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DNA-Templated Strontium-Doped Calcium Phosphate Nanoparticles for Gene Delivery in Bone Cells

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Abstract

Calcium phosphates (CaPs), constituents of the inorganic phase of natural bone, are highly biocompatible and biodegradable. Strontium (Sr) regulates the formation and resorption of bone. Incorporation of Sr into CaPs may target genes of interest to bone cells while regulating their function. In this work, we developed a single-step synthesis method to prepare Sr-doped CaP nanoparticles (SrCaP-DNA NPs) by using DNA as a template for controlling the mineralization and the stability of the colloidal solution. The resulting nanoparticles were monodispersed with well-controlled size, morphology, and composition. By using this method, we were able to fabricate CaP NPs with varying contents of Sr²⁺. We demonstrated that the stability of CaP NPs in extracellular environments increased when Sr²⁺ partially replaced Ca²⁺ in CaP NPs. We showed that the cellular uptake of SrCaP-DNA NPs and the efficiency of gene transfer and alkaline phosphatase activity in human fetal osteoblastic cell line (hFOB1.19) were dependent on the content of Sr²⁺ in NPs. Together with other studies, our results suggest SrCaP-DNA NPs can be optimized for targeted gene transfer to regulate function of bone cells, enabling applications such as bone tissue engineering and treating bone diseases.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsbiomaterials.8b01587. Figures comparing the gene transfer efficiency of the NPs prepared by either SBF or SBF-3.7; SEM images of NPs prepared with higher concentrations of strontium (PDF)

The authors declare no competing financial interest.

Keywords

gene delivery; nanomedicine; calcium phosphate; bone tissue engineering

INTRODUCTION

Calcium phosphates (CaPs) are the inorganic phase of hard tissues such as bone and teeth. They are present throughout the body in the form of amorphous calcium phosphate (ACP) as well as hydroxyapatite.^{1–6} Calcium phosphate nanoparticles have been extensively used for therapeutic delivery applications, due to their biocompatibility and controllable biodegradability.^{7–12} Preparation of CaP nanoparticles for transfection is relatively straightforward and consists of two steps: first, DNA should be mixed with a calcium chloride solution, and then, phosphate-buffered saline solution added to this mixture results in formation of CaP-DNA nanoparticles. The high chemical affinity of calcium phosphate to the phosphate groups in nucleic acids is considered the main reason for adherence of DNA to CaP, which eliminates additional steps required for conjugation of these molecules to CaP nanoparticles. An ideal gene transfection vector should be able to facilitate the cellular uptake and endosomal escape and render sufficient protection of DNA during intracellular trafficking toward nucleus. The inherent pH-dependent solubility of CaPs is advantageous for the intracellular delivery of the macromolecules (e.g., DNA). Calcium phosphate nanoparticles are relatively insoluble at physiological pH and become more soluble in the low acidic environment of endosomal compartments, resulting in the release of DNA into the cytosol.¹³ Further, the electrostatic interaction of Ca^{2+} with negatively charged DNA protects it against nuclease attacks so that DNA can enter the nucleus with minimum degradation. In addition, Ca^{2+} ions enhance the nuclear uptake of DNA through interfering with nuclear protein pores known as nuclear pore complexes.^{14–21}

Similar to calcium, strontium (Sr) is present in the mineral phase of the bone. Sr is a natural bone-seeking element that can be incorporated into the bone at the same rate as Ca.^{22,23} A new compact bone contains three to four times higher Sr than the old compact bone. Sr both stimulates bone formation and inhibits the bone resorption. As a result, it produces higher bone mass and improves its strength and quality.^{24–26} Studies have suggested that the administration of Sr in the form of strontium ranelate can reduce the risk of fracture in osteoporotic patients.²⁷

Taken together, these observations suggest CaP-mediated gene transfer can target the gene of interest to bone cells while further regulating the function of cells if Sr is incorporated into CaP. Previous studies have investigated methods for incorporating Sr into CaP nanostructures.^{28–30} These methods usually involve high temperatures, harmful capping agents or surfactants, and complicated procedures with a limited control over the morphology and composition of the final product, which makes them unsuitable for in situ DNA loading.^{31–33} In this study, we developed a facile and one-pot synthesis method to prepare spherical and monodisperse strontium-doped calcium phosphate nanoparticles (SrCaP-DNA NPs) at physiological conditions (37 °C and pH = 7.4) (Figure 1).

The mineralizing solutions were formulated based on simulated body fluid (SBF). The presence of DNA controlled the mineralization and the stability of resulting colloidal solutions during this process. NPs with varying contents of Sr²⁺ were obtained by tuning the concentration of Sr²⁺ in the mineralizing solution. We evaluated the gene transfer efficiency of SrCaP-DNA NPs in human fetal osteoblastic cell line (hFOB 1.19). This cell line displays many characteristics of human osteoblasts and it was chosen due to the relevance of our system to bone repair and bone diseases. We showed the effect of Sr content on the gene transfer efficiency mediated by SrCaP-DNA NPs. The optimized gene transfer by these NPs was comparable to a common commercial transfection vector, Lipofectamine 2000.

EXPERIMENTAL SECTION

Preparation of Mineralizing Solution.

All materials were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. The mineralizing solutions were prepared based on previously reported procedures.³⁴ All ingredients (Table 1) except CaCl₂ were carefully weighed and dissolved in Milli-Q water in an appropriate container. CaCl₂ was dissolved in Milli-Q water in a separate container and then added to the initial solution in a dropwise manner to prevent spontaneous precipitation. The initial salt solution was continuously mixed using a stir bar during the entire addition process. The resulting solution was filtered with a 0.2 µm Millipore membrane and buffered to pH 7.4 with Tris-HCl and stored at 4 °C.

Synthesis of SrCaP-DNA NPs. In a 50 mL Falcon tube (BD Biosciences; San Jose, CA), 3, 6, and 9 µL of SrCl₂ (1 M) aqueous solution was added to 3 mL of mineralizing solutions (Table 1) to obtain 1, 2, and 3 mM strontium concentrations. Then the mineralizing solutions were mixed with 15 µL of 1 mg/mL of plasmid DNA in TE buffer (Tris-EDTA: Ethylenediamine Tetraacetic Acid). The plasmid DNA, gWIZ Beta-gal (amplified by Aldevron; Fargo, North Dakota), encodes for the reporter enzyme β-galactosidase (β-gal).

The final solution was placed at 37 °C on a Rotomix Type 50800 speed rotator (Barnstead/Thermolyne; Dubuque, IA) at the speed of 115 rpm for 40 min. The resulting precipitation was collected by centrifuging the solution at 2500 rpm for 90 min at 4 °C. After removing the supernatant, NPs were resuspended in serum-free Ham's F12 Medium Dulbecco's Modified Eagle's Medium (DMEM F12) or indicated buffers.

Characterization of SrCaP-DNA NPs.

The size and morphology of SrCaP-DNA NPs were analyzed by scanning electron microscopy (SEM, JEOL 7000). 40 μ L of mineralizing samples was taken and centrifuged at 13,200 r.p.m. for 10 min. The supernatant was removed, and particles were collected and washed with 30 μ L of 70% ethanol twice to make sure the mineralizing solution was completely removed. The washed particles were resuspended in 30 μ L of 70% ethanol. Twenty microliter aliquots of each sample were placed on top of a small silicon wafer and air-dried overnight. The samples were then sputter-coated with platinum and imaged at 10 kV. The elemental compositions of the samples were analyzed using energy dispersive X-ray spectroscopy (EDX) at 15 kV.

The crystallographic properties of the NPs were evaluated by X-ray diffraction method (Bruker D8 Discover, Germany). The diffraction spectra were acquired in the 2 θ range of 15–90° using Cu K α (wavelength = 1.54 Å, 40 kV, 120 mA).

The hydrodynamic diameter and zeta potential of the NPs were measured using a Zetasizer Nano ZS equipped with a zeta potential analyzer (Malvern Instrument; Westborough, MA). The autocorrelation function from the scattered intensities was obtained for each sample and the average diameter of the NPs was determined using Stokes–Einstein equation. NPs were collected by centrifugation and redispersed in 10 mM KNO₃ (pH = 7.4) for the measurement of zeta potential. Three measurements were performed for each sample. All the measurements were conducted at room temperature.

Evaluation of the Efficiency of DNA Incorporation.

At the end of the mineralization, the supernatants were removed and centrifuged at 13,000 rpm for 10 min. The Quant-iT PicoGreen dsDNA reagent (Invitrogen; Carlsbad, CA) was used to measure the concentration of DNA in the supernatant according to the manufacturer's protocol. The standard curves with a known amount of DNA were constructed by using the same solutions as samples. The quantity of the incorporated DNA into the SrCaP-DNA NPs was calculated by subtracting the amount of the DNA in supernatants from the initial amount of DNA added to each sample.

Dissolution of SrCaP-DNA NPs at Different pHs.

The SrCaP-DNA NPs were prepared as described above using mineralizing solution SBF-3.7 (Table 1). NPs were centrifuged at 13,200 rpm for 30 min and their supernatants were replaced with the same volume (3 mL) of serum free DMEM without phenol red. Dissolution profiles of NPs were determined by gradually decreasing the pH of each sample using 0.1 M hydrochloric acid. Samples were mixed well and allowed to equilibrate for 5 min at each pH change. The quantity of DNA released from the NPs was evaluated using

Quant-iT PicoGreen dsDNA assay as described above. Standard curves were constructed at different pH to take into consideration the effect of pH on assays. The pH at which 50% of DNA was released, designated as pH₅₀, was used as an indication of pH responsiveness of SrCaP-DNA NPs. The dissolution experiment was conducted at room temperature.

Cell Culture.

Human osteoblast-like cell line hFOB 1.19 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Ham's F12 Medium Dulbecco's Modified Eagle's Medium (DMEM F12), supplemented with 10% fetal bovine serum (FBS) and 0.3 mg/mL G418 at 37 °C with 5% CO₂.

In Vitro Gene Transfer in hFOB1.19 Cells.

The gene transfer efficiency of SrCaP-DNA NPs was assessed in hFOB1.19 cells. The cells were plated onto 24-well tissue culture plate at 40,000 cells/well and incubated 24 h before transfection. A commercial transfection reagent, Lipofectamine 2000 (Invitrogen, Carlsbad, CA), was used as a positive control, following the manufacturer's protocol. The ratio of DNA to Lipofectamine 2000 was 1 μ g to 2 μ L. Lipofectamine/DNA complexes were incubated with cells for 4 h before being replaced with cell culture medium. The SrCaP-DNA NPs were collected by removing their supernatants and then redispersed in cell culture media (either serum free or containing 10% FBS). Cells were incubated with 500 μ L of NPs with the total of 2 μ g of DNA. After incubation for 4 h, the media was replaced with fresh cell culture medium containing 10% FBS and incubated for an additional 24 h.

Cytotoxicity Assessment.

Cell viability assay was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric method. This method is based on the reduction of the MTT (Sigma; St. Louis, MO) into formazan crystals by viable cells. hFOB 1.19 cells were seeded into the 24-well tissue culture plate at an initial density of 40,000 cells per well and incubated for 24 h. After removing the cell culture medium, 500 μ L of DMEM F12 medium containing SrCaP-DNA NPs were added to each well. In addition, cells treated with Lipofectamine 2000 were used as control. The metabolic activity of the cells was measured 24 h after transfection by the addition of 50 μ L of MTT (5 mg/mL solution in PBS) to each well. The cells were incubated for an additional 2.5 h. After removing the MTT solution, 0.5 mL dimethyl sulfoxide was added to each well and incubated for 30 min to dissolve the formazan crystals. The optical density of each well was measured at 570 nm by using the SpectraMax M5 microplate reader (Molecular Devices; Sunnyvale, CA).

Quantification of SrCaP-DNA NPs Uptake by Flow Cytometry.

Cells were cultured into the 24-well tissue culture plate and incubated for 24 h. Afterward, the cell culture medium was removed and cells were incubated with 500 μ L of culture medium (either serum free or containing 10% FBS) containing SrCaP-DNA NPs in the concentration of 2 μ g of fluorescently labeled DNA (TriLink BioTechnologies, San Diego, CA). After 4 h of incubation, the media was removed and cells were washed three times

with 0.25 mL of Dulbecco's phosphate buffered saline (DPBS) and then detached using 0.25 mL of trypsin-EDTA (0.1% Trypsin, 0.4% EDTA.4Na) at 37 °C for 10 min. Cells were collected and washed three times with DPBS containing 1% FBS and immediately analyzed by flow cytometry (BD FACScan2, San Jose, CA). The extracellular fluorescence was quenched using 0.04 wt %/vol Trypan blue.^{35,36} The level of NP uptake was quantified using FlowJo (Treestar; Ashland, OR) software and expressed as geometric mean of fluorescence intensity (GMFI).

Quantification of Gene Expression.

At the end of 24 h posttransfection, the cell culture media was removed and cells were lysed with 150 μ L of a solution containing 9 mM MgCl₂, 10 μ M 2-mercaptoethanol (2-ME), and 0.1% Triton X-100 in DPBS for 15 min. The process was followed by three freeze-thaw cycles between -80 and 37 °C to completely release proteins from the cells. 50 μ L of another solution containing 10 μ M 2-ME, 9 mM MgCl₂, 0.15 mM chlorophenol red- β -D-galactoside (CPRG), and 0.1% Triton X-100 in DPBS was mixed with 50 μ L of the lysed cell solutions and incubated for 30 min at 37 °C. The absorption at 570 nm was measured using SpectraMax M5 microplate reader. The level of gene expression was quantified by using the value of standard curve generated from the known concentrations of β -gal and presented as nanograms of β -gal per milligram of total protein. We used β -galactosidase as a reporter gene due to its high sensitivity and stability, rapid quantification assays to evaluate transfection, and feasibility for use in lysates of the cells.³⁷ Coomassie protein assay (Biorad; Hercules, CA) was used to measure the total protein, following the manufacturer's protocol. Briefly, 5 μ L of DPBS was mixed with 5 μ L of the lysed cell solution and then mixed with 200 μ L of Coomassie solution. After 5 min incubation at room temperature, the absorption at 595 nm was determined using the SpectraMax M5 microplate reader. The amount of total protein was determined by comparing the absorption of samples with standard curve constructed with the known concentrations of bovine serum albumin.

Assessment of the Lysosomal Permeabilization.

hFOB 1.19 cells were plated at 40,000 cells/well onto the 12 mm coverslips in the 24-well plate and incubated overnight. Then, cells were either treated with dextran 4 kDa (0.5 mL of 2 mg/mL) alone (as a negative control) or cotreated with the SrCaP-DNA nanoparticles and incubated for 4 h. The coverslips were then washed and cells were fixed with 4% paraformaldehyde in DPBS for 20 min at room temperature. The paraformaldehyde was removed and the coverslips were washed and glued onto glass coverslips with hard-set mounting medium. The confocal microscope (LSM 510 Meta, Zeiss, 63 $\times$ oil immersion objective) was used to image the cells.

Alkaline Phosphatase (ALP) Activity Test.

hFOB 1.19 cells were seeded into the 24-well plate at a density of 20,000 cells/well and incubated for 24 h. After removing the culture medium, cells were treated with 500 μ L of DMEM F12 medium containing SrCaP-DNA nanoparticles. ALP activity was measured by a colorimetric method using Alkaline Phosphatase Assay kit (Bioassay Systems, Hayward, CA) following the manufacturer's protocol on days 7 and 14. At the end of designated times, cells were washed three times with PBS and lysed in 0.2% Triton X-100 by shaking for 20

min at room temperature. Then, 20 μL of cell lysate was mixed with working solution containing *p*-nitrophenyl phosphatase (pNPP) in 96-well plate. The absorbance was then measured at 405 nm and ALP activity was normalized by total protein content which was measured using the Coomassie protein assay kit (Biorad; Hercules, CA).

Statistical Analysis.

All experiments were performed in triplicate and repeated independently at least three times. The results represent the average values of triplicates. Error bars indicate the standard deviation (SD) of triplicates unless otherwise indicated. One-way analysis of variance (ANOVA) and Bonferroni post hoc test were performed for statistical analysis. A value of $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Choice of Mineralizing Solutions.

In this study, we chose two mineralizing solutions, SBF and SBF-3.7 (Table 1) for preparing SrCaP-DNA. SBF-3.7 was developed based on SBF but with a higher concentration of Ca^{2+} . SrCaP-DNA was prepared by the addition of varying concentrations of Sr^{2+} to both solutions. Figure 2 shows the average size (Z-Ave) and size distributions, characterized by polydispersity index (PdI), of SrCaP-DNA before (BC) and after (AC) concentrating the nanoparticles by centrifuge (yield $\sim 0.050\text{--}0.075$ mg per mL of the mineralization solution). The concentrating step by centrifuge may be required due to the limitations in the suitable injection volume at clinical applications. SrCaP-DNA NPs derived from both mineralizing solutions exhibited almost similar sizes and PdI values. The size of the NPs was slightly bigger after the centrifugation for both mineralizing solutions while their PdI remained similar. The differences between Z-average values observed at different Sr concentrations was not statistically significant at $\alpha = 0.05$ level ($n = 5$).

We performed an initial evaluation on the gene transfer efficiency of the NPs prepared with SBF and SBF-3.7 solutions with or without 1 mM of Sr^{2+} (Figure S1). SrCaP-DNA NPs prepared from SBF-3.7 showed a significantly higher level of gene transfer efficiency in comparison with those formed from SBF. In comparison with the SBF, we speculate that higher concentration of calcium ions in the SBF-3.7 promoted the nucleation and growth of more stable CaP NPs within the same reaction condition. Encouraged by these results, we used SBF-3.7 mineralizing solution to further study the effect of Sr^{2+} concentration on physicochemical characteristics and gene transfer efficiency of SrCaP-DNA NPs.

Size, Morphology, and Compositions of SrCaP-DNA NPs.

SEM images of SrCaP-DNA NPs synthesized from different Sr^{2+} concentrations are shown in Figure 3. Particles were spherical with a diameter of about 300 nm. There was no distinct difference in the size of the individual particles at various concentrations of Sr^{2+} . However, coalescence of SrCaP-DNA NPs started to appear when prepared with 2 and 3 mM of Sr^{2+} (Figure 3c,d). This was more pronounced when the NPs were mineralized at much higher Sr^{2+} (i.e., 6 to 24 mM) (Figure S2). Therefore, we only used NPs, prepared in the presence of 0 to 3 mM Sr^{2+} .

The chemical compositions of the NPs were analyzed using EDX. The EDX spectra were shown in insets of Figure 3, and the key elements, Ca, P, and Sr in NPs, were labeled. Semiquantitative analysis of the spectra shows that Sr^{2+} was effectively incorporated into NPs. Atomic percentage (atomic %) of the elements are also shown in Figure 3e, as representatives of molar ratios. The Sr content ($\text{Sr}/(\text{Sr} + \text{Ca})\%$) varied from 0% to 32% (Table 2) as the concentration of Sr^{2+} in mineralizing solution changed from 0 to 3 mM. In addition, we calculated the Ca/P and (Sr+Ca)/P molar ratios. It is worth noting that NPs have amorphous structures (as shown in Figure 4b), and therefore, the exact stoichiometry is not maintained. The P content of the NPs remained unchanged, which suggests the substitution of Ca^{2+} by Sr^{2+} in the atomic structure of NPs.

Surface Charge.

The surface charge of NPs, represented by zeta potential, is an important physicochemical property for the stabilization of a given colloidal system.^{38,39} Figure 4a shows the dependence of zeta potential to the concentration of Sr^{2+} in mineralizing solutions. CaP NPs formed in the absence of DNA, showed near-neutral zeta potential of -3 mV (data not shown). On the other hand, SrCaP-DNA NPs exhibited a negative zeta potential between -30 and -18 mV, which was due to the negatively charged DNA adsorbed on the surface of NPs.^{40,41} It is worth noting that DNA incorporation efficiency was also monitored at the end of each mineralization and nearly 100% of the initial DNA was incorporated into the NPs in all mineralizing conditions.

Currently, we do not fully understand the formation of SrCaP-DNA NPs in our process. Previous studies suggest prenucleation clusters exist in SBF solutions. In the presence of a template, these clusters concentrate around the template and progress into a defined size of spherical NPs.⁴² In the current study, increasing the Sr^{2+} ions in the SBF-3.7 thermodynamically favored the formation of more and more prenucleation clusters, which may aggregate around DNA molecules and form NPs. The SFB-3.7 is a supersaturated solution and adding an extra amount of ions such as strontium (due to its chemical similarity to calcium) into these solutions promotes the formation of a higher number of prenucleation clusters, due to an increased ion concentration. In other words, increasing the concentration of Sr^{2+} ions increase the prenucleation cluster density, which improves the likelihood of forming a higher number of stable clusters at the same time. These prenucleation clusters will then grow and result in the higher number of NPs. Since the amount of initial DNA in all mineralizing solutions was constant, the higher number of formed NPs have to share the same amount of DNA. Therefore, in solutions with higher Sr^{2+} contents, each NP has less DNA molecules available, resulting in lower amount of DNA per nanoparticle as compared to the nanoparticles mineralized from solutions with lower concentration of Sr^{2+} .

The surface charge reduction in Figure 4a was attributed to the lower number of DNA molecules per NP. The adsorption of DNA can stabilize NPs due to both electrostatic repulsion and steric hindrance.^{43,44} As a result of this depletion in surface charge at higher concentrations of Sr^{2+} in the mineralizing solution, the electrostatic repulsion forces decreased, and thus NPs became more prone to coalescence as shown in SEM micrographs (Figure 3c,d and Figure S2).

X-ray Diffraction Analysis of SrCaP-DNA NPs.

The crystallographic properties of the SrCaP-DNA NPs were obtained by XRD (Figure 4b). The diffraction patterns showed the broad peaks of amorphous calcium phosphates (ACPs) and lacked the long-range atomic order characteristic of crystalline materials. Unlike the needle-like crystals of hydroxyapatite, which are the most thermodynamically stable form of CaPs, the ACP phases mostly appear to be spherical.⁶

Amorphous CaP NPs present several advantages over crystalline forms. First, crystalline phase of CaP usually renders larger particle sizes and elongated needle-shaped microstructures.⁴⁵ The larger size of particles potentially hinders the effective cellular uptake.

Second, unlike crystalline phases of the CaP (e.g., hydroxyapatite) that have limits for the incorporation of larger ions such as Sr^{2+} in the crystal lattice, the amorphous phase is able to incorporate relatively high Sr in its structure. The incorporation limit in crystalline phases is due to an increase in lattice distortion resulting from the substitution of larger Sr (ionic radius: 1.13 Å) ions with the Ca (ionic radius: 0.99 Å) ions in the crystal structure. Li et al.⁴⁶ showed that higher than 15% Sr^{2+} could destroy the lattice symmetry of the hydroxyapatite and result in the broadening of the X-ray line width. SrCaP-DNA NPs derived in the current study permitted 20–30% of Sr^{2+} , which offers greater flexibility in tuning the differentiation and function of bone cells.

Third, crystalline particles can trigger undesirable inflammatory responses.⁴⁷ The amorphous growth of our SrCaP-DNA NPs resulted in the formation of monodispersed spherical NPs that can be advantageous for future clinical applications.

Dissolution of SrCaP-DNA NPs at Different pH Environment.

We evaluated the dissolution of the SrCaP-DNA NPs by gradually decreasing the pH of each sample using 0.1 M hydrochloric acid. The release of DNA from NPs was used as an indication of the dissolution. As shown in Figure 5a, DNA started to release at about pH = 7.0. We defined the pH at which 50% of DNA was released as pH₅₀, which qualitatively indicates the stability of NPs and the efficiency of DNA release during the acidification process of endosomal compartments. pH₅₀ gradually decreased from 6.8 to 6.4, as the content of Sr increased from 0% to 32%. These results indicate that SrCaP-DNA NPs are less soluble at physiological pH compared to CaP-DNA NPs (without Sr). All the SrCaP-DNA NPs were stable at pH = 7.4, and soluble at low acidic pH of endosomal compartments. This pH-dependent property makes SrCaP-DNA NPs ideal as intracellular delivery vectors through endosomal pathways. They remain intact at physiological pH (pH = 7.4) and dissolve when they encounter a rapid decrease in pH within endolysosomal compartments.

In Vitro Assessment of Cytotoxicity.

The cytotoxicity of the SrCaP-DNA NPs was evaluated by measuring the metabolic activity of cells. Lipofectamine 2000 (a commercial gene transfer agent) was used as controls. The results were normalized with respect to the negative controls that were not treated with any

agents (Figure 5b). It is evident that SrCaP-DNA NPs caused little cell death and displayed substantially lower cytotoxicity than Lipofectamine 2000.

When SrCaP-DNA NPs were dissolved, they released their ionic constituents. Rapid increase in intracellular calcium ions can lead to cell death.⁴⁸ Previous findings^{49,50} suggest that CaP NPs cause relatively low calcium disturbance. In contrast, the dissolution of large CaP particles results in irregular calcium spiking and cell death. Little cell death was observed when hFOB 1.19 cells were treated with SrCaP-DNA NPs. We did not measure the intracellular calcium level. It is unclear whether SrCaP-DNA did not induce irregular spikes of intracellular calcium due to the size of NPs as suggested by Neuman et al.⁴⁹ or the substitution of Ca^{2+} by Sr^{2+} in NPs or both. Further studies are required to investigate the intra-cellular calcium level induced by CaP-DNA and SrCaP-DNA.

Cellular Uptake and Gene Transfer Efficiency Induced by SrCaP-DNA NPs.

We evaluated the *in vitro* gene transfer efficiency of SrCaP-DNA NPs using human fetal-osteoblast (hFOB 1.19) cell line in the presence and absence of 10% serum. Several factors contribute to the effective gene transfer including the level of uptake of particles and subsequent intracellular release and transport of DNA to nuclei. We initially examined the extent of cellular uptake of SrCaP-DNA NPs using both fluorescent microscopy (Figure 6a) and flow cytometry (Figure 6b). SrCaP-DNA NPs were effectively internalized by cells, and the level of intracellular DNA was inversely correlated to the content of Sr. The presence of serum reduced the cellular uptake at higher Sr content of NPs.

The extent of gene transfer exhibited strong dependence on the content of Sr in SrCaP-DNA NPs; the presence of serum did not affect the gene transfer efficiency significantly (Figure 6c). The highest gene transfer efficiency was observed in relatively lower content of Sr, which was comparable to the level obtained by using commercial reagent, Lipofectamine 2000 (Figure 6c).

We observed substantial reduction in surface charges of SrCaP-DNA NPs in high content of Sr (Figure 4a). This reduction in zeta potential led to coalescence and aggregation of NPs (Figure 3c,d). As a result, the cellular uptake of NPs was expected to decrease (Figure 6b), leading to the subsequent reduction in gene transfer efficiency. Also, SrCaP-DNA NPs with the high content of Sr became more stable in an acidic environment, which possibly hindered the release of DNA either into cytosol or nuclei. We examined the intracellular release of DNA and did not observe a significant difference between different SrCaP-DNA NPs (data not shown). Compared to the reduced uptake of NPs, we consider the reduced intracellular release of DNA played a less significant role in the lower gene transfer efficiency of SrCaP-DNA NPs with higher content of Sr.

In vitro evaluation of the gene transfection efficiency of the nanoparticles requires consideration of both physical and biological factors and observation of a single parameter (e.g., cellular uptake) is not sufficient to fully understand the transfection mechanism. Following their uptake, NPs should intracellularly deliver the plasmid DNA to its final destination in the nucleus, where encoded information is transcribed to mRNA. It is equally important to understand the mechanism of cellular uptake of nanoparticles, their intracellular

trafficking and different factors affecting these mechanisms in order to achieve high gene transfection efficiency. Additionally, the fate of the NPs after their cellular uptake includes exocytosis, getting transported to secondary endosomes, degradation inside lysosomes, or getting transported to other intracellular compartments including cytosol.⁵¹ Even though the cellular uptake is relatively high in the presence of serum in Figure 6b, these nanoparticles still need to successfully pass through all the intracellular barriers in order to reach to nucleus and exert their function. Some studies have reported clear differences between intracellular trafficking regimes in cells transfected in the absence or presence of serum and showed both positive and negative effects on transfection efficiency.^{52–54} Such discrepancies might be in part attributed to the various mechanisms by which the nanoparticles travel through different cellular compartments, type of the cell lines, structural characteristics of nanoparticles, and transfection conditions used in the experiments. Additional mechanistic studies will be required to address the intracellular trafficking of CaP nanoparticles and its effect on gene transfection efficiency in the absence or presence of serum.

Previous studies have examined the effect of Sr on both osteoblasts and osteoclasts by using hydroxyapatite (HA) doped with Sr.^{55,56} Their results demonstrate that Sr promotes the differentiation of osteoblasts but inhibits the proliferation of osteoclasts depending on the content of Sr in HA. The optimal Sr content for inducing the proliferation of osteoblasts is about 14% while the stimulation of osteoclasts increases with the increase of Sr content. Our studies demonstrate that increasing Sr content more than 21% leads to reduced gene transfer efficiency. Therefore, an optimal Sr content in SrCaP-DNA NPs is essential for regulating function of bone cells through Sr itself and the target gene.

Assessment of the Endosomal Permeabilization.

We further investigated whether SrCaP-DNA NPs were capable of inducing the endosomal/lysosomal leakage by confocal microscopic analysis (Figure 7). Fluorescent dextran (FITC-dextran, MW: 4 kDa) was codelivered with nanoparticles and cells were incubated for 4 h. Fluorescent dextran is a membrane-impermeable dye and has been used previously as a tracer to monitor the integrity of endosomal/lysosomal compartments.⁵⁷ This molecule enters the cells by fluid phase pinocytosis and is able to fuse into the endosomal/lysosomal compartments containing nanoparticles.^{58,59} In the absence of nanoparticles (Figure 7a), the endocytosed FITC-dextran appeared as punctate structures in cells, as shown by the abrupt fluorescence intensity change along the depicted line, indicating that dye remained in distinct and intact endosomal/lysosomal compartments. On the other hand, in the presence of nanoparticles, cells showed a diffuse pattern of fluorescence as displayed by the relatively low intensity of the fluorescence along the depicted line (Figure 7b). This diffused characteristic indicates the leakage of cargo from endosomal/lysosomal compartments.

Escaping from endosome either by fusion with its membrane or through endosome disruption is considered a critical step in intracellular delivery of CaP nanoparticles. It was envisaged that calcium ions in the form of nascent CaP precipitates and other lysosomolytic molecules are able to facilitate endosomal escape.⁶⁰ The outcome of these previous studies^{60,61} indicates that CaP, regardless of phase or crystallinity, is less soluble at

physiological pH and becomes more soluble at low acidic pH of endosomal/lysosomal compartments, which facilitates the endosomal escape.

In Vitro Alkaline Phosphatase Activity Test.

ALP activity is considered an important parameter for the mineralization of bone and a useful biochemical marker of bone formation.⁶² We further investigated the effect of SrCaP-DNA nanoparticles on the production of alkaline phosphatase by hFOB 1.19 osteoblasts after 7 and 14 days of culture and normalized the results by the total protein content of each sample (Figure 8). After culturing for 7 days, the relative ALP activities of hFOB 1.19 osteoblasts were higher on the sample groups treated with nanoparticles as compared with controls. We also observed statistically significant ($p < 0.05$) higher amount of the ALP activities on all the sample groups containing Sr in the nanoparticle composition as compared to CaP-DNA nanoparticles without Sr. There were no significant differences between the ALP activities of sample groups with various concentrations of Sr (1–3 mM Sr). After culturing for 14 days, we observed the same ALP activity trend between sample groups although the ALP activities were higher as compared to the 7 days culture time. The data show that Sr-doped CaP nanoparticles can enhance the positive effect of the CaP nanoparticles and stimulate osteoblast activity in hFOB 1.19 cells. Previous studies have shown that Sr substitution for calcium into hydroxyapatite (HA) thin films has a beneficial effect on the osteointegration and bone regeneration of MG63 osteoblast-like cells and prevents undesirable bone resorption.^{63,64} A study by Xu et al.⁶⁵ using an Ag-loaded Sr hydroxyapatite/chitosan porous scaffold showed the enhanced osteogenic effects on human bone marrow mesenchymal stem cells (hBMSCs) in Sr-doped scaffolds as they increased the ALP activity and osteogenic-related gene expression. It has been speculated that the positive effects of Sr in Sr-hydroxyapatite are most likely due to other ions such as Ca and P and strontium substitution can enhance this effect.⁶⁶ Previously, an *in vitro* study has shown that Sr substitution for Ca into (HA) in the range of 3–7 atom % stimulates osteoblast activity and differentiation.⁵⁵ We have extended this range up to 19 atom % and demonstrated the positive effect of Sr substitution on osteoblast activity as shown by higher amount of ALP activity with respect to pure CaP nanoparticles. It is worth noting that there was no statistically significant difference in ALP activities of the sample groups between 1 and 3 mM Sr. This could be due to only needing a small amount of Sr substitution for stimulating ALP activity in vitro, and after certain concentrations it reaches a plateau. The results of current work indicate that the Sr concentration of 12 atom % is sufficient to promote ALP activity of hFOB 1.19 osteoblasts.

CONCLUSIONS

We have developed a single-step synthesis method for preparing highly uniform and spherical SrCaP-DNA NPs. DNA molecules functioned as a template for the initiation of biomineralization and controlled the stability of colloidal solutions. The method is cost-effective and does not need any surfactants or other complex post-treatment processes. SrCaP-DNA NPs are amorphous and exhibited lower cytotoxicity than the common commercial lipoplexes. The composition of the NPs can be tuned by proper choice of mineralizing solutions. We demonstrated that SrCaP-DNA NPs exhibited gene transfer

efficiency in the human fetal osteoblast cell line comparable to Lipoplexes, when the Sr content was less than 21%. As Sr content increased, gene transfer efficiency was reduced, likely due to the reduction of uptake. Our results together with other studies show SrCaP-DNA NPs have great potential for regulating functions of bone cells through both Sr^{2+} and the target gene.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Schematic illustration of the preparation of SrCaP-DNA NPs.

Figure 2.

(a) Size and (b) Pdl of SrCaP-DNA NPs prepared from SBF and SBF-3.7 mineralizing solutions with different Sr concentrations (0 to 2 mM), before (BC) and after centrifuging (AC). The differences between Z-average values for different Sr concentrations was not statistically significant at $\alpha = 0.05$ level ($n = 5$).

Figure 3. (a–d) Morphology and (e) composition (atomic percentage as a representative of molar ratios) of SrCaP-DNA NPs prepared from SBF-3.7 with various Sr concentrations (0 to 3 mM Sr for (a) to (d) images, respectively). Elemental analyses of NPs by EDS are shown as insets. Scale bar is 1 μm .

Figure 4.

(a) Zeta potential of the SrCaP-DNA NPs in 10 mM KNO₃ (pH = 7.4). (b) X-ray diffraction patterns of the SrCaP-DNA NPs. All the synthesized NPs exhibited the characteristic of amorphous phase. Single asterisks denote statistically significant differences between zeta potential values of samples prepared from solutions with 0 and 1 mM Sr concentrations, as compared to samples prepared with 2 and 3 mM Sr ($n = 5$, $\alpha = 0.05$).

Figure 5.

(a) Dissolution of SrCaP-DNA NPs at different pH environments. The pH of solution was adjusted using 0.1 M hydrochloric acid and the released DNA was determined using Quant-iT PicoGreen dsDNA reagent. (b) Relative metabolic activity of the hFOB1.19 cells with respect to the negative controls (nontreated cells) 24 h after the treatment with SrCaP-DNA NPs or controls. There was no statistically significant difference in metabolic activity of the cells treated with NPs and negative control, whereas statistically significant difference was observed between the metabolic activity of the cells treated with Lipofectamine as compared to all other samples tested here ($\alpha = 0.05$).

Figure 6.

(a) Fluorescent microscopic analysis of the uptake of cells treated with SrCaP-DNA NPs for 4 h in the presence of 10% FBS. The cell nuclei were stained with DAPI (blue) and DNA was shown in green. The scale bar is 20 μ m. (b) Cellular uptake of NPs measured by flow cytometry in the absence and presence of 10% FBS. The extent of uptake was expressed as the geometric mean of fluorescence intensity (GMFI). (c) Gene transfer efficiency in hFOB1.19 cell line transfected with SrCaP-DNA NPs. The transfection expressed as the amount of reporter gene encoding β -galactosidase (β -gal) normalized by total protein.

Figure 7.

Assessment of the endosomal permeabilization in hFOB1.19 cells treated with SrCaP-DNA nanoparticles for 4 h using confocal microscopy. Cells were incubated with either (a) dextran 4 kDa (0.5 mL of 2 mg/mL) alone (as a negative control) or (b) cotreated with the nanoparticles. The diffused characteristic is shown by the relatively low intensity of the dye along the depicted line. The images are analyzed by ImageJ software.

Figure 8.

Alkaline phosphatase (ALP) activity in the hFOB1.19 osteoblasts treated with SrCaP-DNA nanoparticles was measured using the *p*-nitrophenyl phosphate assay and normalized to protein content after incubation for 7 and 14 days. Data presented as means $\pm$ SEM, $n = 3$.

* $P < 0.05$.

Table 1.

Composition of the Mineralizing Solutions

	concentration		(mM)
	SBF	SBF-3.7	
CaCl ₂ ·H ₂ O	2.5	3.7	
KH ₂ PO ₄	1	1	
NaCl	141	141	
KCl	4	4	
MgSO ₄ ·6H ₂ O	0.5	0.5	
MgCl ₂ ·6H ₂ O	1	1	
NaHCO ₃	4.2	4.2	

Table 2.

Relative Amount of Strontium in the Solid Products

Sr²⁺ concentration in SBF-3.7 (mM)	(Sr/(Sr + Ca)%) ratio in the solid product	(Sr+Ca)/P ratio in the solid product	Ca/P ratio in the solid product
0	0	1.23	1.23
1	20	1.26	1.16
2	28	1.28	0.93
3	32	1.29	0.89