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Enhanced antitumor efficacy of folate modified amphiphilic nanoparticles through co-delivery of chemotherapeutic drugs and genes

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ABSTRACT

Folate (FA) modified amphiphilic linoleic acid (LA) and poly (β-malic acid) (PMLA) double grafted chitosan (LMC) nanoparticles (NPs) with optimum grafting degrees of hydrophobic LA and hydrophilic PMLA were developed for the co-delivery of paclitaxel (PTX) and survivin shRNA-expressing plasmid (iSur-pDNA). The resultant NPs exhibited particle size of 161 nm and zeta potential of 43 mV. FA modification and the increasing grafting degrees of LA and PMLA were correlated with the suppressed protein adsorption, the inhibited release of PTX, and the accelerated dissociation of pDNA. PTX loading, cellular uptake, nuclear accumulation of pDNA, *in vitro* gene silencing efficiency, and cell growth inhibition were promoted by FA modification and higher grafting degree of LA, but impeded by increasing grafting degree of PMLA. In tumor-bearing mice, co-delivery of PTX and iSur-pDNA exhibited enhanced antitumor efficacy and prolonged survival period as compared with single delivery of PTX or iSur-pDNA. These results indicated that amphiphilic LMC NPs could serve as a promising platform for the co-delivery of antitumor drugs and genes, and highlighted the importance of adjusting the hydrophobic and hydrophilic grafting degrees.

1. Introduction

Due to the complexity of their signaling network, tumor cells may develop several pathways to escape from death induced by chemotherapeutics [1,2]. The curative effects of conventional chemotherapy hitting single target in tumor cells are therefore severely limited. The combination of chemotherapy and gene therapy provides a promising modality to improve the therapeutic index through simultaneous modulation of multiple signaling pathways in tumor cells [3]. Numerous delivery systems involving the co-delivery of antitumor drugs and genes have been developed including liposomes, dendrimers, and inorganic nanoparticles (NPs), intending to obtain a synergistic effect of the drug and gene in tumor therapy [4–6]. However, interferential influences between agents and toxicity induced by multi-component carriers are inevitable problems for these co-delivery systems [2,7–9]. Comparatively, a biodegradable amphiphilic copolymer can self-assemble core–shell NPs with a hydrophobic core and a cationic hydrophilic shell in an aqueous solution, which has the ability of simultaneously loading a hydrophobic antitumor drug and a poly anionic gene in a single-component carrier [10], thereby avoiding aforementioned issues.

The hydrophobic and hydrophilic modification of biodegradable chitosan has many advantageous effects for the delivery of antitumor drugs or plasmid DNA (pDNA). Hydrophobic modification, such as polylactide modification [11], stearic acid modification [12], and alkylation [13], can promote the encapsulation and cellular uptake of hydrophobic antitumor drugs and improve the protection from nuclease degradation, cellular uptake, and endosomal escape of pDNA. Hydrophilic modification, including PEGylation [14] and arginine modification [15], is able to facilitate the drug accumulation in tumor cells and intracellular release of pDNA. However, to our knowledge, few researches have concerned the application of amphiphilic chitosan derivatives as co-delivery systems.

In our previous studies, amphiphilic linoleic acid (LA) and poly (β-malic acid) (PMLA) double grafted chitosan (LMC) NPs were developed as a safe and effective delivery system for paclitaxel (PTX) or pDNA [16,17]. Considering the fact that hydrophobic LA core and cationic PMLA shell of LMC NPs could be respectively evoke hydrophobic and electrostatic interactions, amphiphilic LMC

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Abbreviations

BSA	bovine serum albumin
DCC	dicyclohexylcarbodiimide
DMAP	4-dimethylaminopyridine
DMEM	Dulbecco's Modified Eagle's Medium
EB	ethidium bromide
EDC·HCl	N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride
EPR	enhanced permeability and retention
FA	folate
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
IC_{50}	half maximal inhibitory concentration
LA	linoleic acid
MTT	methyl tetrazolium
NHS	N-hydroxysuccinimide
NPs	nanoparticles
PBS	phosphate buffered solution
pDNA	plasmid DNA
PMLA	poly (β-malic acid)
PMLABz	poly (β-benzyl malate)
PTX	paclitaxel
RhB	rhodamine B
SD	standard deviation
SDS	sodium dodecyl sulfate
TIR	tumor inhibition ratio
LMC	LA and PMLA double grafted chitosan
LMC1	LMC grafted with 20.1% LA and 4.1% PMLA
LMC2	LMC grafted with 31.1% LA and 4.2% PMLA
LMC3	LMC grafted with 65.9% LA and 4.1% PMLA
LMC4	LMC grafted with 67.0% LA and 8.4% PMLA
LMC3/pDNA NPs	iSur-pDNA loaded LMC3 NPs
LMC3/PTX NPs	PTX loaded LMC3 NPs
LMC/PTX/pDNA NPs	PTX and iSur-pDNA co-loaded LMC NPs
FA-LMC3	FA modified LMC3
FA-LMC3/pDNA NPs	iSur-pDNA loaded FA-LMC3 NPs
FA-LMC3/PTX/pDNA NPs	PTX and iSur-pDNA co-loaded FA-LMC3 NPs
FA-LMC3/PTX/pGL NPs	PTX and pGL3-control vector co-loaded FA-LMC3 NPs.

NPs were therefore adopted here as a co-delivery system carrying PTX and survivin shRNA-expressing pDNA (iSur-pDNA) simultaneously. Although the optimum hydrophobicity and hydrophilicity of LMC NPs have been elucidated for pDNA delivery [17], they are unavailable for the co-delivery of antitumor drugs and pDNA. Therefore, in this study, LMC NPs with various grafting degrees of LA and PMLA were prepared to probe the optimized hydrophobicity and hydrophilicity for a co-delivery system. Folate (FA) was further conjugated to endow LMC NPs with the active-targeting capacity. PTX and iSur-pDNA were loaded into LMC NPs through hydrophobic and electrostatic interactions, respectively. Assays of pDNA binding affinity, protein adsorption, pDNA protection, *in vitro* release, cellular uptake, intracellular distribution, and *in vitro* cell growth inhibition were performed. Finally, *in vivo* antitumor efficacies of various NPs were evaluated in tumor bearing mice.

2. Materials and methods

2.1. Materials, cell lines, and animals

Chitosan with molecular weight of 100 kDa and deacetylation degree of 85% was obtained from Golden-Shell Biochemical Co., Ltd. (Zhejiang, China). Fluorescein isothiocyanate (FITC), ethidium bromide (EB), and Hoechst 33258 were purchased from Sigma (St. Louis, MO, USA). FA, rhodamine B (RhB), 4-dimethylaminopyridine (DMAP), and dicyclohexylcarbodiimide (DCC) were supplied by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). N-Hydroxysuccinimide (NHS) and N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC·HCl) were obtained from Yuanju Bio-Tech Co., Ltd. (Shanghai, China). DNase I was purchased from Worthington (Lakewood, NJ, USA). PTX was purchased from Hisun Pharmaceutical Co., Ltd. (Zhejiang, China). The iSur-pDNA and pGL3-control vector were amplified in *Escherichia coli* and isolated with EndoFree Plasmid Mega Kit (Tiangen Biotech Co., Ltd., Beijing, China). All other chemical reagents were of analytic grade.

Human liver cell line L02 and human hepatoma cell line QGY-7703 were purchased from Chinese Academy of Sciences (Shanghai, China). Both cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, NY, USA) containing 10% fetal bovine serum.

Female Kunming mice (6 weeks, 20 ± 2 g) were purchased from Slaccas Experimental Animal Co., Ltd. (Shanghai, China). All animal experiments were performed following the protocol approved by the Institutional Animal Care and Use Committee, Fudan University.

2.2. Preparation and characterization of LMC and FA-LMC

LMC was prepared according to our previous report [16]. In brief, poly (β-benzyl malate) (PMLABz) was synthesized through polymerization of lactic acid and lactone (1:10, m/m). PMLABz and oxalyl chloride (1:1.5, v/v) were reacted in dichloromethane at 40 °C under N_2 for 12 h to obtain PMLABz acyl chloride. LA acyl chloride was prepared using the similar method. Chitosan (1 g) was dissolved in 20 mL of methanesulfonic acid, followed by an addition of LA acyl chloride. After agitation for 1 h, PMLABz acyl chloride was added and the mixture was stirred for another 4 h before 30 g of ice water mixture was added to terminate the acylation reaction. The detailed feed amounts of LA acyl chloride and PMLABz acyl chloride for LMC1-4 were listed in Table 1. The grafting degrees of LA and PMLA were determined by ^{1}H nuclear magnetic resonance (^{1}H NMR, Bruker, Germany) [16]. FA modified LMC (FA-LMC) was synthesized via Schiff base reaction. Briefly, 40 mg of EDC·HCl, 40 mg of NHS, 10 mg of DMAP, and 89 mg of FA were added into 50 mL of water. After stirred for 4 h, the mixture was reacted with 80 mL of LMC (10 mg/mL) for another 12 h to obtain FA-LMC.

2.3. NPs formation and characterization

To form LMC NPs, 15 mL of LMC (2 mg/mL) was sonicated at 200 W for 15 min in an ice bath. LMC NPs were incubated at 37 °C for 30 min before use. PTX and pDNA were loaded into LMC NPs via sonication and electrostatic adsorption methods, respectively. As for PTX encapsulation, LMC NPs and FA-LMC3 NPs were further sonicated for 10 min following the addition of 50 μL of PTX solution in ethanol (60 mg/mL). The PTX loaded LMC NPs and FA-LMC3 NPs was purified by silica column as previously described [16]. As for pDNA encapsulation, iSur-pDNA (100 μg/mL) were added into PTX loaded LMC NPs and FA-LMC3 NPs at a weight ratio of 12:1 and kept under vortex for 15 s to obtain LMC/PTX/pDNA NPs and FA-LMC3/PTX/pDNA NPs.

Particle size and zeta potential of LMC/PTX/pDNA NPs and FA-LMC3/PTX/pDNA NPs suspended in HCl solution (pH 5.5) were measured with Zetasizer Nano (Malvern, Worcestershire, UK). Loading capacity and encapsulation efficiency of PTX were measured as described by Zhao et al. [16]. The association of pDNA with the NPs was evaluated by the gel retardation assay on a 1% (w/v) agarose gel, and the electrophoresis was performed at 120 V for 40 min. After the NPs were centrifugated

Table 1
Grafting degrees, particle sizes, and zeta potentials of LMC/PTX/pDNA NPs and FA-LMC3/PTX/pDNA NPs. Indicated values were mean ± SD ($n = 3$).

Sample	Feed amount of acyl chloride (g)		Grafting degree (%)[a]		Particle size (nm)	Zeta potential (mV)
	PMLA	LA	PMLA	LA		
LMC1/PTX/pDNA NPs	1.3	0.6	4.1	20.1	206.7 ± 2.7	45.6 ± 2.3
LMC2/PTX/pDNA NPs	1.3	1.2	4.2	31.1	172.8 ± 2.0	44.8 ± 1.6
LMC3/PTX/pDNA NPs	1.3	1.8	4.1	65.9	151.7 ± 2.6	43.3 ± 2.6
LMC4/PTX/pDNA NPs	2.6	1.8	8.4	67.0	219.3 ± 7.4	41.4 ± 0.4
FA-LMC3/PTX/pDNA NPs	1.3	1.8	4.1	66.0	161.9 ± 5.3	43.0 ± 1.0

[a] The grafting degrees were determined by ^{1}H NMR.

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