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Efficacy of MRI visible iron oxide nanoparticles in delivering minicircle DNA into liver via intrabiliary infusion

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ABSTRACT

Gene therapy is a very promising technology for treatment of liver diseases. Minicircle DNA (MCDNA) is a versatile gene vector, which possesses excellent features in bio-safety and duration of transgene expression. However, its application has been hampered by the lack of an efficient gene delivery system. In this study, we developed a magnetic resonance imaging (MRI) visible nanoparticle to monitor MCDNA gene delivery and explore the potential of gene therapy *in vivo*. The nanoparticle was constructed via self-assembly of stearic acid modified low molecular polyethyleneimine (stPEI) and superparamagnetic iron oxide (SPIO) nanocrystals. The multiple SPIO nanocrystals with a controlled clustering structure in the nanoparticles were designed to achieve high MRI sensitivity. Furthermore, the MCDNA was combined with the nanoparticles via electronic interaction. Through intrabiliary infusion, the stPEI-SPIO/MCDNA nanoparticles were efficiently delivered to liver that was visualized by MRI *in vivo* and confirmed by histology. Moreover, the MCDNA nanoparticles exhibited non-cytotoxicity with no obvious inflammation in the transfection sites. These results indicate that stPEI-SPIO nanoparticle is able to serve as both an efficient DNA vector delivery system and a sensitive agent for MRI visualization.

1. Introduction

Liver is vulnerable to many physiological and environmental factors, such as virus infection, metabolic genetic disorders and so on. Recently, gene therapy has been extensively exploited for treatment of various diseases, and liver-targeted gene delivery has attracted a lot of attention. Although virus-based genetic vehicles are able to introduce high level of transgene expression, the clinical application is limited by the immunogenicity and genotoxicity [1]. Minicircle DNA (MCDNA) as a new-generation gene vector contains a circular expression cassette free of extraneous plasmid sequences [2]. It possesses excellent long-term sustained transgene expression and bio-safety, and these dominances make it promise for hepatopathy treatment [3–5]. However, the *in vivo* expression level of naked nucleotide molecules is very low owing to rapid nuclease degradation, poor cellular uptake and high rate of systemic clearance [6]. Therefore, it is essential to develop an efficient and safe delivery system for MCDNA-based gene therapy. In addition, a reliable method is necessary to monitor and evaluate the long-standing location and distribution of the gene carrier, preferably in a non-invasive manner *in vivo* [7,8].

Molecular imaging methods and nanotechnology generate non-invasive diagnosis strategies and effectively gene/drug delivery vectors [9]. Incorporating remedial agents with imaging system, theranostic nanoparticles are invented for both diagnosis of diseases and drug/gene therapy [10,11]. Herein, magnetic resonance imaging (MRI) technique as a functional clinical imaging methodology possesses excellent spatial resolution and deep tissue penetration [12,13]. With the development of contrast agents, such as

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gadolinium or superparamagnetic iron oxide (SPIO), MRI is the best modality for molecular imaging of gene delivery [14]. Compared with the gadolinium contrast agents, SPIO has better biocompatibility and higher imaging sensitivity to visualize the gene carriers [15]. Polyethylenimine (PEI) is one of the excellent transfect reagents and has the potential to modify SPIO nanoparticles to form a multifunctional platform [16,17]. Nevertheless, the contradictory among the PEI's molecular weight, transfection efficiency and cytotoxicity is still a major barrier for treatment [16]. Recently, stearic acid modified low molecular weight PEI (stPEI) with high gene transfection efficiency and non-cytotoxicity has been reported [18]. Furthermore, the hydrophobic apartment can be used to load hydrophobic materials, such as SPIO nanocrystals. However, how to achieve high targeting efficacy and safety for liver gene therapy is still a challenge. As a promising liver-therapeutic method, intrabiliary infusion of gene delivery displays many advantages over systemic administration [19,20]. The local delivery or direct infusion of the gene carriers into hepatic cells can significantly decrease macrophages phagocytosis, and avoid the opsonization of the genetic nanocomplexes in blood [6,19].

In this study, a functional nanoparticle, stPEI-SPIO, was constructed by the stPEI encapsulating high-quality SPIO nanocrystals via self-assemble progress. Then, the delivery of exogenous gene into mouse hepatic cells was performed by intrabiliary infusion and monitored via MRI *in vivo*. Finally, the successful gene expression was confirmed by histology.

2. Materials and methods

Stearic acid, N, N-carbonyldiimidazole (CDI) and polyethylenimine (Mw 600 g/mol, PEI600) were obtained from Aladdin Reagent Co., China. 1,2-hexadecanediol (97%), iron(III) acetylacetonate, oleylamine (>70%), oleic acid (90%) and benzyl ether (99%) were purchased from Aldrich Chemical Company. Dulbecco's modified Eagle's medium (DMEM), Phosphate-buffered saline (PBS), ethidium bromide and agarose gel were purchased from GIBCO Company (USA). The normal murine embryonic liver cells (BNL.CL.2 cells) were purchased from Cell Resource Center of SIBS (Shanghai, China). BALB/c female mice (8–10 weeks age, average body weight 20 g) were purchased from Guangdong Province Laboratory Animal Center (Guangzhou, China). All animal protocols were approved by the Institutional Animal Care and Usage Committee (File Num. SIAT-IRB-120312-A0027).

2.1. Preparation and characterization of minicircle LacZ gene

Minicircle plasmid ZY824.Pmc.CMVj.nls.LacZ and *Escherichia coli* strain of ZYCY10P3S2T were prepared by Zhi-ying Chen and the MCDNA expressing β-galactosidase gene (MCLacZ) was made per the previous method [3]. Briefly, competent cells were transformed with minicircle plasmid. Then single colony was mixed with 400 ml TB culture media with kanamycin (50 μg/ml). The mixtures were added with 400 ml LB, 16 μL NaOH (1 N) and 400 μL Arabinose (20%) in order to induce minicircle DNA formation. The MCLacZ was collected and purified using EndoFree Plasmid Kits (QIAGEN, USA). Then, the double digestion system consisted of 2 μL MCLacZ, 1 μL BamHI, 1 μL NsiI and 32 μL MilliQ water was executed to characterize the purity followed by the electrophoresis.

2.2. Preparation and characterization of stPEI-SPIO nanoparticles

Firstly, the synthesis of stearic acid modified PEI was prepared following the previous publication [21]. Herein, N, N′-carbonyldiimidazole was used as the condensation agent. The reaction was carried out in chloroform. Then, the purified product, stPEI, was obtained as a white solid after precipitation in cold ether and confirmed by ^{1}H NMR ($CDCl_3$, Bruker 500 MHz, Bellerica, MA) with approximate 15% modification.

The high-quality SPIO nanocrystals were synthesized following a reported method [22]. Briefly, 5 mmol 1,2-hexadecanediol, 1 mmol $Fe(acac)_3$, 3 mmol oleylamine, and 3 mmol oleic acid were mixed in 10 ml benzyl ether. The reaction was protected under argon, and then the mixture was heated up to 300 °C for 1 h. Finally, the purified SPIO nanocrystals were obtained via precipitation in ethanol and kept in hexane. These nanocrystals were characterized by transmission electron microscopy (TEM, FEI Tecnai 20) and dynamic light scattering (DLS, Malvern Nanosizer).

The stPEI-SPIO nanoparticles were prepared following a previously published protocol [23]. Briefly, 10 mg SPIO nanocrystals were dried under argon and re dispersed in chloroform with stPEI (5 mg). The mixed solution was added into the water drop by drop with sonication. After that, the mixture was under shaking for 24 h and the remaining chloroform was removed by rotary evaporation. The stPEI-SPIO nanoparticles were analyzed through DLS and TEM. The physical stability of stPEI-SPIO nanoparticles was analyzed by DLS in the different aqueous solutions with various sodium chloride concentrations or various pH values. The elemental analysis of iron in the stPEI-SPIO samples was determined by means of the atomic absorption spectrum (AAS, Analytik Jena ZEEnit 700 P). The MR relaxivity of these nanoparticles was measured via an 3 T clinical MRI scanner (MAGNETOM Tim, Siemens Healthcare, Germany) at room temperature [23,24]. T_2-weighted images of these nanoparticles were acquired by using a spin-echo acquisition with TR = 5000 ms and various TE values (from 12 ms to 150 ms). The relaxivity R_2, $1/T_2$ relaxation time (s^{-1}), was calculated by fitting the curves of signal intensity vs. TE, and presented along with iron concentration (mM Fe).

2.3. The characterization of stPEI-SPIO/MCLacZ nanocomplexes

The stPEI-SPIO/MCLacZ nanocomplexes with N/P ratios from 1 to 40 (the ratio of nitrogen atoms in the macromolecule to phosphorus atoms in the MCLacZ) were prepared by mixing an appropriate amount of nanoparticles with MCLacZ (0.5 μg) in water [25]. These nanocomplexes were incubated at room temperature for 20 min. After that, the electrophoretic mobility of these nanocomplexes was characterized by agarose gel electrophoresis at 90 V for 30 min. Then, the gel was imaged using a Dolphin-Doc Plus gel documentation system (Wealtec, USA). Furthermore, the stPEI-SPIO/MCLacZ nanocomplexes were characterized through DLS, TEM and atomic force microscopy (AFM, MFP-3DAsylum Research, USA).

In addition, for gene release assay, the stPEI-SPIO/MCLacZ nanocomplexes were treated with 150 μg heparin for 20 min at room temperature, and then the sample was subjected to electrophoresis as described above.

2.4. Cell culture and transfection

The BNL.CL.2 cells (5×10^3 cells per well) were cultured on 96 well plates with 100 μL DMEM media with 10% fetal bovine serum (FBS), 100 units/mL of penicillin and 100 units/mL streptomycin. Then the cells were transfected with stPEI-SPIO/MCLacZ nanocomplexes containing 0.5 μg MCLacZ at different N/P ratios from 5 to 20. After 6 h of incubation, the medium was replaced with 100 μL fresh DMEM. At 48 h post transfection, the cytotoxicity was determined via a Cell Counting Kit-8 kit (Dojindo, Janpan) by measuring the absorbance at 450 nm using the Synergy 4 multimode plate reader (BioTek). The β-galactosidase expression was measured using the Beta-Glo assay system kit (Promega, USA) and the luminescent signal was detected using multimode plate reader (Synergy 4, BioTek). Furthermore, immunocytochemistry (ICC) staining following the previously reported process [26], with the primary monoclonal Anti-β-galactosidase antibody (Promega), and the Prussian blue staining (Sigma) were analyzed for each sample.

2.5. Western blotting

The proteins of BNL.CL.2 cells were quantified by a protein assay (Bio-Rad, Hercules, CA) based on the Bradford method [27]. Firstly, the proteins (100 μg per well) were separated by 10% sodium dodecyl sulfate-polyacryl-amide gel electrophoresis (SDS-PAGE) at 100 V for 2 h, then transfected to membrane. Secondly, the samples were incubated with primary monoclonal Anti-β-galactosidase antibody diluted 1:1000 (Promega) and anti-β-Actin-Peroxidase antibody diluted 1:20,000 (Sigma) in a diluent of 5% dried skim milk at 4 °C overnight, followed by determination of protein activity using a horseradish peroxidase-conjugated secondary antibody and ECL detection (Thermo).

2.6. In vitro MR imaging

For *in vitro* MRI study, the transfected cells were washed twice with PBS. After that, the 5×10^5 cells were harvested and suspended in 200 μL 1% agar gel [23]. T_2-weighted images were acquired using a spin-echo sequence on the 3 T clinical MRI scanner (TR = 5000 ms, various TE values from 10.6 ms to 74.2 ms, FOV = 25 × 62 mm and slice thickness = 1.0 mm).

2.7. Transfection in vivo

The BALB/c female mice were administrated with stPEI-SPIO/MCLacZ nanocomplexes or physiological saline via intrabiliary infusion [20]. The method was described as follow. Mice were anesthetized via intraperitoneal injection of pentobarbital sodium with the dose of 40 mg/kg body weight. Then, the gallbladder, cystic duct, common hepatic duct and common bile duct were exposed. Tailor-made needle (27-gauge needle, BD) with apparatus infusions (custom-made ivory white hose with inside diameter = 1 mm) connected to hose was pricked quickly into the gallbladder after performed the hypodesis of the duodenum common bile duct junction. Subsequently, the proximal site of the hose was also clamped loosely with 6-gauge silk suture of surgery. Then, the stPEI-SPIO/MCLacZ nanocomplexes with 10 μg MCLacZ plasmid in 132 μL physiological saline were injected slowly into the gallbladder and the mice injected with the same volume of physiological saline were the control groups. After administration, the hose was removed and the suture was taken.