Ca-Mg phosphate nano-crystals for high efficiency gene delivery and expression in mammalian cells

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ABSTRACT

Co-precipitation of DNA with calcium phosphate which is based on hydroxyapatite, is one of the most commonly used non-viral vectors. Although inefficiency in particle-mediated uptake of DNA by the cells has been considered as a major barrier of low transgene expression in vitro and in vivo, an effective way of manipulating particle growth kinetics at the molecular level has not been focused so far, which could overcome the hurdle dramatically. Here, we report on the development of a highly efficient synthetic device for gene delivery and expression into mammalian cells, based on controllable growth of nano-apatite particles. Mg^{2+} incorporation into the apatite particles caused significant inhibition of particle-growth, resulting in retention of nano-sized particles which could contribute remarkably to the cellular uptake of DNA and its subsequent expression (10 to 100-fold) compared with classical calcium phosphate co-precipitation, one of the most widely used transfection methods.

Keywords: calcium phosphate, hydroxyapatite, magnesium, nano-apatite, gene delivery, transfection

Non-viral gene-delivery techniques are preferable to the viral vectors for basic research and clinical medicine [1]. Despite existence of a wide variety of non-viral techniques particularly relying on synthetic lipids (liposomes), peptides (poly-L-lysine), dendrimers (polyamidoamine) and other polymers, such as polyethylenimine, limited understanding of the molecular and cellular basis in gene transfer hinders the development of a smart technology. Co-precipitation of DNA with calcium phosphate is one of the most commonly used non-viral vectors, having potential applications in gene therapy [2, 3]. A time-dependent control in particle growth kinetics was shown to modulate transfection and short time incubation resulted in finer particles and thus better performance in transgene expression [4]. Although the method is fairly straightforward, relying on direct mixing of the components, instead of the laborious dropwise mixing followed in the old system, transfection activity of the former was not better than the latter [5]. Here, we report on the generation of Ca-Mg phosphate precipitates which like Ca phosphate precipitates, adsorbed DNA, but unlike the latter, could prevent the growth of the particles to a significant extent, resulting in huge cellular uptake of DNA, followed by notably high transgene expression.

RESULTS AND DISCUSSION

Generation and chemical characterization of Ca-Mg phosphate particles

Addition of 0 to 140 mM Mg^{2+} along with 125 mM Ca^{2+} to HBS (pH 7.05) containing 0.75 mM inorganic phosphate, followed by incubation at room temperature, resulted in microscopically visible particles. The IR spectrum of Ca phosphate particles (generated in absence of Mg^{2+}) suggests formation of hydroxyapatite and the X-ray diffraction patterns also shows typical apatitic features [6]. To know the chemical composition of all types of the particles, elemental analysis was performed for sample 1, 2, 3,4, 5, 6, 7 and 8, representing, respectively, 0, 20, 40, 60, 80, 100, 120 and 140 mM Mg^{2+} added for particle generation (described above).With increase in Mg^{2+} concentrations in solution, particle-associated Mg^{2+} level increased upto ~3% with concomitant decrease in Ca^{2+} level whereas phosphorus (P) level remains almost fixed for sample 1 to 3 (~12%) and sample 4 to 8 (~16%), indicating precipitation of 2 different types of apatite [6]. The calculated molar ratio values (Table 1) indicate formation of hydroxyapatite with the formula $Ca_{10-x}Mg_x(PO4)_6(OH)_2$ for sample 1 to 3 and octacalcium phosphate (0CP) with the

Sample	Mg : Ca : P		
1	0.0	10.1	6
2	0.36	9.83	6
3	0.64	9.39	6
4	0.84	7.76	6
5	1.13	7.67	6
6	1.16	7.37	6
7	1.3	7.21	6
8	1.43	7.04	6

Table 1: Calculation of molar ratio values for Mg, Ca and P

formula $Ca_{4-x}Mg_x(PO4)_3$ for sample 4 to 8, thereby suggesting that a high Mg^{2+} level drives the reaction to the formation of OCP.

Regulating growth kinetics and sizes of particles

Turbidity determination of a particle suspension could be used to analyse time-dependent particle growth, following nucleation in a supersaturated solution [4]. As shown in Fig. 1, at 1min following mixing all of the components in HBS (described above), turbidity declined continuously with increasing Mg^{2+} concentrations in the solution, suggesting clearly that incorporated Mg^{2+} slows down the growth of the particles to a significant extent. With incubation for additional periods (5 to 30 min), turbidity plot showed an up and down profile which could be explained with the notion that an increasingly high concentrations of Mg^{2+} (20 to 60 mM) could further induce the precipitation reaction depending on the incubation time, thus causing an increment in turbidity for an increase in particle numbers and that with a more significant amount of Mg^{2+} (80 to 140 mM), inhibition of particle growth played the major role for the sharp decrease in turbidity.

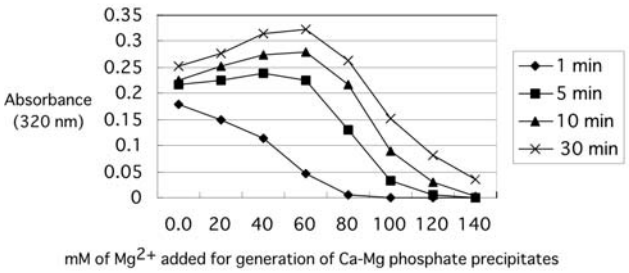

Figure 1: Monitoring precipitation kinetics. Turbidity measurement at 320 nm was used as an indicator of precipitation or particle growth. Just after mixing two solutions, one containing 1.5 mM inorganic phosphate in 300 μl of 2× HBS (pH 7.05) and the other containing 250 mM Ca^{2+}, in addition to 0.0 to 280 mM Mg^{2+} in 300 μl water, spectroscopic reading (320 nm) was taken at 1 to 30 min.

In order to make a better understanding of how Mg^{2+} inclusion into the particles contributes immensely to the reduction of the growth and consequently the sizes of the particles, we estimated the mean diameters of all types of particles during their growing stages. As shown in Fig. 2, at a period of time from 1 to 30 min following initiation of precipitation reaction, an increasing dose of Mg^{2+} dramatically reduced the particle diameters from micro to nano level. Moreover, the figure enables us to predict a clear and reliable growth kinetics indicating that an increasingly high Mg^{2+} incorporation could transform a fast growing particles to more slowly growing ones having size distribution in the nano-meter range. The strong inhibitory effect of Mg^{2+} on particle growth could be explained by creation of a distorted atomic structure in hydroxyapatite upon replacement of Ca^{2+} with Mg^{2+}, that subsequently slows the growth of the particles.

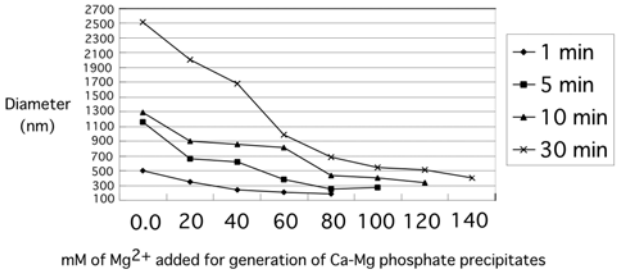

Figure 2: Estimation of particle sizes. Determination of particle diameter was performed in the same manner described above using a DLS device.

High rate cellular uptake of DNA carried by nano-apatite

Particle size is a crucial factor for successful gene transfer into mammalian cells; fine particles mediate an efficient gene transfer, whereas coarse ones do not [4]. Rapid growth of the particles resulting in sharp increase in diameter (Fig. 2) is thus a big hurdle which must be eliminated for efficient gene delivery and expression into the cells. Since Ca-Mg phosphate could block the growth and limit the sizes of the particles at a desirable level, we investigated DNA uptake in the cells, mediated by the particles. DNA was labelled with PI, a cell-impermeable DNA intercalating dye, by adding PI in DNA/Ca^{2+} solution prior to mixing with HBS at 1:1 weight ration of DNA to PI. As shown in Figure 3, internalization of DNA by Mg^{2+} -free particles was inefficient and gradually decreased depending on passage of time to the lowest level due to the growth of the particles (see fig. 1 and 2). On the contrary, strong fluorescence of PI -labelled DNA was observed inside the cells for Mg^{2+} -containing particles (Fig. 3) which were sufficiently resistant to growth (see fig. 1 and 2), indicating that DNA/Ca-Mg phosphate particles are efficiently endocytosed owing to their potential ability of blocking particle growth. The decline in uptake efficiency level for the particles generated with a high Mg^{2+} dose (Fig. 3) indicates formation of insufficient amount of nano-particles (see fig. 1) since Mg^{2+} beyond a level, could abolish precipitation reaction [7].

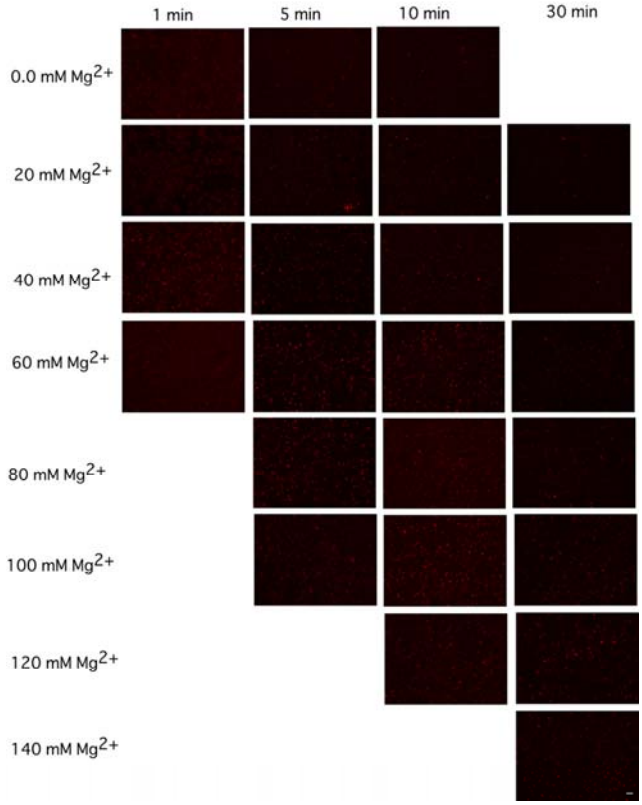

Figure 3: Nano-apatite-mediated DNA uptake in HeLa cells. Following mixing of the two solutions, one containing 1.5 mM inorganic phosphate in 300 μl of 2×HBS and the other containing 6 μg DNA labeled with PI at a DNA/PI weight ratio of 1, 250 mM Ca^{2+}, in addition to 0.0 to 280 mM Mg^{2+} in 300 μl water, 100 μl of each particle suspension was collected at a specified period of time (1 to 30 min) and added onto the cells being cultured in a well of 24-well plate in presence of 10% FBS-supplemented DMEM. After incubation at 37^0C for 4 hr, cells were rinsed with 5 mM EDTA in PBS to remove the extracellular particles and observed under a fluorescence microscope. (Scale bar, 50 μm.)

Notable level of transgene expression mediated by nano-precipitate

To reach the final goal of our strategy, we checked expression profile of a luciferase gene based on DNA/Ca-Mg phosphate particles isolated according to a specified timetable (Fig. 4 and 5). Surprisingly, depending on the level of Mg2+, particle generation time and cell type, at least 10 to 100-fold higher luciferase expression could be detected compared with Mg2+ - free particles. Such a high transfection efficiency could be solely attributed to the intrinsic property of Ca-Mg phosphate to significantly block the growing process and consequential generation of nano-sized particles (Fig. 2) needed for efficient cellular uptake of DNA. The profound effect of particle sizes on DNA delivery and subsequent expression could be clearly seen when the particles are allowed to grow for 30 min; Mg2+ inclusion caused a remarkable transition of particle diameter from 2.5 μm to 500 nm and finally enhanced gene expression efficiency by at least 40-times.

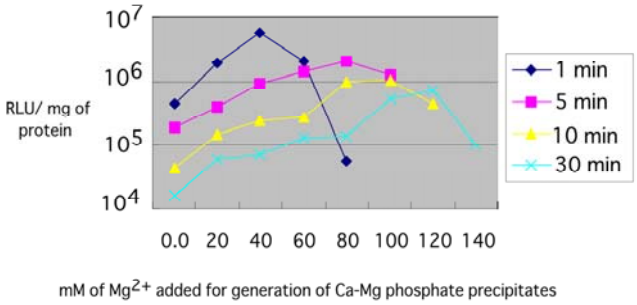

Figure 4: Enhancement of luciferase expression in HeLa cells by nano-precipitates. Mixing of the two solutions, one containing 1.5 mM inorganic phosphate in 300 μl of 2× HBS and the other containing 6 μg DNA labeled with PI at a DNA/PI weight ratio of 1, 250 mM Ca^{2+}, in addition to 0.0 to 280 mM Mg^{2+} in 300 μl water, was immediately followed by incubation at room temperature according to a specified timetable (1 to 30 min) and 100 μl of the resulting particle suspensions was collected and added onto the cells being cultured in a well of 24-well plate in presence of 10% FBS-supplemented 1 ml DMEM. After incubation for 4 hr, cells were rinsed with fresh medium and recultured for 1 day and luciferase expression was detected by a luminometer using luciferase detection kit.

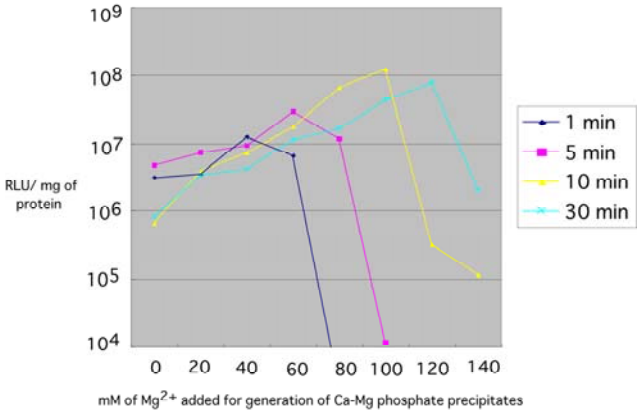

Figure 5: Enhancement of luciferase expression in NIH 3T3 cells by nano-precipitates. Mixing of the two solutions, one containing 1.5 mM inorganic phosphate in 300 μl of 2×

HBS and the other containing 6 μg DNA labeled with PI at a DNA/PI weight ratio of 1, 250 mM Ca^{2+}, in addition to 0.0 to 280 mM Mg^{2+} in 300 μl water, was immediately followed by incubation at room temperature according to a specified timetable (1 to 30 min) and 100 μl of the resulting particle suspensions was collected and added onto the cells being cultured in a well of 24-well plate in presence of 10% FBS-supplemented 1 ml DMEM. After incubation for 4 hr, cells were rinsed with fresh medium and recultured for 1 day and luciferase expression was detected by a luminometer using luciferase detection kit.

Thus, instead of providing tremendous efforts for limited transfection activity by collecting the precipitates just after initiation of precipitation [4], Mg2+-regulated particle growth profiling could confer a highly flexible way of nano-apatite preparation/ and enable to establish a super-efficient gene delivery system for mammalian cells.

Considering the high impact of a traditionally and widely used transfecting agent like Ca phosphate precipitate in basic research laboratories, biotech companies for production of recombinant cell lines and recently in gene therapy [2, 3], our newly developed technology based on Ca-Mg phosphate nano-precipitate, would emerge as a tool of utmost importance in the above applications replacing the old one.

References

1. Luo, D. & Saltzman, W. M. Synthetic DNA delivery systems. Nature Biotechnology 18, 33-37, 2000.

2. Fasbender, A., Lee, J.H., Walters, R.W., Moninger, T.O., Zabner, J. & Welsh, M.J. Incorporation of adenovirus in calcium phosphate precipitates enhances gene transfer to airway Epithelia in vitro and in vivo. J. Clin. Invest 102, 184-193, 1998.

3. Toyoda, K., Andresen, J., Zabner J.,Faraci, F. & Heistad, D. Calcium phosphate precipitates augment adenovirus-mediated gene transfer to blood vessels in vitro and in vivo. Gene Therapy 7, 1284-1291, 2000.

4. Jordan, M., Schallhorn, A. & Wurm FM. Transfecting mammalian cells : optimization of critical parameters affecting calcium-phosphate precipitate formation. *Nucleic acids research* 24, 596-60I, 1996.

5. Seelos, C. A critical parameter determining the aging of DNA-calcium-phosphate precipitates. Analytical Biochemistry 245, 109-111, 1996.

6. Chowdhury, *et al*. High-efficiency gene delivery for expression in mammalian cells by nanoprecipitates of Ca-Mg phosphate. Gene 341, 77-82, 2004.

7. Kibalczyc, W., Christoffersen, J., Christoffersen, M. R., Zielenkiewicz, A. and Zielenkiewicz, W. The effect of magnesium-ions on the precipitation of calcium phosphates. J. Cryst. Growth 106, 355-366, 1990.