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A facile approach to construct hybrid multi-shell calcium phosphate gene particles*

Zhi-xue XU, Ran ZHANG, You-xiang WANG†‡, Qiao-ling HU†

(*MOE Key Laboratory of Macromolecular Synthesis and Functionalization, Department of Polymer Science and Engineering, Zhejiang University, Hangzhou 310027, China*)

†E-mail: yx_wang@zju.edu.cn; huql@zju.edu.cn

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Abstract: The calcium phosphate (CaP) particles have attracted much attention in gene therapy. How to construct stable gene particles was the determining factor. In this study, hybrid multi-shell CaP gene particles were successfully constructed. First, CaP nanoparticles served as a core and were coated with DNA for colloidal stabilization. The ξ-potential of DNA-coated CaP nanoparticles was −15 mV. Then polyethylenimine (PEI) was added and adsorbed outside of the DNA layer due to the electrostatic attraction. The ξ-potential of hybrid multi-shell CaP particles was slightly positive. With addition of PEI, the hybrid multi-shell particles could condense DNA effectively, which was determined by ethidium bromide (EtBr) exclusion assay. The hybrid particles were spherical and uniform with diameters of about 150 nm at proper conditions. By simple modification of PEI, the hybrid multi-shell CaP gene particles were successfully constructed. They may have great potential in gene therapy.

Key words: Calcium phosphate, Polyethylenimine (PEI), Hybrid, Gene
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1 Introduction

Gene therapy has become a research focus during the past several decades (El-Aneed, 2004; Ren *et al*., 2005b; 2006; Wagner and Kloeckner, 2006). Non-viral gene vectors including lipids, peptides, polycations, and inorganic particles have been paid much attention to due to their remarkable advantages, such as low immunogenicity, ease of large-scale production, and large nucleic acid loading capacity (Radler *et al*., 1997; Uherek and Wels, 2000; Wang and Shen, 2005; Ren *et al*., 2006; Wang *et al*., 2007).

Among them, the calcium phosphate (CaP) particles have been one of the research focuses (Kakizawa *et al*., 2004; Bisht *et al*., 2005; Sokolova and Epple, 2008). Conventionally, CaP particles were prepared via co-precipitation method. The mixture of deoxyribonucleic acid (DNA) and calcium chloride solution was added to phosphate buffered saline (PBS) solution. However, the CaP particles via co-precipitation method were unstable and grew with time, which limits their use in gene therapy. How to construct stable CaP gene particles is the determining factor. Many methods have been adopted to improve their stability. Polyethylene glycol-b-polyanion was added to the CaP gene particles by Kakizawa *et al*. (2004; 2006). Driven by electrostatic attraction, the hybrid particles showed high stability and efficient transfection (Kakizawa *et al*., 2004; 2006). DNA was also used to coat and stabilize the CaP particles (Welzel *et al*., 2004; Sokolova *et al*., 2006; 2007). The DNA-coated particles showed high transfection efficiency in in-vitro cell culture experiments. However, when used in vivo, the outside DNA layer was easily attacked by nuclease; thus, the in-vivo transfection

‡ Corresponding author
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efficiency might be influenced.

The ideal gene delivery systems should fulfill a series of drug delivery functions in the extracellular and intracellular transports of DNA. Polyethylenimine (PEI) was commonly used as non-viral vector due to its strong DNA condensation capacity and intrinsic endosomolytic activity (Petersen *et al*., 2002; Lungwitz *et al*., 2005; Ren *et al*., 2005a; Wang *et al*., 2006). It can carry DNA across the cell membrane, harness the molecular motors to actively move along the microtubule network, and finally enter the nucleus (Thomas and Klibanov, 2002; Bausinger *et al*., 2006). The PEI/DNA polyplexes showed effective transfection at high N/P ratio, whereas excessive PEI leads to high toxicity (Fischer *et al*., 1999). In the present study, only a small quantity of PEI was added to the suspension of DNA-coated CaP particles as shown in Fig. 1. Due to the electrostatic attraction, PEI was adsorbed to the outside wall of the DNA layer to form hybrid multi-shell gene particles. The preparation and properties of hybrid multi-shell particles were investigated in this study.

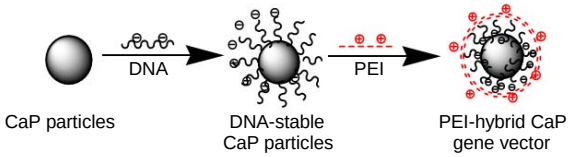

Fig. 1 Formation illustration of PEI-hybrid multi-shell calcium phosphate gene particles

2 Materials and methods

2.1 Materials

PEI (branched, 25 kDa) was obtained from Sigma-Aldrich (Steinheim, Germany). Calcium chloride and sodium phosphate were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). DNA (fish sperm, sodium salt) was purchased from AMRESCO (Shanghai, China). Three-time distilled water was used. All reagents were used as received.

2.2 Preparation of DNA-coated CaP nanoparticles

CaP particles were prepared by adding calcium chloride (6.25 mmol/L) solution into an equal volume of sodium phosphate (3.74 mmol/L) at pH 8.0. The mixture was vigorously vortexed. Then various quantities of DNA were instantly added to prepare DNA-coated CaP particles. As a control, CaP gene particles were also prepared via co-precipitation method by adding the mixture of calcium phosphate and DNA into a phosphate precursor. The DNA concentration of co-precipitated particles was 58 μg/ml.

2.3 Preparation of hybrid multi-shell gene particles

The calcium phosphate nanoparticles served as core and were coated with DNA (58 μg/ml) for colloidal stabilization. Then PEI of various concentrations was added. Due to the electrostatic attraction, PEI was adsorbed to the outside wall of the DNA layer to construct hybrid multi-shell gene particles. It is usually thought that 1 μg DNA contains 3 nmol of anionic phosphate. The N/P ratio (the molecular ratio of amine groups of PEI to phosphate groups of DNA) was calculated according to the adding dosages of PEI and DNA. In this study, the hybrid gene vectors were prepared at N/P ratios of 0.5, 1, 1.5, and 2, respectively. The hybrid gene particles were marked as CaP/DNA/PEI-0.5, CaP/DNA/PEI-1, CaP/DNA/PEI-1.5 and CaP/DNA/PEI-2 correspondingly.

2.4 Characterization of CaP gene particles

The sizes of particles were determined by dynamic light scattering (DLS). Measurements were carried out with a 90Plus/BI-MAS particle size analyzer (Brookhaven, Holtsville, NY, USA). Scattering light was detected at 90° angle and determined at room temperature. For data analysis, the refractive index (1.330) was used. The ξ-potential was measured by Zetasizer 3000HS (Malvern, UK).

Atomic force microscope (AFM) images were conducted on an AFM (SPA400, Seiko, Japan) by tapping mode in air. The freshly cleaved mica was dipped into the suspension of CaP gene vectors and then taken out. The sample was dried overnight in vacuum for measurement.

Ethidium bromide (EtBr) exclusion assay was used to evaluate the DNA condensation ability of polycation (Petersen *et al*., 2002). The CaP gene particles were prepared as mentioned above. Then EtBr (the weight ratio of EtBr to DNA was 1:4) was added. The suspension was shaken and analyzed by fluorescence spectrum (LS55, Perkin-Elmer, Connecticut, USA) at an excitation of 510 nm and an emission of

610 nm. Results are given as relative fluorescence values where 1 is the fluorescence of DNA interacting with EtBr and 0 is the remaining fluorescence of non-intercalating EtBr.

3 Results and discussion

3.1 Property of DNA-coated CaP particles

By adding the mixture of DNA and calcium chloride solution to PBS solution, CaP particles via co-precipitation method were prepared as contrast. The DLS results indicated that the particle size was increased quickly. It reached to about 850 nm after a 30-min incubation. A large amount of precipitate was observed even after long incubation. It has proved that a successful transfection of cells strongly depends on the particle size (Zhang *et al*., 2002; Roy *et al*., 2003). Large aggregates make it difficult to internalize into the cells and reduce the cell uptake efficiency. Therefore, construction of stable CaP gene particles has become the research focus.

DNA-coated CaP particles were then constructed. At pH 8.0, an equal volume of 6.25 mmol/L calcium chloride solution was mixed with 3.74 mmol/L sodium phosphate. The mixture was vigorously vortexed and then added with DNA. The negatively charged DNA interacts with calcium phosphate surface to coat the inorganic nanoparticles and thus prevents their aggregation (Okazaki *et al*., 2001). DLS results indicated that DNA concentration had a great effect on the property of DNA-coated CaP particles (Table 1). At DNA concentrations of 58 and 72 μg/ml, spherical particles with diameters of about 200 nm were observed (Fig. 2). DLS indicated that higher or lower DNA concentration increased the size of particles. We assumed that at the lower DNA concentration, the surface was not sufficiently covered with DNA to prevent agglomeration of the CaP crystals. At higher concentrations, bridging agglomeration of the CaP particles occurred. This agglomeration may be driven by the interaction of complementary nucleobases of DNA (Sokolova *et al*., 2007). To obtain stable DNA-coated CaP nanoparticles, the optimal concentration of DNA was chosen to 58 μg/ml.

EtBr produces strong fluorescence when intercalating with DNA. However, only very weak fluorescence was observed when it is free in solution.

Table 1 Effect of DNA concentration on the property of DNA-coated CaP particles determined by DLS

DNA concentration (μg/ml)	Diameter* (nm)	PDI
16	645±202	0.22
30	392±170	0.17
58	240±118	0.22
72	246±133	0.21
150	390±92	0.22
280	367±202	0.22

* Mean±standard deviation; PDI: polydispersity

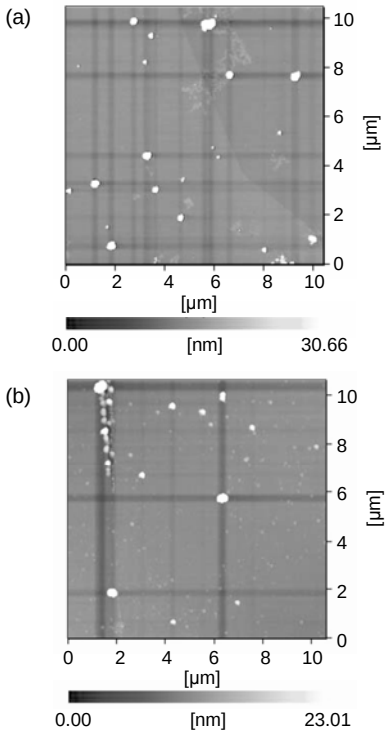

Fig. 2 Planar AFM images of DNA-coated CaP gene particles with DNA concentrations of (a) 58 μg/ml and (b) 72 μg/ml

When DNA is packed by polycations into the core, the EtBr is unable to intercalate within the DNA (Dutton *et al*., 1995). Thus the relative fluorescence values of polyplexes are low.

The CaP gene particles via co-precipitation method were also evaluated as contrast. The result is shown in Fig. 3. The relative fluorescence of co-precipitation gene vectors was about 0.31, which indicated that most of DNA was packed in the CaP particles. The relative fluorescence of DNA-coated CaP particles was near 1 at DNA concentration of 58 μg/ml and their ξ-potential was about −15 mV. DNA was mainly covered at the shell of CaP particles and

could easily interact with EtBr. So we induced the nucleases to be also easily contacted with the DNA in vivo. The in-vivo transfection efficiency is affected by a few factors, among which the incorporation of DNA into nanoparticles is a key point.

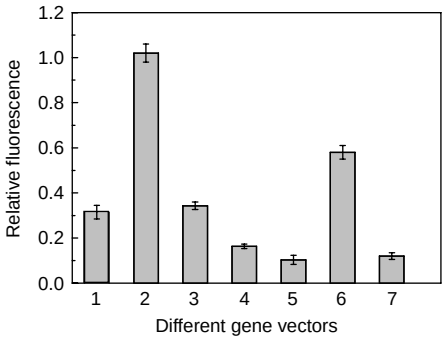

Fig. 3 Relative fluorescence values of different CaP gene particles
Gene vectors: 1, coprecipitation gene particles; 2, DNA-coated CaP particles; 3, CaP/DNA/PEI-0.5; 4, CaP/DNA/PEI-1; 5, CaP/DNA/PEI-2; 6, PEI/DNA-1; 7, PEI/DNA-10

3.2 Property of hybrid multi-shell gene particles

At DNA concentration of 58 μg/ml, stable DNA-coated CaP particles were prepared. Then PEI was added and adsorbed to the outside wall of the DNA layer due to the electronic attraction. The hybrid particles CaP/DNA/PEI-0.5, CaP/DNA/PEI-1, CaP/DNA/PEI-1.5, and CaP/DNA/PEI-2 were prepared. The dosage of PEI might have a great effect on the property of novel hybrid multi-shell particles.

EtBr exclusion assay was used to evaluate the DNA condensation ability. As contrast, the PEI/DNA polyplexes were prepared at N/P ratios of 1 and 10, respectively. The result is shown in Fig. 3. The PEI/DNA-1 polyplexes were not able to effectively condense DNA or maintain significantly high relative fluorescence at about 0.58. PEI/DNA-10 polyplexes showed excellent DNA condensation ability with relative fluorescence at about 0.12. However, for hybrid CaP gene particles, a small quantity of PEI led to the rapid reduction of the relative fluorescence. It even reached 0.16 for CaP/DNA/PEI-1 hybrid particles, very similar to PEI/DNA-10 polyplexes. All the results indicate that the hybrid multi-shell gene particles could effectively condense DNA as well as PEI/DNA-10 polyplexes. Our previous study indicated that the cell viability decreased with increasing PEI dosages (Wang *et al*., 2006). Comparing with PEI/DNA-10 polyplexes, the lower amount of PEI was needed to prepare CaP/DNA/PEI-1 gene vectors. Therefore, the cytotoxicity of hybrid multi-shell particles was reduced.

The size distribution of hybrid CaP/DNA/PEI particles was determined by DLS. The result indicated the mean diameters of CaP/DNA/PEI-0.5, CaP/DNA/PEI-1, and CaP/DNA/PEI-2 particles are about 200, 150, and 500 nm, respectively (Fig. 4). The dosage of PEI also had a great effect on the sizes of hybrid particles. The morphology of hybrid gene vectors was determined by AFM (Fig. 5). CaP/DNA/PEI-1 particles are spherical and uniform with

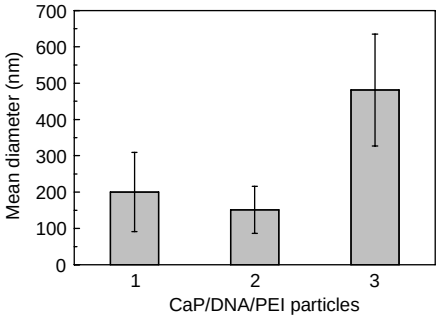

Fig. 4 Effect of PEI dosage on the property of CaP/DNA/PEI particles determined by DLS
CaP/DNA/PEI particles: 1, CaP/DNA/PEI-0.5; 2, CaP/DNA/PEI-1; 3, CaP/DNA/PEI-2

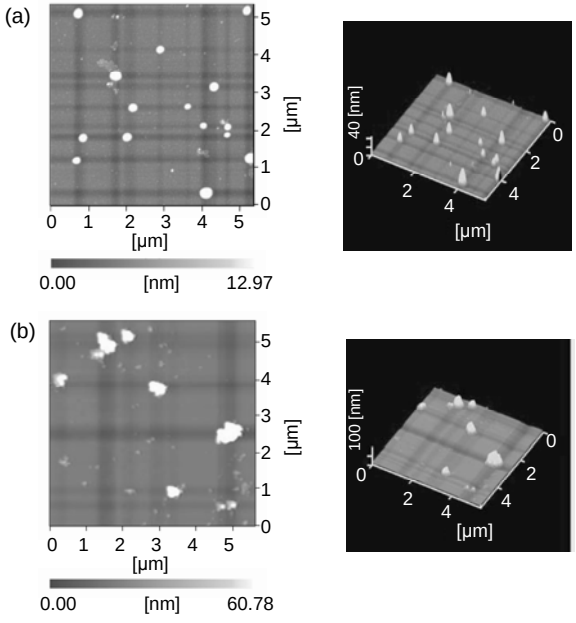

Fig. 5 Planar (left) and three-dimensional (right) AFM images of CaP/DNA/PEI gene particles
(a) CaP/DNA/PEI-1; (b) CaP/DNA/PEI-2

diameter of about 150 nm, whereas huge aggregates were observed for CaP/DNA/PEI-2 particles. We deduced that, with increasing addition of PEI, the excessive PEI chains on one particle were probably bound to another particle by interparticle cross-bridging (Sharma *et al*., 2005). Thus large aggregates were formed.

The ξ-potential of CaP gene particles was also compared (Fig. 6). The co-precipitative CaP gene vectors were slightly negative with most of the DNA packed in the core, while the ξ-potential of DNA-coated CaP gene particles reached to about −15 mV. The hybrid CaP/DNA/PEI-1 particles were about 4.8 mV. Taken together with the result of EtBr exclusion assay, we deduced that PEI formed the shell out of the DNA layer and showed excellent DNA condensation. It is likely that, when the hybrid particles were used in vivo, the nuclease was also difficult to contact with DNA. On the other hand, the cytotoxicity of hybrid multi-shell gene vectors was hopefully reduced due to the small quantity of PEI.

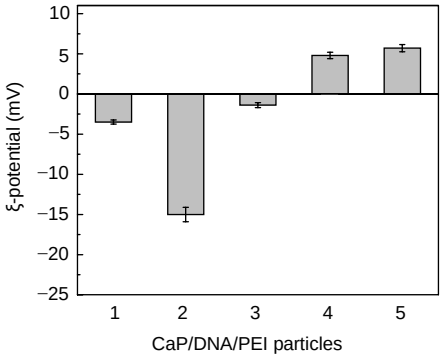

Fig. 6 ξ-potential of different CaP gene particles
CaP/DNA/PEI particles: 1, coprecipitation gene particles; 2, DNA-coated CaP particles; 3, CaP/DNA/PEI-0.5; 4, CaP/DNA/PEI-1; 5, CaP/DNA/PEI-2

In conclusion, in the present study, a novel hybrid multi-shell particles were successfully constructed using a facile approach and may have great potential in gene therapy.

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