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Carbonate apatite-facilitated intracellularly delivered siRNA for efficient knockdown of functional genes

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ABSTRACT

Gene therapy through intracellular delivery of a functional gene or a gene-silencing element is a promising approach to treat critical diseases. Elucidation of the genetic basis of human diseases with complete sequencing of human genome revealed many vital genes as possible targets in gene therapy programs. RNA interference (RNAi), a powerful tool in functional genomics to selectively silence messenger RNA (mRNA) expression, can be harnessed to rapidly develop novel drugs against any disease target. The ability of synthetic small interfering RNA (siRNA) to effectively silence genes *in vitro* and *in vivo*, has made them particularly well suited as a drug therapeutic. However, since naked siRNA is unable to passively diffuse through cellular membranes, delivery of siRNA remains the major hurdle to fully exploit the potential of siRNA technology. Here pH-sensitive carbonate apatite has been developed to efficiently deliver siRNA into the mammalian cells by virtue of its high affinity interactions with the siRNA and the desirable size of the resulting siRNA/apatite complex for effective cellular endocytosis. Moreover, following internalization by cells, siRNA was found to be escaped from the endosomes in a time-dependent manner and finally, more efficiently silenced reporter genes at a low dose than commercially available lipofectamine. Knockdown of cyclin B1 gene with only 10 nM of siRNA delivered by carbonate apatite resulted in the significant death of cancer cells, suggesting that the new method of siRNA delivery is highly promising for pre-clinical and clinical cancer therapy.

1. Introduction

Genes encode proteins through messenger RNA (mRNA) to carry out the major functions of a biological system and a disorder either acquired or genetic is usually associated with the suppression or the overexpression of certain genes. Regulation of the gene expression particularly through the delivery of exogenous gene(s) or gene-silencing element(s) could assist in restoring the regular physiological functions for treatment of a genetic or an acquired disease. RNA interference (RNAi) being one of the mechanisms to selectively silence mRNA expression can be harnessed to rapidly develop novel drugs against target genes [1–6]. There are two basic ways of implementing RNAi for selective gene inhibition: 1) cytoplasmic delivery of short interfering RNA (siRNA) and 2) nuclear delivery of gene expression plasmid to express a short hairpin RNA (shRNA) [7]. Silencing by synthetic siRNA, an RNA duplex of 21–23 nucleotides, is more advantageous than shRNA partly due to the difficulty of constructing shRNA expression systems prior to the selection and verification of the active sequences [7] and the requirement of the expression system to cross the nuclear membrane for shRNA expression [8]. The ability of siRNA to potently, but reversibly, silence genes in vivo has made them a highly promising drug therapeutic with several different clinical trials ongoing and more poised to enter the clinic in the future [2,5]. However, due to the strong anionic phosphate backbone with consequential electrostatic repulsion from the anionic cell membrane, siRNA is unable to passively diffuse across the membrane [9]. For the intracellular delivery of siRNA, both viral or non-viral vectors have been investigated. Although the viral vectors are highly efficient, they are limited to shRNA delivery and remain highly immunogenic and carcinogenic. On the other hand, the non-viral systems are promising alternatives for siRNA delivery since they are relatively safe and cost-effective. Being usually polycationic, they are able to form complexes with anionic nucleic acid, protecting it from nuclease attack and facilitating cellular uptake through electrostatic interactions with negatively charged plasma membrane or through specific interactions

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; DLS, dynamic light scattering; TEM, transmission electron microscope; GFP, green fluorescence protein; RNAi, RNA interference; siRNA, small interfering RNA; shRNA, short hairpin RNA.

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between the ligand attached to the complex and the receptor on cell membrane [8]. Among the non-viral vectors, both polyplexes as well as lipoplexes have been found efficient for siRNA delivery with significant gene-silencing effect both *in vitro* and *in vivo* [10–38]. However, synthetic non-viral systems are inefficient and an increase in performance is often associated with an increase in cytotoxicity [39]. The major obstacle for siRNA delivery in the non-viral route is the degradation of a significant portion of the internalized siRNA by lysosomal nucleases [40]. The endosomal escape of siRNA is, therefore, a crucial step in successful gene silencing.

We have recently developed an efficient delivery system based on some fascinating properties of carbonate apatite-ability of preventing crystal growth for generation of nanoscale particles as needed for efficient endocytosis and fast dissolution kinetics in endosomal acidic compartments to facilitate the release of delivered therapeutics from the particles and endosomes [41–50]. Here, we show that pH-sensitive carbonate apatite particles having high affinity interactions with siRNAs, mediate efficient endocytosis and subsequent endosomal escape of the siRNAs, leading to the silencing of reporter gene expression more effectively than commercially available lipofectamine. Moreover, nanoparticle-assisted delivery of validated siRNA against cyclin B1 results in significant inhibition of cancer cell growth.

2. Materials and methods

2.1. Reagents

Plasmid pGL3 (Promega) containing a luciferase gene under SV40 promoter and pEGFP-N2 (CLONTECH Laboratories, Inc.) containing green fluorescence protein gene under CMV were propagated in the bacterial strain XL-1 Blue (as described in Molecular Cloning) and purified by QIAGEN plasmid kits. LysoTracker™ Red DND-99, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and DMEM were purchased from Molecular Probes, Sigma and Gibco BRL, respectively. The lipofectamine 2000 transfection reagent was obtained from Invitrogen™ corporation, California, USA. Luciferase GL3 siRNA (Target sequence 5′-AACTTACGCTGAGTACTTCGA-3′), GFP-22 siRNA (Target sequence 5′-CGGCAAGCTGACCCTGAAGTTCAT-3′), siRNA against cyclin B1 (Target sequence 5′-AACACTTATACTAAGCACCAA-3′) and all Stars Neg. siRNA Fluorescein were purchased from QIAGEN. siRNAs were delivered in the lyophilized form and upon delivery the siRNAs were diluted to obtain a 20 μM solution using RNAse-free water provided by Qiagen. The siRNA solution was then allocated into multiple reaction tubes for storage as repeated thawing might affect siRNA's silencing efficiency. The siRNAs were stored at −20 °C as recommended by Qiagen.

2.2. Cell culture

HeLa cells were cultured in 75-cm^2 flasks in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% fetal bovine serum (FBS), 50 μg penicillin ml–1, 50 μg streptomycin ml–1 and 100 μg neomycin ml–1 at 37 °C in a humidified 5% CO_2-containing atmosphere.

2.3. Formation of siRNA/carbonate apatite particles and transfection of cells

Cells from the exponentially growth phase were seeded at 50,000 cells per well into 24-well plates the day before transfection. 3–6 μl of 1 M $CaCl_2$ was mixed with 100 pM–100 nM of siRNA in 1 ml of fresh serum-free HCO_3^- (44 mM)-buffered DMEM medium (pH 7.5), followed by incubation at 37 °C for 30 min for complete generation of siRNA/carbonate apatite particles. Medium with generated siRNA-containing particles was added with 10% FBS to the rinsed cells. After 4 h incubation, the medium was generally replaced with serum-supplemented medium and the cells were cultured up to 24–72 h depending on the assay. In some experiments, siRNA/apatite particles were continuously incubated with the cells for 48 h. siRNA/lipofectamine formulation and transfection were done according to the procedure provided by Invitrogen.

2.4. Particle size measurements

The distribution of particle size was measured using FPAR-1000 fiber-optics particle analyzer (Otsuka Electronics, Osaka, Japan) equipped with a 660 nm diode laser. The measurement was carried out by a scattering angle of 90° at 25 °C. The size distribution was obtained by CONTIN method.

2.5. Observation of siRNA/apatite complexes with transmission electron microscope (TEM)

siRNA/apatite complexes prepared with 100 nM of siRNA and 5 mM of $CaCl_2$ were observed under H-7500 transmission electron microscope (Hitachi, Tokyo, Japan) at an acceleration voltage of 80 kV. After loading of the particles on the copper grid with collodion membrane, the grid was dried in the air.

2.6. Determination of siRNA loading efficiency

The amount of fluorescein-labeled siRNA adsorbed onto apatite nanoparticles was determined from the fluorescence intensity of the pellet obtained after centrifugation of siRNA/apatite complexes. Following generation of siRNA/apatite particles as described above, using 5 mM Ca^{2+} and 1–200 nM of fluorescein siRNA and centrifugation at 15,000 rpm for 3 min, the resulting pellet was washed 3 times with the same medium and dissolved in 100 μl of 10 mM EDTA-PBS. The whole dissolved particle solution was taken to an assay plate and quantified for the fluorescence intensity by a fluorescence microplate reader. Free fluorescein-labeled siRNA (1 pM to 200 nM) in PBS was quantified and plotted to make the calibration curve. siRNA loading was quantified with the help of the calibration curve.

2.7. Estimation of the dissolution of carbonate apatite particles in acidic solution

200 ml of siRNA/apatite complex suspension was prepared from DMEM containing 100 nM of fluorescein-labeled siRNA and 6 mM of exogenously added $CaCl_2$. The pH of the suspension was decreased by adding 1 N HCl and 1 ml of the suspension at each pH was taken. Optical density at 320 nm of the collected sample was measured with SmartSpec™ 3000 spectrophotometer (Bio-Rad).

2.8. Cellular uptake and intracellular siRNA measurement in HeLa cell line

siRNA/apatite and siRNA/lipfectamine 2000 particles were prepared in the presence of florescein siRNA (100 nM) and were added onto the HeLa cells with or without (in case of Lipofectamine 2000) 10% serum and kept for 4 h in the incubator for cellular internalization. Extracellular particles were removed by EDTA prior to observation of the cells by a fluorescence microscope (Olympus-IX71). For quantitative analysis, HeLa cells were lysed using the lysis buffer (NP40) following 4 h incubation of siRNA/apatite and siRNA/lipofectamine 2000 particle suspensions with the cells and the intracellular fluorescein intensity was determined using a microplate reader (DTX 880, Multimode Detector by BECKMAN COULTER). Controls were the intensities for the delivery of free fluorescein siRNA and the cells without any particle.