Preparation of Calcium Phosphate Nanocapsule Including Deoxyribonucleic Acid–Polyethyleneimine–Hyaluronic Acid Ternary Complex for Durable Gene Delivery

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ABSTRACT: Our plasmid delivery systems comprising deoxyribonucleic acid (DNA), polyethyleneimine (PEI), and hyaluronic acid (HA) have already achieved the high-extragene expression in tumor tissues. Repeated transfection with certain cytokine genes effectively induced tumor regression and complete disappearance of the tumor in some cases. Frequent injection is sometimes difficult depending on the tumor site. However, single injection often leads to an unsatisfactory efficacy owing to the short duration of the gene expression. In this study, we prepared calcium phosphate (CaP) nanocapsule including plasmid DNA complexes as a durable gene transfection system, which would be slowly degraded, and release DNA complex in the body. CaP nanocupsule including DNA complexes with a diameter of approximately 200 nm was prepared by immersing HA-coated DNA–PEI complex in simulated body fluid. It showed gene expression in cultured cells with duration longer than 2 weeks. By this slow-releasing system, significant tumor-growth suppression and, finally, complete tumor disappearance were observed after single injection into the tumor. Capsulated DNA complex with Ca thus seems promising as a sustained gene expression device. © 2013 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:179–184, 2014

Keywords: gene therapy; non-viral gene delivery; durable gene expression; plasmid DNA; DNA complex; nanoparticles; calcium phosphate; nanocapsuls

INTRODUCTION

Gene therapy has been proposed as a novel strategy for the treatment of refractory disease. Viruses have been used as vehicles for therapeutic nucleic acids, and various kinds of recombinant viruses were designed to efficiently deliver the genes to the target cells. Fairly high efficacy was reported in some clinical trials, but there still remain problems such as random recombination, immunogenicity, or neutralization by the antibody.[1–3] As a safer alternative, polycations and cationic lipids have been explored as nonviral transfection mediators.[4] The deoxyribonucleic acid (DNA) molecules can electrostatically associate with these cationic reagents to form small particles.[5] However, these cationic reagents can lead to high gene expression only in cultured cells, and their *in vivo* transfection efficiency is very limited.

Low gene expression efficiency of the nonviral vectors would be mainly attributed to their positive surface charge, which invites an adverse interaction with blood components or cells,[6–8] and too large size of the complex particles.[9] We found that hyaluronic acid (HA) could deposit onto the DNA–polycation (or cationic lipid) complexes to recharge their surface to negative, and effectively diminished the adverse interactions.[8,10] Too large size of the particle has long been the other big problem for an efficient delivery. Small polymer ion complexes can be obtained only at very low concentration. But when they are condensed, they are easily aggregated, and inactivated. HA coating could stabilize the dispersion of the DNA complexes, and DNA–polyethyleneimine(PEI)–HA ternary complex could, thus, be freeze-dried without any cryoprotectant maintaining their size and gene transfection activity under precise conditions. It enabled the preparation of very small DNA complex particles by the following method.[11] Small DNA–PEI–HA ternary complex was prepared at highly-diluted solutions. It was lyophilized, and then rehydrated with small amount of water to afford condensed suspension of very small DNA complex particles. It achieved highly-effective *in vivo* gene transfection.[11] HA coating also functioned as targeting ligand to malignant cells,[10] and to improve the transcription efficiency of the DNA complex.[10]

Therapeutic efficacy of the small DNA–polycation–HA complexes was then examined. Complexes comprising plasmid DNA coding cytokines were prepared and injected into tumor-bearing mice. It showed strong suppression of the tumor growth, and the small tumors completely disappeared.[11] In those experiments, repeated injection of the complex was required to achieve the satisfactory therapeutic effect, and single dose rarely induced high response, probably owing to the short duration of the gene expression by such artificial vectors. However, frequent injection is sometimes difficult, in medical practice depending on the injection site. Slow-release system is, thus, desired to attain a high therapeutic effect by a few times

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of administration. As for slow-releasing device, a biocompatible and biodegradable material is required. Calcium phosphate (CaP)-based compounds such as hydroxyapatite, having similar inorganic components to bone and tooth, have recently been used as drug-sustained releasing reservoir.[12–14]

We have developed several kinds of durable drug releasing reservoir consisting of an apatite cement that would be degraded by osteoclast to slowly release the contents.[15] CaP-coated surfactant micelle was also synthesized. Simvastatin-containing micelles coated by the inorganic salt showed much higher efficacy on the osteoporosis model mouse than the drug itself.[16] In this study, we developed novel CaP nanocapsules including DNA–PEI–HA ternary complexes, and their durable gene expression, and therapeutic efficacy on tumor-bearing mice was examined.

EXPERIMENTAL

Materials

Hyaluronic acid sodium salt (from a microorganism), and linear PEI (PEI"MAX"; Mw 40,000, DPn 580 as a HCl salt) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan) and Polyscience, Inc. (Warrington, PA), respectively. YOYO-1 iodide (YOYO) was obtained from Invitrogen Corporation (Eugene, OR). 4′,6-Diamidino-2-phenylindole, dihydrochloride (DAPI) were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Green fluorescent protein (GFP) coding plasmid with cytomegavirus promoter was obtained from CLONTECH Laboratories, Inc. (Mountain View, CA) Plasmid coding mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) was produced with pcDNA3.1 vector and the gene isolated from mouse lymphocytes.[17] B16 cells were obtained from Cell Bank, RIKEN BioResource Center, and Health Science Research Resources Bank.

Preparation of CaP Nanocapsules Including DNA–PEI–HA Ternary Complexes

Typically, an aqueous solution of DNA (300 μg/28.08 mL; [PO] = 32.3 μM) was mixed with a solution of HA (0.91 mL; [COOH] = 12 mM) and then with PEI solution (0.91 mL; [NH] = 12 mM). The mixing ratio of DNA, PEI, and HA was at P:N:COOH = 1:12:12 in mole. All the solutions were prepared in pure water. After standing for 30 min, it was mixed with 100 μL of 10% dextran (final [DNA] = 10 μg/mL), and then freeze dried. The resulting white spongy complex was rehydrated with 200 μL pure water, and then mixed with double volume of 1.5 times concentrated simulated body fluid (1.5 SBF), which was prepared according to the method described by Kokubo and coworkers.[18–20] The mixture was kept at 4°C for one night. CaP was deposited outside the DNA–PEI–HA complex to form nanocapsules including the DNA complex ([DNA] = 500 μg/mL). DNA complex mixed with 1.5 times phosphate-buffered saline (1.5 PBS) instead of 1.5 SBF was also prepared similarly as a control without encapsulation.

Electrophoresis

The suspension of DNA–PEI–HA treated with or without SBF ([DNA] = 500 μg/mL) was diluted with pure water (final [DNA] = 15 μg/mL). Half volume of 30% NaCl solution was then added to the DNA complex suspension and incubated for 24 h at 37°C to dissociate the DNA complex. The samples were subjected to electrophoresis ([agarose gel] = 1%) at 100 V for 45 min, and dissociation behavior of the DNA complex was observed.

Scanning electron microscopy and energy dispersive X-Ray spectroscopy (SEM–EDS) Analysis

The DNA complex prepared and treated with 1.5 SBF as above was dropped onto adhesive carbon tape and vacuum dried overnight. The surface was analyzed by SEM–EDS (JSM-7600F; JEOL Ltd., Tokyo, Japan) operated at 5 kV.

X-Ray Diffraction Analysis

The DNA complex was prepared and treated with 1.5 SBF as above, and the X-ray powder diffraction profiles were measured with a powder X-ray diffractometer (RINT-ULTIMA III; Rigaku Company, Tokyo, Japan). Conditions were as follows: target, Cu; filter, Ni; voltage, 40 kV; current, 40 mA; scanning speed; 2°/min.

Microscopic Observation of the DNA Complex Particles

Fluorescence microscopic observation was performed with an IX70 microscope (Olympus Corporation, Tokyo, Japan) equipped with a 100× oil immersion objective lens and a high-sensitivity Hamamatsu SIT TV camera (Hamamatsu Photonics K.K., Sizuoka, Japan)

connected to a DVD recorder. The DNA complex samples prepared as in the electrophoresis study ([DNA] = 15 μg/mL) was mixed with an equal volume of DAPI solution (4 μM) to visualize the DNA molecule, and subjected to an observation.

Measurement of the Size of the DNA Complex Particles

The DNA complexes were prepared and treated with 1.5 SBF as above, diluted by PBS to [nucleotide] = 20 μM, and then measured for their sizes by a dynamic light scattering (DLS) method with a particle analyzer (Zetasizer Nano ZS, Malvern Instruments Ltd., Worcestershire, UK).

In Vitro Transfection

Plasmid DNA coding GFP was employed to measure the gene expression efficiency. DNA solution (2 μg in 187.2 μL; [PO] = 32.3 μM) was mixed with 6.1 μL of HA solution ([COOH] = 12 mM), and with 6.1 μL of PEI solution ([NH] = 12 mM), in this order. After standing for 30 min, it was mixed with 0.67 μL of 10% dextran (final [DNA] = 10 μg/mL), and freeze-dried. The lyophilized DNA complex was rehydrated with 66.7 μL pure water. It was then mixed with 133.3 μL of 1.5 SBF or 1.5 PBS, and left standing at 4°C for one night.

B16 cells, a mouse melanoma cell line, were seeded in 24-well plates at 8.7×10^4 cells per well, and cultured in 1 mL minimum essentital medium (MEM) supplemented with 10% fetal bovine serum (FBS), penicillin G sodium (100 unit/mL), and streptomycin sulfate (0.1 mg/mL) for 1 day. The medium was then replaced with 1 mL of fresh medium with FBS and the antibiotics. Two-hundred microliter of the DNA complex suspension containing 2 μg of DNA was added to each well, and the cells were incubated at 37°C in a 5% CO_2 incubator. The medium was replaced with 1 mL of fresh one every day.

In vitro transfection was performed also with fluorescently labeled DNA to evaluate the cellular uptake efficiency. DNA coding GFP was labeled with YOYO at a YOYO–nucleotide ratio of 0.02. It was mixed with HA and PEI, and resulting fluorescent DNA complex was then added to the cells (2 μg of