measured with Zetasizer Nano (Malvern, Worcestershire, UK). The binding affinity of UA-GT NCs for siRNA was evaluated by 4% (w/v) agarose gel electrophoresis at 56 V for 1 h. Morphology of UA-GT NCs was observed with scanning electron microscopy (SEM) (Vega TS5136, Tescan, Czech). The cytotoxicity of UA-GT NCs with (w/) siScr in QGY-7703 cells was evaluated by methyl tetrazolium (MTT) assays.

GT/PAH-Cit/siRNA NCs (GT NCs), UA-TC/PAH-Cit/siRNA NCs (UA-TC NCs), and UA-GT/PSS/siRNA NCs (UA-GT NCs w/PSS) were prepared at the polymer/crosslinker/siRNA ratio of 150:15:1 (w/w/w) as non-buffering, non-targeting, and non-sensitive controls, respectively.