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Inhibition of murine breast cancer growth and metastasis by survivin-targeted siRNA using disulfide cross-linked linear PEI

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ABSTRACT

Biodegradable disulfide-containing polyethyleneimine (PEI) derivatives showed great potential as siRNA vectors for the treatment of cancer due to the reduction-sensitive property. In this study, we developed and characterized a hyperbranched disulfide cross-linked PEI (lPEI-SS) based on linear PEI (lPEI) by ring-opening reaction of propylene sulfide. We evaluated the efficiency of lPEI-SS as a siRNA vector in vitro with luciferase reporter gene system, and investigated the anti-tumor efficacy of survivin-targeted siRNA (siRNA^{sur}) on 4T1 murine breast cancer model using lPEI-SS synthesized here. Results from cytotoxicity and hemolysis assay proved that lPEI-SS showed favorable cell and blood compatibility. lPEI-SS/siRNA polyplexes prepared under the optimized condition were compact spherical particles with the average size of 229.0 nm and zeta potential of 42.67 mV. Cellular uptake of lPEI-SS/siRNA polyplexes was significantly improved due to the higher branching degree of lPEI-SS over the parent lPEI. lPEI-SS/siRNA^{sur} exhibited great anti-proliferation effect on 4T1 cell line, which was found to be caused by the induction of apoptosis. Most importantly, results of tumor volume, tumor weight and histological observation demonstrated that lPEI-SS/siRNA^{sur} polyplexes effectively inhibited the tumor growth and metastasis of 4T1 murine breast cancer model.

1. Introduction

Breast carcinoma is the leading cause of cancer death in women, and the second mostly diagnosed cancer after lung cancer(Jemal et al., 2011). Currently, using gene therapy to treat breast cancer has aroused great interest and been proven to be quite effective. Survivin is a member of the inhibitors of apoptosis protein (IAP) family, and is highly overexpressed in breast cancer (Jha et al., 2012). Moreover, the overexpression of survivin has been proved to play an important role in the diagnosis, treatment and prognosis of breast cancer(Lv et al., 2010). Therefore, survivin has been considered as a potential pharmacological target for breast cancer therapy. Several reports have shown that targeting survivin could effectively inhibit the growth as well as metastasis of breast cancer (James et al., 2014; Li et al., 2013; Peng et al., 2008b; Yang et al., 2013; Yin et al., 2013), which is a promising strategy for breast cancer therapy.

Developing non-viral gene vectors able to transfer plasmid DNA or siRNA into cells safely and efficiently is the essence of gene therapy (O'Connor and Glynn, 2010). Among the various non-viral vectors, polyethyleneimine (PEI) has an obvious advantage over the other cationic polymers due to its high charge density and so called "proton sponge effect". However, the high efficiency of PEI is usually accompanied with high toxicity which hinders its clinical application, and the high toxicity of PEI was proved to be partially due to non-degradability (Luten et al., 2008). Hence, many biodegradable PEI derivatives based on low molecular weight (LMW) PEI have been developed to acquire the high efficiency and limit the cytotoxicity as well. Among the many degradable linkages such as ester(Luu et al., 2012; Park et al., 2012), amide (Xiong et al., 2007), ketal (Shim and Kwon, 2008), and disulfide (Bauhuber et al., 2012), disulfide is of great superiority to fabricate bioreducible PEI derivatives for DNA or siRNA delivery, because (1) the bond strength of disulfide is appropriate, (2) degradation kinetics of disulfide is quick, and most importantly, (3) disulfide is reduction-sensitive. Different concentrations of glutathione (GSH) between the intracellular (3–10 mM) and extracellular regions (~2.8 μM) lead to the extra-and intracellular redox potential gradient (Ottaviano et al., 2008), thus disulfide could utilize such redox potential gradient to tightly condense DNA or siRNA outside the cells and easily release them inside the cells, which solves the contradiction between tight polyplexes formation outside cells and the easy dissociation of polyplexes inside cells(Wang et al., 2006). Therefore, integrating disulfide bond into PEI polymers is supposed to provide not only biodegradable PEI derivatives with low toxicity but also favorable reduction-sensitive polymers.

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Up to date, many reducible disulfide-containing PEI derivatives have been reported, and their design, development and application as nucleic acid vector have been extensively reviewed (Cho, 2012; Ryu and Kim, 2014; Son et al., 2012). There have been two approaches to introduce disulfide into polymers(Son et al., 2012), one is using disulfide-containing cross-linkers, the other is processed by initial prethiolation of polymers (polymer-SH) and subsequent oxidation of the thiolated polymers (polymer-SS). Because prethiolation strategy could facilitate further modification of polymer-SH with thiol-containing bioactive moieties such as targeting peptides (Son et al., 2010, 2011), it was more popular to fabricate disulfide-containing polymers. The prethiolation method has been used to synthesize both hyperbranched PEI-SS based on branched LMW PEI (M.W.: 800 (Xia et al., 2012); 1200 (Son et al., 2010, 2011); 1800 (Liu et al., 2010); 2000 (Xiao et al., 2013b)) and linear PEI-SS based on linear LMW PEI (M.W.: 2600 (Breunig et al., 2007, 2008); 3100(Breunig et al., 2007); 4600 (Breunig et al., 2007)).

So far, however, branched PEI-SS based on linear LMW PEI via the prethiolation method has not been reported yet. It is generally considered that branched PEI (bPEI) has more advantage over linear PEI (lPEI) of the same molecular weight in binding with nucleic acid and enhancing cellular uptake (Breunig et al., 2008); whereas lPEI is more efficient than bPEI in releasing/dissecting nucleic acid. Therefore, we assumed that constructing branched PEI-SS by cross-linking linear PEI might be able to integrate the respective advantage of bPEI and lPEI and address the contradiction between extracellular tight binding and intracellular easy dissection of nucleic acid (Wang et al., 2006). Therefore, in this study, we synthesized branched disulfide-bonded PEI (lPEI2200-SS) based on linear LMW PEI (M.W.: 2200), studied the efficiency of lPEI2200-SS as a siRNA vector, and evaluated the anti-tumor efficacy of survivin-targeted siRNA loaded with the bioreducible PEI developed here.

2. Materials and methods

2.1. Materials

5 kDa poly (2-ethyl-2-oxazoline) s (PEOZs) was from Alfa Aesar (Tianjing, China). Propylene sulfide, N-(2-hydroxyethyl) piperazine-N′-ethanesulfonic acid (HEPES), 5-diphenyltetrazolium bromide (MTT), ethidium bromide (EtBr), and dithiothreitol (DTT) were from Sigma-Aldrich (St. Louis, MO, USA). Annexin V-FITC Apoptosis detection kit was provided by KeyGEN Biosciences Company (Nanjing, China). Luciferase assay kit was from Promega Corporation (Madison, WI, USA). Trypsin, fetal bovine serum (FBS), DMEM and RPMI 1640 medium were all provided by Hyclone Company (Logan, Utah, USA). FAM-labeled siRNA was from Shanghai GenePharma Co., Ltd. (Shanghai, China).

2.2. Synthesis of siRNA

Two kinds of siRNAs which target luciferase and survivin gene respectively, namely $siRNA^{luc}$ and $siRNA^{sur}$, were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The sequences of $siRNA^{luc}$ and $siRNA^{sur}$ were as follows:

$siRNA^{luc}$: 5′-CUUACGCUGAGUACUUCGATT-3′.

$siRNA^{sur}$: 5′-GAACAUCAUCAUCCAGGAC-3′.

2.3. Synthesis and structural confirmation of lPEI2200 and lPEI2200-SS

Linear PEI with a molecular weight of 2200 (lPEI2200) was synthesized from commercial poly (2-ethyl-2-oxazoline)s (PEOZs) (M.W. = 5 kDa) by acid-catalyzed hydrolysis, as reported previously (Thomas et al., 2005). lPEI2200-SS was synthesized following previous method(Peng et al., 2008a) with some modifications. Typically, 0.45 mmol lPEI2200 obtained by the aforementioned method was dissolved in the mixture of methanol (10 mL) and triethylamine (2 mL) in a flask, and the flask was purged with nitrogen for three times. Then 20 mL of methanol containing propylene sulfide (2.25 mmol) was added by syringe. The mixture was kept in 60 °C oil bath for 24 h while stirring. The resulted solution was evaporated to dryness under reduced pressure, dissolved in 30 mL DMSO, kept in dark and stirred for 48 h at room temperature (r.t.). Finally, lPEI2200-SS was obtained by dialyzing the mixture against distilled water (MWCO 1000) and lyophilization. lPEI2200-SS was structurally confirmed by ^{1}H NMR (D_2O, 400 MHz).

2.4. Cell line and cell culture

Mouse mammary tumor cell line 4T1 and human breast adenocarcinoma cell line MCF-7, which were labeled with luciferase reporter gene and can stably express the firefly luciferase, were obtained from the Department of Pathology in Institute of Medicinal Biotechnology in Peking Union Medical College. MCF-7-luc and 4T1-luc cells were cultured in humidified atmosphere containing 5% CO_2 at 37 °C in DMEM and RPMI 1640 medium containing 10% FBS, respectively. Cells in logarithmic growth phase were used to conduct all cell experiments in this study.

2.5. Cytotoxicity of lPEI2200-SS

Cytotoxicity of lPEI2200-SS polymers was evaluated by MTT assay with both 4T1-luc and MCF-7-luc cells. Briefly, 4T1-luc and MCF-7-luc cells were seeded onto 96-well plates at a density of 4×10^3/well and 6×10^3/well, respectively. After incubated overnight for adherence, the previous medium was replaced with FBS-supplemented medium containing various concentrations of lPEI2200-SS or lPEI2200. After 4 h of incubation, the medium was removed and 100 μL fresh medium containing 0.5 mg/mL MTT was added to each well. Cells were further incubated for 4 h, and medium was replaced with 150 μL DMSO to solubilize the converted formazan. Absorbance was measured at 570 nm with a microplate photometer (Molecular Devices, USA). Cells without exposure to the polymers were used to represent 100% cell viability. Experiments were performed in triplicate.

2.6. pH titration

pH titrations were performed as reported before(Thomas et al., 2005). Shortly, 2 mL solution of lPEI2200-SS or lPEI2200 (0.4 mg/mL) was adjusted to pH 12 with NaOH. Sequential 5 μL of 1 M HCl was added, and the pH after each addition was measured with a pH meter till the pH was reached the value of 2.5.

2.7. Hemolysis assay

Rat erythrocytes were collected from heparin-treated blood, washed with 0.01 M isotonic PBS (pH 7.4) for 4 times (700 g for 10 min at 4 °C). The erythrocyte pellet was diluted 10-fold with 0.01 M isotonic PBS (pH 7.4) to a concentration of 10^9/mL. A 75 μL aliquot of erythrocyte suspension was added to a 96-well plate containing 75 μL serial solutions of lPEI2200 or lPEI2200-SS. The final concentrations of lPEI2200 and lPEI2200-SS varied from 6 to 25 μg/mL. After incubated for 1 h at 37 °C with constant shaking, unlysed erythrocytes were removed by centrifugation (700 g for 10 min at 4 °C), then 80 μL supernatant was transferred to a new 96-well plate and hemoglobin absorption was measured at 450 nm using SpectraMax 190 Absorbance Microplate Reader (Molecular Devices, USA). 1% Triton-X 100 was used as the positive control (100% lysis). Experiments were performed in triplicate.