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A novel potential biocompatible hyperbranched polyspermine for efficient lung cancer gene therapy

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ABSTRACT

The clinical successful application of gene therapy critically depends upon the development of non-toxic and efficient delivery system. Although polycationic non-viral vectors hold great promise in nanomedicine, the exploring of application in clinics still remains a big challenge. To develop a non-toxic and efficient non-viral gene delivery system, two kinds of endogenous substance, citric acid (CA) and spermine (SPE), were used to prepare a new low charge density hyperbranched polyspermine (HPSPE) by one-pot polymerization. The biocompatibility evaluated by hemolytic activity and red blood cell (RBC) aggregation indicated that HPSPE was highly biocompatible without causing hemolysis and RBC aggregation compared with PEI as well as SPE. The MTS assay also demonstrated that the cell viability of HPSPE was above 90% even at 200 μg/mL at different time (24 and 72 h), which much higher than PEI 25 K. Besides, HPSPE showed high transfection efficiency without any toxic effect after aerosol delivery to the mice. Moreover, aerosol delivery of HPSPE/Akt1 shRNA significantly reduced tumor size and numbers and efficiently suppressed lung tumorigenesis ultimately in K-*ras*^{LA1} lung cancer model mice. These results suggest that low charge density as well as endogenous substance skeleton endow HPSPE with great potential for toxicity-free and efficient gene therapy.

1. Introduction

Gene therapy has been proved to be a promising strategy for the treatment of various congenital or acquired diseases, including cancer. Over the past decade, based on the in-depth understanding of the molecular mechanisms of cancer and the development of gene delivery vectors, cancer gene therapy has gained intensive attention as a new alternative to conventional drugs (Morille et al., 2008). However, successful application of gene therapy requires the development of efficient and safe delivery vehicles. Concerns on security issues of viral vectors, such as the infectivity, immunogenicity and inflammation, have frustrated their further application (Kim et al., 2007). To address this dilemma, polycationic non-viral vectors have emerged as robust nanovectors for gene delivery.

Researchers have designed a variety of polycations including polyamidoamine (PAMAM) as well as polyethylenimine (PEI), which is often used as "golden standard" of polycations (Sun et al., 2013). Yet high cytotoxicity issue exhibited in synthetic cationic polymers still remains great challenge in clinical application. Besides, high charge density of those vectors caused strong non-specific interaction between polyplexes and negatively charged red blood cell (RBC) (Domanski et al., 2004; Ziemba et al., 2012) once applied *in vivo*. Therefore, all of these limitations, rooted in safety, have encouraged researchers to focus on biocompatible human endogenous amino compounds for developing safe system.

Spermine (SPE), which is involved in cellular metabolism of all eukaryotic cells, is safe and naturally present in body tissues (Allen,

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1983; Jiang et al., 2011b). In the normal physiological environment, SPE is a biogenic tetra-amine with two primary and secondary amino groups, which have the potential to condense DNA reversibly (Jiang et al., 2011a). Owing to these attractive properties above, SPE has been extensively investigated for constructing polymers to deliver exogenous gene into targeted cells. However, to the best of our knowledge, studies involving polymers (based on SPE) that built completely by human endogenous element were seldom reported, thereby making it difficult to put these polymers into therapeutic applications eventually.

As one of the most important organic acids, citric acid (CA) is primary found in citrus fruits, such as lemon, grapefruit and orange (Abdel-Salam et al., 2014). CA is a type of α-hydroxy acid which has been widely used in food, beverage, chemical industries for many years (Xu et al., 2014). Being a metabolic product of the Krebs cycle, CA is also found in all animal tissues. In recent years, various studies have indicated that CA has many pharmacological effects and has been applied to drug delivery and gene therapy (Namazi et al., 2011; Slizewska, 2013; Welling et al., 2013; Yang et al., 2004; Zhang et al., 2009). Typically, it has been demonstrated that CA can either kill malignant cells or facilitate cell death (Bucay, 2007, 2009, 2011, 2012; Kruspig et al., 2012; Pitt et al., 2014). Therefore, underlying mechanisms of citrate-mediated cell death can promote the further development of anticancer treatment.

Here inspired by those outstanding features above mentioned, we propose to employ CA as skeleton to link SPE for developing a novel hyperbranched polyspermine (HPSPE). This new HPSPE is composed of two kinds of endogenous substance completely, thereby is expected to contribute to the improvement of cytotoxicity and serum tolerant function. In this study, HPSPE was synthesized and its potential as a safe and efficient gene delivery carrier was investigated. Also, cytotoxicity and transfection efficiency were evaluated *in vitro* and *in vivo*. Furthermore, the therapeutic effect of HPSPE as a lung cancer gene carrier was studied in K-*ras*^{LA1} lung cancer model mice.

2. Materials and methods

2.1. Materials

CA (anhydrous) and triphenyl phosphate (TPP) were obtained from Aladdin (Aladdin Industries Inc., Nashville, USA). SPE was purchased from Sigma–Aldrich (St. Louis, MO, USA) and pyridine (Py) was from sinopharm chemical reagent Co., Ltd. (China). Dulbeco's Modified Eagle Medium (DMEM) was purchased from KeyGEN Biotech (KeyGEN, China) and fetal bovine serum (FBS) was purchased from HyClon (Thermo Fisher Scientific, USA). Lipofectamine® 2000 was obtained from Invitrogen (Life Technologies, USA). Trypsin–EDTA solution (0.25%) was obtained from Gibco (Burlington, ON, Canada). All other chemicals were analytical grade and used without further purification. [3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolm; MTS] was purchased from Promega (Promega, USA). Plasmids were propagated in *Escherichia coli*, extracted by the alkali lysis technique, and purified by a E.Z.N.A.® Fastfilter Endo-free Plasmid Maxi kit (Omega, USA).

2.2. Preparation and characterization of HPSPE

The HPSPE was synthesized by polymerization reaction between activated carboxyl groups of CA and amine groups of SPE. Briefly, CA (0.68 mmol) and SPE (1.34 mmol) were dissolved in anhydrous DMSO. Then TPP (2.04 mmol) and Py (2 mL) were added to the solution after the monomers were dissolved completely. After mixing, the reaction was performed for 12 h. The solution was dialyzed (MWCO = 3500) for 3 days against DMSO, then against deionized water for 3 days at 4 °C. After dialysis, the HPSPE was lyophilized.

The construction of the prepared HPSPE was estimated by ^{1}H nuclear magnetic resonance (^{1}H NMR, Bruker AV-300, USA). The molecular weight of the HPSPE was measured using gel permeation chromatography (GPC, LC-20 AB, Shimadzu, Japan). The column temperature was maintained at 40 °C and the flow rate was controlled at 1 mL/min, and the mobile phase was 0.2 M ammonium acetate (pH 4.5).

2.3. Acid-base titration

The buffering capability of HPSPE was determined by acid–base titration over the pH range from 10.0 to 4.0. Briefly, 2 mg of HPSPE and PEI 25 K were dissolved in 10 mL of 150 mM NaCl solution. The solution was brought to a starting pH of 10.0 with 0.1 M NaOH and then was titrated with 0.1 M HCl using a pH meter (OHAUS, USA).

2.4. Preparation of HPSPE/DNA complexes

All HPSPE/DNA complexes were freshly prepared before subsequent use and characterized using the method developed by Jiang et al. (2008). Complexes at different weight ratios were prepared by adding DNA to equal volume of HPSPE solutions containing various amounts HPSPE with gentle vortexing and then incubated at room temperature for 30 min. In the following experiments, all the ratios represented the weight ratio of HPSPE to DNA.

2.5. Gel retardation assay

Electrophoresis was used to confirm DNA condensation ability of HPSPE. Complex formation was induced at various weight ratios from 0.1 to 20 with 12 μL final volume. After incubated at room temperature for 30 min, the complex solutions were loaded in a well for electrophoresis assay on a 1% agarose gel with Goldview staining and Tris-acetate (TAE) running buffer at 100 V for 30 min. The image was captured through gel image system (Tanon 1600, China).

2.6. Measurement of particle size and zeta potential

The particle sizes and zeta potentials of the HPSPE/DNA complexes at different weight ratios were determined using ZetaPlus particle size and zeta potential analyzer (Brookhaven Instruments, USA). HPSPE/DNA complexes were prepared in water at weight ratios from 5 to 30. The volume of each sample was 2 mL, containing a final DNA concentration of 20 μg/mL.

2.7. Observation of transmission electron microscopy

The morphology of the HPSPE/DNA complex was observed by TEM (JEM-200CX, JEOL, Japan). A drop of HPSPE/DNA complex was placed on a copper grid. After air-drying, the sample was examined under the electron microscope.

2.8. Protection and release assay

Protection and release of DNA in complexes were measured using electrophoresis by the method of Jiang et al. (2007). Briefly, 1 μL of DNase I or PBS in DNase I/Mg^{2+} digestion buffer was added to 4 μL of complexes solution (weight ratio: 5) or to 0.4 μg of naked plasmid DNA, and incubated at 37 °C with shaking at 100 rpm for 30 min. Then all samples were treated with 4 μL EDTA (250 mM) for 10 min at 65 °C for DNase I inactivation and mixed with 1% sodium dodecyl sulfate (SDS), dissolved in 1 M NaOH at a final