Colloids and Surfaces B: Biointerfaces 118 (2014) 111–116

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Modification of nanostructured calcium carbonate for efficient gene delivery

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ARTICLE INFO

Article history:
Received 22 November 2013
Received in revised form 8 February 2014
Accepted 4 March 2014
Available online 29 March 2014

Keywords:
Gene delivery
Nanoparticles
Calcium carbonate
Calcium phosphate
Co-precipitates

ABSTRACT

In this study, a facile method to modify nanostructured calcium carbonate ($CaCO_3$) gene delivery systems by adding calcium phosphate (CaP) component was developed. $CaCO_3$/CaP/DNA nanoparticles were prepared by the co-precipitation of Ca^{2+} ions with plasmid DNA in the presence of carbonate and phosphate ions. For comparison, $CaCO_3$/DNA nanoparticles and CaP/DNA co-precipitates were also prepared. The effects of carbonate ion/phosphate ion (CO_3^{2-}/PO_4^{3-}) ratio on the particle size and gene delivery efficiency were investigated. With an appropriate CO_3^{2-}/PO_4^{3-} ratio, the co-existence of carbonate and phosphate ions could control the size of co-precipitates effectively, and $CaCO_3$/CaP/DNA nanoparticles with a decreased size and improved stability could be obtained. The *in vitro* gene transfections mediated by different nanoparticles in 293T cells and HeLa cells were carried out, using pGL3-Luc as a reporter plasmid. The gene transfection efficiency of $CaCO_3$/CaP/DNA nanoparticles could be significantly improved as compared with $CaCO_3$/DNA nanoparticles and CaP/DNA co-precipitates. The confocal microscopy study indicated that the cellular uptake and nuclear localization of $CaCO_3$/CaP/DNA nanoparticles were significantly enhanced as compared with unmodified $CaCO_3$/DNA nanoparticles.

1. Introduction

Gene therapy shows great promise for the treatment of genetic disorder diseases such as cancers. The growing interest in gene therapy has become a strong incentive to develop the gene vectors with high transfection efficiency and minimized toxicity [1–4]. Although viral vectors are more effective, their toxicity and production problems have greatly limited their applications. Compared with viral vectors, non-viral vectors have lower immune response and higher safety, and thus have attracted increasing attention despite their relatively low transfection efficiency. Nevertheless, the biocompatibility of the widely investigated non-viral vectors based on cationic polymers and cationic liposomes is still not satisfactory [1].

Among different non-viral gene delivery strategies, the technique of co-precipitation of Ca^{2+} with DNA in the presence of inorganic anions present in bodies is an attractive option because of its good biocompatibility and biodegradability [5–9]. For example, calcium phosphate (CaP) co-precipitation technique is a well-established method to prepare CaP/DNA co-precipitates that can effectively protect the entrapped DNA from degradation and mediate gene transfection [5–8]. Despite the advantage of this technique, i.e., safety and non-toxicity, this method suffers from the less consistent transfection efficiency and difficulty in controlling transfection conditions [5,6]. As an effective modification of this method, the co-precipitation of Ca^{2+} with DNA in the presence of CO_3^{2-} anions is attractive option because the ease in controlling the co-precipitation condition and the improved performance in gene delivery [9]. Nevertheless, the growth of $CaCO_3$/DNA precipitates also causes unfavorable effects on cellular internalization and gene transfection [10]. In order to control the particle size and crystallization, improve the thermodynamic stability of particles, and thus further enhance the delivery efficiency, various modification strategies have been developed for the gene delivery systems based on inorganic compounds. According to previous studies, the careful optimization of transfection experiment parameters such as the initial concentrations of the ions, temperature and the mode of mixing could minimize the variability in transfections [5,6]. The presence of additional components, such as ions [11,12] and macromolecules [13–15], could affect the crystallization of inorganic compounds such as $CaCO_3$ and CaP. As a result, through

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http://dx.doi.org/10.1016/j.colsurfb.2014.03.007
0927-7765/

optimizing the composition of the delivery system, the particle size can be effectively controlled, which results in enhanced transfection efficiency [16–18]. In our previous study, alginate was introduced to the co-precipitation system to form alginate/$CaCO_3$/DNA nanoparticles to achieve an improved colloid stability and gene delivery efficacy [10].

In this study, we further developed a facile method to modify the $CaCO_3$ based nanoparticles for gene delivery. In our study, $CaCO_3$/CaP/DNA nanoparticles were fabricated by co-precipitation of Ca^{2+} ions with DNA in the presence of carbonate and phosphate ions simultaneously. The co-existence of carbonate and phosphate anions could efficiently retard the crystallization, and the size of the nanoparticles prepared could be well controlled at less than 200 nm in aqueous media. The *in vitro* gene delivery showed that the $CaCO_3$/CaP/DNA nanoparticles exhibited significantly improved gene delivery efficiency as compared with both $CaCO_3$/DNA and CaP/DNA co-precipitates.

2. Experimental

2.1. Materials

Anhydrous calcium chloride, anhydrous sodium carbonate and sodium phosphate tribasic dodecahydrate of analytical grade were supplied by Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China) and used as received. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Amresco. Dimethylsulfoxide (DMSO) was from Sigma. Hoechst 33258, YOYO-1 iodide and Lipofectamine 2000 were purchased from Invirogen.

Human embryonic kidney cell line (293T) and human cervical carcinoma cell line (HeLa) were obtained from China Center for Typical Culture Collection (Wuhan, China). The medium for cell culture was Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS), 2 mg/mL $NaHCO_3$, and 100 U/mL penicillin/streptomycin. Cells were incubated at 37 °C in humidified air/5% CO_2.

Luciferase plasmid (pGL3-Luc) was amplified in *Escherichia coli* and extracted and purified by QIAfilter Plasmid Mega Kit (QIAGEN). Plasmids were suspended in water and stored at −20 °C.

2.2. Preparation and characterization of $CaCO_3$/CaP/DNA nanoparticles

1 μg of pGL3-Luc (1 μg/μL) was added to 16 μL of $CaCl_2$ solution (0.5 M), and then diluted with deionized water to make solution A with a volume of 50 μL. Na_2CO_3 solution (0.01 M) and Na_3PO_4 solution (0.01 M) with a particular volume ratio (31, 15, 10, 7, 3 and 1, respectively) was mixed to obtain 16 μL of the mixed solution, and then diluted with deionized water to make solution B with a volume of 50 μL. Solution A was rapidly added into solution B under stirring to obtain $CaCO_3$/CaP/DNA nanoparticles. The solution containing $CaCO_3$/CaP/DNA nanoparticles was immediately used for gene transfection.

For comparison, $CaCO_3$/DNA nanoparticles were prepared using 16 μL of Na_2CO_3 solution (0.01 M) in the absence of Na_3PO_4. CaP/DNA nanoparticles were prepared using 16 μL of Na_3PO_4 solution (0.01 M) in the absence of Na_2CO_3. Other conditions were the same as that for the preparation of $CaCO_3$/CaP/DNA nanoparticles.

The size and zeta potential of the nanoparticles were measured by a Zetasizer Nano ZS (Malvern Instruments). Prior to measurements, 800 μL of deionized water was added to 200 μL of the freshly prepared nanoparticles containing solution (containing 2 μg of pGL3-Luc) for dilution. Data were given as mean ± standard deviation (SD) based on three independent measurements.

2.3. Determination of encapsulation efficiency of DNA

500 μL of nanoparticles containing solution (containing 5 μg of pGL3-Luc) was prepared as mentioned before and centrifuged at 4 °C for 1 h at 18,000 rpm. After centrifugation, the amount of non-precipitated free DNA remaining in the supernatant of solution was determined by the Quant-iT™ PicoGreen® dsDNA Assay Kit (Molecular Probes) according to the manufacturer's protocol using a spectrofluorophotometer (RF-5301 PC, Shimadzu). Data were given as mean ± standard deviation (SD) based on three independent measurements.

The encapsulation efficiency of DNA was calculated as follows:

$$\text{encapsulation efficiency} = \frac{W_T - W_F}{W_T} \times 100\%$$

where W_T is the total weight of DNA fed and W_F is the weight of non-encapsulated free DNA.

2.4. In vitro transfection

The cells in 1 mL of complete medium (DMEM containing 10% FBS) were seeded directly in the well of a 24-well plate (5×10^4 cells per well) and incubated for 24 h. Then the solution (100 μL with 1 μg pGL3-Luc) containing $CaCO_3$/CaP/DNA nanoparticles with a particular CO_3^{2-}/PO_4^{3-} molar ratio (31, 15, 10, 7, 3 and 1, respectively) were added to each well, and the cells were co-incubated with the nanoparticles in the complete medium containing 10% FBS at 37 °C for 48 h. The gene expression was evaluated after co-incubation for 48 h.

To assay the expression of luciferase, the medium was removed and the cells were rinsed gently by phosphate buffered saline (PBS, 0.1 M, pH 7.4). After thorough lysis of the cells with reporter lysis buffer (Promega) (200 μL/well), the luciferase activity was determined by detecting the light emission from 20 μL cell lysate incubated with 100 μL of luciferin substrate (Promega) in a luminometer (Lumat LB9507, Berthold). The protein content of the cell lysate was determined by BCA protein assay kit (Pierce). The optical density (OD) value was determined at 570 nm using a microplate reader (Bio-rad 550). The data were given as mean ± standard deviation (SD) based on three independent measurements.

For comparison, the luciferase expressions mediated by $CaCO_3$/DNA nanoparticles, CaP/DNA co-precipitates, and Lipofectamine 2000/DNA complexes were measured. Lipofectamine 2000/DNA complexes were prepared following the manufacturer's Instructions. 1 μg of pGL3-Luc was used in each well (5×10^4 cells per well).

The statistical significance between two sets of data was calculated using Student's *t*-test. A p value <0.05 was considered statistically significant.

2.5. Study on cellular uptake of nanoparticles

1 μg of pGL3-Luc was mixed with 2.5 μL of YOYO-1 water stock solution (10 μM) and incubated for 15 min at 37 °C. Then the YOYO-1 labeled pGL3-Luc was used to prepare $CaCO_3$/CaP/DNA, $CaCO_3$/DNA, and CaP/DNA samples.

HeLa cells in complete medium (DMEM containing 10% FBS) were seeded directly in the well of a 24-well plate (5×10^4 cells per well). After incubation at 37 °C for 24 h, the nanoparticles containing solution (100 μL containing 1 μg YOYO-1 labeled pGL3-Luc) were added. After the cells were co-incubated with the nanoparticles in the complete medium containing 10% FBS at 37 °C for 4 h, the medium was removed and the cells were washed twice with PBS to remove the nanoparticles. The cell nuclei were stained with Hoechst 33258 solution for 20 min at 37 °C. Subsequently, the cells were washed with PBS three times and then incubated with