pH-sensitive nano-crystals of carbonate apatite regulate delivery and release kinetics of DNA for efficient expression in mammalian cells

E. H. Chowdhury & Toshihiro Akaike
Department of Biomolecular Engineering, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan
Tel: (45)-924-5790, Fax: (81)-45-924-5815, E-mail: takaike@bio.titech.ac.jp

ABSTRACT

Due to some major limitations of viral-mediated delivery, non-viral synthetic systems have become increasingly desirable. However, synthetic systems are notably inefficient compared to the viral ones in gene delivery and expression. Here we report on the development of the simplest, but highly efficient gene delivery device based on generated nano-apatite crystals having high affinity to DNA but fast dissolution kinetics in acidic vesicles, following efficient endocytosis, for effective release of DNA and thus result in 5 to 100-fold higher transgene expression than the existing ones. Fluoride (F^-) or strontium (Sr^{2+}) which is known to decrease the solubility of carbonate apatite, dramatically reduced the transfection efficiency, suggesting that DNA release through particle dissolution, is a crucial factor in gene delivery pathway. Additionally, flexibility in modulating crystal dissolution kinetics enabled to control intracellular DNA release and an intermediate rate of DNA release enhanced survival of DNA and subsequent expression.

Keywords: calcium phosphate, carbonate apatite, hydroxyapatite, endosomes, acid solubility, DNA release, transfection

DNA delivery to mammalian cells has become a powerful and popular research tool for elucidating gene structure, regulation and function and is highly expected for gene therapy and DNA vaccination strategies to treat and control diseases. Due to the limitations of viral-mediated delivery, including toxicity, limited DNA carrying capacity, production and packaging problems, recombination and high costs, non-viral synthetic systems have become increasingly desirable in the above applications. However, synthetic systems are very inefficient compared with viral ones in gene delivery for protein expression (termed as transfection) [1]. Despite intensive efforts for the last 3 decades, there has been a lack of proper understanding on the molecular and cellular barriers in gene delivery pathway, that could rather assist in developing a superior non-viral technique. Calcium phosphate precipitation which is based on hydroxyapatite and has been being widely used for over 30 years [2], is a good example where no report could be found on regulation of crystal growth at the molecular level so as to generate nano-size particle for effective DNA delivery or elucidation of escape of bound DNA from the apatite particles for final expression. Here we reveal how simply and unexpectedly we could generate inorganic crystals of carbonate apatites which, like hydroxyapatites, adsorbed DNA, but unlike the latter, could prevent the growth of its crystals to a significant extent and possessed high dissolution rate in endosomal (acidic) pH, leading to tremendously high transgene expression. Moreover, we show how simply release of DNA could be controlled so as to avoid nuclease-mediated hydrolysis of DNA, leading to tremendously high transgene expression.

RESULTS AND DISCUSSION

Generation and chemical characterization of nano-crystals

Addition of only 3 mM Ca^{2+} to the HCO3- - buffered cell culture medium (DMEM, pH 7.5) and incubation at 37^0C for 30 min, resulted in microscopically visible particles. Generation of these particles only in HCO_3^- -, but not in Hepes-buffered media or solution (pH 7.5) containing the same amount of total Ca^{2+} (4.8 mM) and phosphate (0.9 mM), indicates the possible involvement of carbonate along with phosphate and Ca^{2+} in particle formation. Elemental analysis proved the existence of C (3%), P (17%) and Ca^{2+} (32%) and FT-IR spectra (Fig. 1a) identified carbonate, as evident from the peaks between 1410 and 1540 cm-1 and at approximately 880 cm-1, along with phosphate in the particles, as shown by the peaks at 1000-1100 cm-1 and 550-650 cm-1. X-ray diffraction patterns (Fig. 1b) indicated less crystalline nature, represented by broad diffraction peaks of the particles (Fig. 1c) – an intrinsic property of carbonate apatite [3]. Since carbonate, when present in the apatite structure, limits the size of the growing apatite crystals [3] we carried out scanning electron microscopic observation of generated carbonate apatite (Fig. 1c) which revealed reduced growth of the crystals, most of which had diameters of 50 to 300 nm.

High rate cellular uptake of DNA

We verified this size limiting effect of carbonate by

observing cellular uptake by Southern blotting of the total DNA isolated following 4 hr transfection, since large particles are phagocytosed less efficiently than small ones [4]. DNA was carried into the cells by carbonate apatite at least 10 times more efficiently

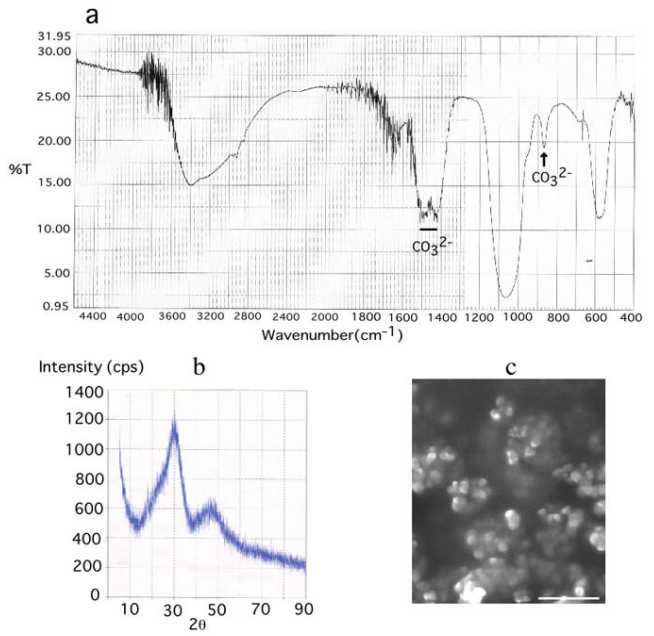

Figure1. (a) FT-IR spectrum. (b) X-ray diffraction patterns and (c) Scanning electron microscopy of generated particles.

than hydroxyapatite following initial incubation of particle-bound 200 ng and 2 μg of pGL3-contro vector (a luciferase gene-containing plasmid DNA), respectively (Fig. 2a). To evaluate the role of carbonate apatite as a carrier of genetic material, we compared transfection efficiency of different techniques including two frequently used ones- CaP co-precipitation method (mentioned above) and lipofection. In HeLa cell, luciferase expression level for carbonate apatite-mediated transfection was over 25-fold higher than for lipofection and CaP co-precipitation method (Fig. 2b). Transfection efficiency was also tremendously high in NIH 3T3 cells conferring over 50-times higher transfection efficiency compared to the existing methods (Fig. 2c). Transgene expression was also significantly higher in mouse primary hepatocytes (not showing).

Intracellular crystal dissolution and DNA release profile

Next, we investigated for any possible role of carbonate apatite on intracellular processing of DNA. Treatment with bafilomycin A1, a specific inhibitor of v-ATPase (a proton pump for acidification of endocytic vesicles) resulted in drastic reduction of transfection efficiency in HeLa cells (Fig. 3a). Similar finding could be observed when the cells were treated throughout the experiment with NH_4Cl (50 mM) – a compound known to raise endosomal pH (not shown here). These results indicated that acidic environment was necessary [5] probably for solubilization of carbonate apatite [6] to facilitate release of DNA from the apatite. To establish this notion, we generated fluoridated carbonate apatite to see the effect of solubility

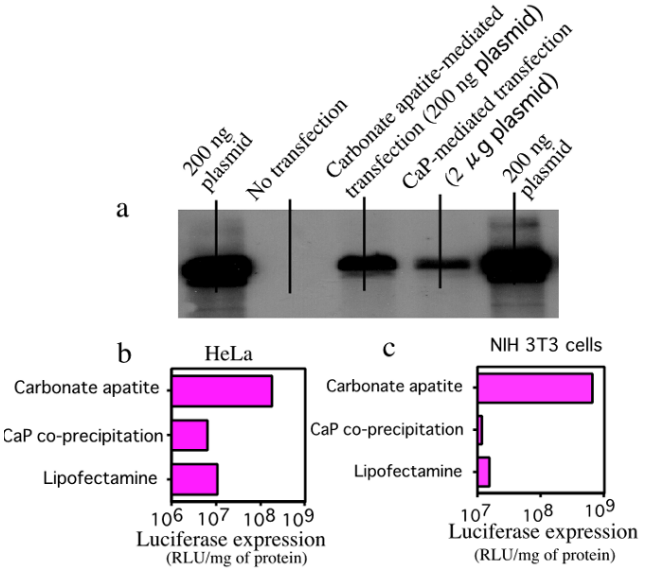

Figure2. (a) Cellular uptake of apatite-bound pGL3-control vector and comparison of luciferase expression in HeLa (b) and NIH 3T3 (c) cells following transfection with the same plasmid vector.

of the particles on transfection efficiency, since incorporation of fluoride reduces the solubility of the apatite [7]. Surprisingly, transfection efficiency was reduced gradually to a significant extent (100 fold) with increasing fluoride level in carbonate apatite (Fig. 3b). To rule out the possibility that reduced transfection efficiency of fluoridated carbonate apatite was due to reduced cellular uptake of DNA, we performed transfection with pEGFP, labeled with PI. With similar level of intracellular plasmid DNA, while almost 50% of the cells showed GFP expression for carbonate apatite, no GFP-positive cell was observed for fluoridated carbonate apatite (Fig. 3c). To establish a relationship between transfection efficiency and dissolution rates of the apatites, turbidity (320 nm) measurement was done as an indicator of their solubilization, following an acid load in solution of generated apatites. Carbonate apatite generated in presence of increasing concentrations of NaF, showed gradual decrease in dissolution rates, as evident from changes in turbidity, following adjustment of pH from 7.5 to 7.0 with 1 N HCl (not showing here), which is consistent with gradually reduced transfection efficiency of fluoridated carbonate apatites (Fig. 3b). With decreasing pH from 7.0 to 6.8, carbonate apatite was completely solubilized within 1 min, whereas fluoridated carbonate apatite was partially

dissolved (Fig. 3d). To further establish that decreased transfection efficiency was only due to decreased solubility of fluoridated carbonate apatite, but not by any other fluoride-mediated effects, we examined the effects of strontium which, when incorporated into carbonate apatite, reduce the solubility of the apatite, but to a lesser extent than fluoride [8, 9] As expected, addition of strontium chloride during preparation of carbonate apatite reduced its dissolution rate but to a level less than that observed for fluoride (Fig 3d). Moreover, transfection efficiency was gradually decreased in harmony with dissolution behavior, with increasing concentrations of strontium chloride added during generation of DNA/carbonate apatite particles (Fig. 3b). Taken together, our findings suggest that intracellular release of DNA through dissolution of apatite should play a major role in carbonate apatite-mediated transfection.

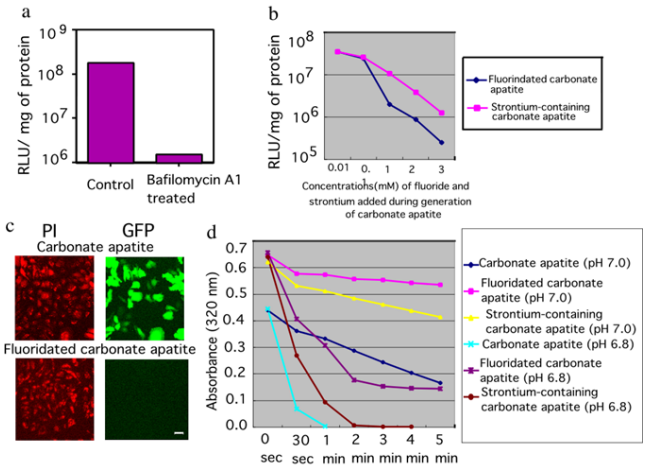

Figure3. (a) Transfection of HeLa cells in presence of bafilomycin A1. (b) Changes in luciferase expression for increasing concentrations of fluoride(0.01 to 3 mM) and strontium (0.01 to 3 mM) added during generation of DNA/carbonate apatite particles. (c) Uptake of PI-labeled plasmid DNA (pEGFP-N2) and GFP expression for carbonate apatite and fluoridated carbonate apatites (Bar indicates 50 μM). (d), Dissolution rates of carbonate apatite, fluoridated carbonate apatite and strontium-containing carbonate apatite at pH of 7.0 and 6.8.

Control in crystal dissolution and survival of DNA

Treatment of the cells with EDTA after 4 hr of DNA uptake resulted in over 2 fold reduction in luciferase expression level, which suggests that DNA uptake for a longer period, contributed to the efficient gene expression and compensated for DNA hydrolysis by nuclease, as also clarified from the persistence of fluorescence intensity of PI-labeled DNA (not showing here). To enhance the efficiency of transgene expression for short term uptake of DNA, we investigated the effect of slightly reducing the solubility of carbonate apatite by low amount of fluoride. Surprisingly, addition of only 1 μM NaF for the generation of carbonate apatite, caused over 15- fold enhancement in luciferase expression and a similar level of efficiency could also be observed for treatment of a cytotoxic drug,

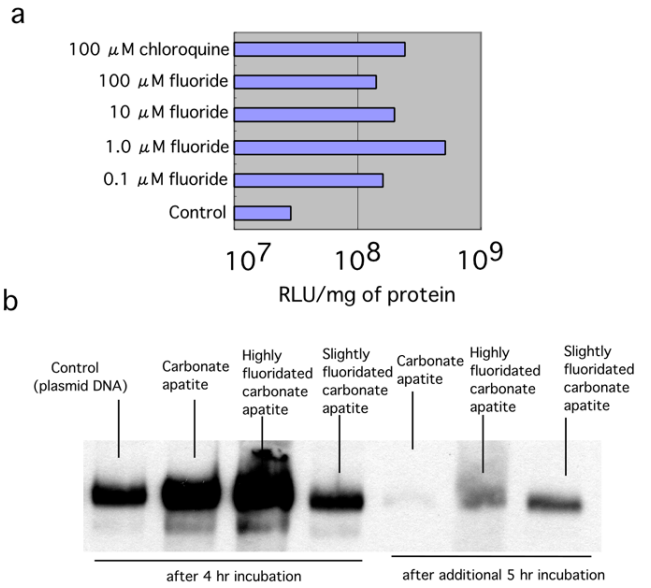

Figure 4. (a) Changes in luciferase expression for μM concentrations of F^- added during formation of DNA/carbonate apatite particles. After incubation of cells with the particles, cells were washed with EDTA and grown for 1 day, as described above. 100 μM chloroquine was added during incubation of the cells with DNA/carbonate apatite particles. (b) Southern hybridization between the DNA isolated from the transfected HeLa cells and the probe of the DNA used to transfect the cells by different apatites.

chloroquine (Fig. 4a) known to raise endosomal pH and protect DNA against nuclease. These observations suggest that an intermediate rate of DNA release during proton consumption and endosome buffering by the low F--containing crystals, could avoid significant nuclease degradation, as also evident from persistent fluorescence of PI (not showing here), thus providing more intact DNA for transcription and translation. To make more quantitative analysis regarding the fate of DNA carried by the apatites of different crystallinity, Southern hybridization was performed following isolating total DNA at different time periods from the cells transfected by the apatites. As shown in fig. 4b, fluoridated carbonate apatites are very effective in preventing DNA degradation whereas DNA carried by carbonate apatite was almost completely degraded. Although uptake of DNA was very high for highly fluoridated carbonate apatite after 4 hr incubation, percentage of intact DNA after a longer period was significantly low (Fig. 4b). This could be explained by the

assumption that a major portion of the total DNA was still bound to the particles at that time, which could not be isolated as free DNA.

Thus, we have developed an effective technology for gene delivery to mammalian cells, having wide and potential applications from laboratories to clinical medicine [10, 11]. Moreover, it provides insights to create a new era for inorganic crystal-based gene and drug delivery.

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