Organosilicate Nanoparticles as Gene Delivery Vehicles for Bone Cells

by

Suniti Moudgil

B. S., Chemical Engineering
University of Florida, 1998

Submitted to the Department of Chemical Engineering in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy in Chemical Engineering

at the

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Abstract

While bone has a substantial capacity to heal itself, there are approximately 1 million fractures that occur in the U.S. annually that are difficult to heal. These include fractures that occur at sites of low vascularity, fractures that result in a large amount of tissue loss, and fractures that result from bone fragility syndromes such as osteoporosis. There has been a great deal of interest in the tissue engineering of bone in order to treat such fractures. One major aspect of the tissue engineering approach involves the addition of growth factors or proteins to synthetic grafts to accelerate bone regeneration. However, delivering these proteins at the appropriate times and therapeutic levels poses significant challenges. Alternatively, delivering the genes that encode for these proteins could offer a more effective treatment, since proper incorporation of the appropriate genes into cellular nuclei would allow the cells to manufacture authentic protein products.

The motivation of this research was to design new materials for gene delivery to bone cells. Conventional non-viral vectors are plagued by toxicity and low transfection efficiencies. The purpose of this work was to design bioactive nanoparticles that could enter the osteoblast membrane without inducing toxicity. These materials were silicate-based, since doped silicates have been shown to possess osteogenic properties. A method to synthesize monodisperse, spherical organosilicate nanoparticles using a surfactant-stabilized sol-gel technique was developed. Surface dopants such as Ca, Mg and Na were found to influence cellular response to nanoparticles. In addition to particle composition, particle size was also found to have a significant effect on osteoblast uptake and cell proliferation.

The metabolic response of these cells after particle ingestion was also characterized in order to ensure that the osteoblasts retained their phenotype. The expression of various proteins involved in bone formation, such as alkaline phosphatase, osteocalcin, osteopontin and fibronectin was quantified. The results indicated that osteoblasts retained their phenotype after organosilicate nanoparticle ingestion. The levels of various cytokines expressed during inflammatory response remained low due to the biocompatibility of amorphous silica.

An optimized Ca-SiO₂ nanoparticulate system was developed with maximum particle uptake that enhanced cell viability. A model gene delivery system was created by complexing these nanoparticles with plasmid DNA that encoded for green fluorescent protein (GFP). The effects of nanoparticle size, composition and surface charge on complex size, DNA binding affinity and subsequent GFP expression in osteoblasts were investigated in detail.
In addition to primary and immortalized osteoblasts, we have studied the effect of this gene delivery system on two other control cell lines: fibroblasts (connective tissue cells) and hepatocytes (non-connective tissue cells). The Ca-SiO\textsubscript{2}-DNA complexes displayed significantly higher transfection efficiencies in osteoblasts and fibroblasts relative to hepatocytes compared to lipofectamine-DNA complexes. In addition, Ca-SiO\textsubscript{2}-DNA complexes enhanced osteoblast cell proliferation while achieving successful transfection. In clinical applications, such characteristics would allow viable cells to remain at the fracture site while delivering growth factors via transfected cells. Properties such as calcium loading and particle size could be tailored to achieve the desired amount of growth factor delivery. The ability of Ca-SiO\textsubscript{2}-DNA complexes to transfec\textup{t} bone cells selectively without inducing cytotoxicity suggested that this nanoparticulate system has exciting potential for clinical applications for bone regeneration.

Thesis Supervisor: Jackie Y. Ying

Title: Professor of Chemical Engineering
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Chapter 1 - Background and Research Motivation

1.1. Bone Regeneration and Fracture Repair

Bone is a dynamic tissue that is constantly renewing itself through a natural remodeling process. This lifelong process involves the resorption of old bone by osteoclast cells and the deposition of new bone by osteoblast cells. When a bone breaks, the same remodeling process is involved in fracture healing. While normal bone possesses a substantial capacity to regenerate itself following a fracture, orthopedic medicine lacks an effective treatment for the nearly 1 million fractures that occur each year in the United States that heal with difficulty [1]. These injuries include: fractures that occur at sites of low vascularity, fractures that result in large tissue loss, and fractures that arise from bone fragility syndromes such as osteoporosis [2]. The cost of treatment for such fractures is extremely high; for example, the annual cost to treat fractures caused by osteoporosis in the U.S. alone is approximately $10 billion [3].

While external or internal fixation is the most common treatment for normal fractures, bone graft augmentation is the current gold standard for acute fracture repair whereby fixation alone is not sufficient for complete bone restoration [4]. Specifically, autogenous bone grafts are the most common. However, disadvantages of autografting include: the need for a second surgical site, donor site morbidity, and the limited availability of autogenous bone, especially in elderly patients [5]. While allografts can also be used from other donors, they are less efficient and can potentially transfer disease and trigger host immune responses [6].

1.2. Synthetic Bone Grafts

In order to overcome these challenges, much research has been focused on synthetic bone grafts to aid the regeneration of bone [7,8]. In particular, orthopedic medicine has focused on bioactive ceramics, such as calcium phosphates [9,10] and bioactive glasses [11-13], which encourage bone cell adhesion and skeletal regeneration. Figure 1.1 shows the insertion of a calcium phosphate cement for tibial fracture augmentation. In addition to ceramic grafts, polymers, metals, and various composite materials have also been studied extensively [15-17].
Despite these developments, synthetic bone grafts are currently used in only 10% of the 2.2 million bone graft procedures worldwide [4]. This is because these materials often do not stimulate bone regeneration rapidly enough or at large enough length scales, as they do not possess the proteins and osteoinductive cells required for complete repair [4].

1.3. Tissue Engineering of Bone

In order to accelerate bone regeneration, the addition of growth factors to synthetic bone grafts has also been studied. Growth factors are proteins capable of stimulating cell proliferation, migration, differentiation, and matrix synthesis. The stimulating effect of a number of growth factors has been demonstrated in a variety of tissues of the musculoskeletal system (Table 1.1), and the gene that encodes for most of the known growth factors has been determined [18].

In particular, osteogenic proteins such as bone morphogenetic proteins (BMPs) have received attention as a promising therapeutic alternative for fracture repair [19,20]. However, delivering these proteins at the appropriate time and at the appropriate therapeutic level poses a significant challenge [21]. Alternatively, delivering genes that encode for these proteins could be a more effective treatment since proper incorporation of the appropriate genes into cellular nuclei would result in the authentic production of proteins [22,23].
Table 1.1. Effect of growth factors in various tissues of the musculoskeletal system [18].

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1.4. Gene Therapy in Orthopedic Medicine

Gene therapy involves the transfer of genetic material into cells to alter their function, that is, to allow cells to produce proteins to treat and potentially cure acute and chronic conditions [24]. In order for the target cells to manufacture the protein products of the introduced gene, the genetic material or DNA must be delivered to the nucleus of the cells. While naked DNA has been shown to successfully transfect certain cell types [25,26], the transfection efficiency for this approach is relatively low, especially in systemic applications. In general, DNA requires the aid of a viral or non-viral vector in order to enter the cell membrane and transfect the nucleus [27]. Although viral vectors are highly efficient in gene delivery, their major disadvantages include toxicity and the difficulty associated with large-scale production [24].

For non-viral vectors, negatively charged DNA molecules are complexed with a delivery vehicle or transfection agent, such as DEAE dextran [29], calcium phosphate [29,30], lipids [31,32], proteins, and other polymers [33-35]. The resulting complexes are then internalized by the cell, generally through endocytosis (Fig. 1.2). Following uptake, the complexes enter acidic endosomal compartments that may degrade DNA and its associated...
complexes. DNA that has survived endocytosis and cytoplasmic nucleases can then enter the nucleus. Once inside the nucleus, the biological processes of gene transcription and expression would proceed [36].

![Figure 1.2. Schematic of the major intracellular barriers that DNA encounters before entry into the nucleus, including complex formation, initial uptake, and endosomal release.](image)

Because of the multiple barriers that the DNA encounters between the cell membrane and the nucleus, the transfection efficiency of non-viral systems is fairly low, and a number of strategies have been used to improve the transfection efficiency of gene delivery vehicles by targeting these various barriers [24,36]. Fortunately, while a high transfection efficiency may be necessary for gene therapy treatment of systemic diseases, this may not be the case for bone regeneration. In the case of bone repair, a relatively small amount of growth factors produced by a few transfected cells can be of significant value [38]. For example, research directed toward enhancing bone regeneration using collagen matrices as carriers for specific genes have shown promising therapeutic results despite exhibiting a relatively low transfection efficiency [38,39].

While such matrices have shown some clinical potential with DNA alone, the incorporation of non-viral transfection agents into these matrices would aid DNA transfection and result in more rapid bone regeneration. However, since conventional non-viral agents exhibit toxicity, they cannot be incorporated into clinical implants. A non-viral gene delivery system that is capable of transfecting bone cells without any toxicity could be combined with a matrix system for more effective local delivery. Furthermore, a non-viral system that
targets bone cells could be administered systemically, thus reducing the need for invasive surgery. In either case, achieving successful transfection without toxicity would allow viable bone cells to remain at the fracture site during treatment, thus accelerating fracture healing.

1.5. Research Objectives

In order for materials to enter the cell membrane, they need to be in the submicron size range. Many groups have studied the effect of particle size on the cellular uptake and endocytosis of different cells lines, and have shown that smaller particles are generally favorable for cellular uptake [40-44]. Nanoparticles are ideal candidates for biological delivery vehicles, since they are able to bypass cells of the immune system [45,46]. The focus of this research is to synthesize bioactive nanoparticles that can enter osteoblasts without inducing toxicity. These particles are then bound to DNA and tested for their ability to deliver DNA to osteoblasts.

1.5.1. Doped Silicates as Orthopedic Biomaterials

Doped silicates such as Bioglass® have been recognized for their ability to bond quickly and strongly to bone [11]. The surface chemistry of these silicates promotes the formation of a calcium phosphate layer that is able to integrate with living bone, as shown in Figure 1.3. However, the clinical applications of bioactive glasses as bulk implants have been limited because of their weak mechanical properties. A nanoparticulate formulation of a similar composition may provide a bioactive delivery system for bone cells.

Figure 1.3. Insertion of Bioglass® (BG) implant into a rat tibial defect results in surface formation of calcium phosphate (CaP) layer, followed by osteoblast (O) adhesion and bone (B) formation [11].
1.5.2. Organosilicate Nanoparticles for Gene Delivery to Osteoblasts

Rather than synthesizing doped silicates using conventional high-temperature methods, sol-gel processing can be employed to produce silicate monoliths and particles. This low-temperature approach provides for greater chemical flexibility and functionalization. Osteoblasts, like most mammalian cells, are on the order of microns, so they would likely behave very differently with nanoparticles vs. bulk materials. The particle size may also be used to control cellular response. While tailoring the composition of organosilicate monoliths affects their \textit{in vitro} bioactivity, bulk gels may not be fully reacted, which is detrimental in cell culture. To attain a fully reacted material, the sol-gel synthesis parameters could be modified to attain fully condensed particles in ultrafine domain sizes.

In this research, organosilicate nanoparticles of various compositions and particle sizes are obtained using sol-gel processing. Bioglass®-like surface compositions can be achieved by adding various salts to the synthesis scheme. Since Bioglass® elicits a bioactive response as a bulk material, nanoparticles of similar compositions are also expected to be biocompatible with no cytotoxicity. These particles may be optimized for osteoblast cell viability by manipulating their particle size and composition.

Specifically, the effects of Ca-SiO$_2$, Mg-SiO$_2$, and Na-SiO$_2$ nanoparticles on cellular uptake and proliferation are studied in this research. Ca surface doping elicits the most dramatic increase in cellular proliferation and uptake. The amount of cellular uptake can be correlated to calcium surface loading and particle size, and the optimized system for cellular uptake will be a likely candidate for a successful gene delivery system for osteoblasts.

The effect of nanoparticle uptake on cellular metabolic response will also be characterized in order to ensure that the cells retain their osteoblastic phenotype. Since amorphous silica is a bioinert material, cells that have ingested nanoparticles are expected to secrete proteins at similar levels as cells that are not incubated with particles. Alkaline phosphatase (ALP) expression will be quantified along with expression of bone-specific proteins such as osteopontin and osteocalcin. Collagen and fibronectin synthesis will also be measured. Any increase in cytokine levels associated with particle ingestion will be determined as well.

Once an optimized particulate system is developed that shows no cytotoxicity and maximizes cellular uptake, it will be complexed with DNA to transfect osteoblast cells.
Factors that may influence transfection efficiency include complex size as well as the particles’ efficiency to bind and release DNA. Smaller complex sizes may result in greater initial uptake. However, small complexes that condense DNA too strongly may not release the DNA effectively to the nucleus. These characteristics can be tailored through calcium loading and other surface functionalization of organosilicate particles.

Lastly, complexes that transfect osteoblasts may also transfect other connective tissue cells like fibroblasts. The selectivity of nanoparticles towards connective tissue vs. a non-connective tissue cell line, e.g., hepatocytes, will be tested in vitro through cell culture studies. The transfection efficiencies of nanoparticle-DNA complexes in osteoblasts and fibroblasts as compared to hepatocytes will be investigated.

1.6. References


[37] Niidome, T., and Huang, L., Gene Ther. 9, 1647 (2002).
Chapter 2 – Synthesis, Cytotoxicity and Cellular Uptake of Organosilicate Nanoparticles

2.1. Introduction

Doped silicate materials such as Bioglass® have received much attention because of their ability to bond quickly and strongly to bone [1]. Various compositions of these silicates have been found to encourage osteoblast proliferation, and the bioactivity of these materials has resulted in their clinical use as dental and cochlear implants [2]. We will attempt to synthesize nanoparticles of similar compositions for use as gene delivery vehicles that can enter bone cells without inducing cytotoxicity.

2.1.1. Doped Silicate Synthesis via Sol-Gel Processing

The first bioactive glasses were prepared via a melt-derived fusion method [3]. While glasses prepared by this method have shown promise, more recent research has employed the use of sol-gel chemistry [3,4]. Sol-gel processing is a wet-chemical synthesis technique that can be used to fabricate inorganic oxides via the hydrolysis and polycondensation of metal alkoxides. This approach provides a great deal of flexibility in tailoring structural characteristics, such as compositional homogeneity, pore size, surface area, grain size, and particle morphology. The first step in this reaction involves the hydrolysis of alkoxides,

\[\equiv Si-OR + H_2O \rightarrow \equiv Si-OH + ROH\]

Polymerization results from the condensation reactions as follows,

\[\equiv Si-OR + HO-Si\equiv \rightarrow \equiv Si-O-Si\equiv + ROH\]

\[\equiv Si-OH + HO-Si\equiv \rightarrow \equiv Si-O-Si\equiv + H_2O\]

Lower pH conditions favor the hydrolysis reaction and gel formation, while higher pH conditions favor condensation and particle formation. Organic functional groups may be introduced via the sol-gel reaction of organically modified silicate precursors, \(R'_n Si(OR)_{4-n}\) [5]. The presence of organic groups results in a more hydrophobic material, and consequently, greater protein adsorption when the material is exposed to a physiological environment. Enhanced protein adsorption may be more desirable in a bulk orthopedic implant since protein adsorption is a precursor to osteoblast adhesion and matrix deposition [6]. It may not be desirable for injectable nanoparticles, however, since excess protein adsorption may result in subsequent clearance from the circulation [7]. Nevertheless, the
increase in surface hydrophobicity from organic functionalization may allow the nanoparticles to pass through the phospholipid bilayer of the cell membrane more easily than hydrophilic silica [8].

2.1.2. Initial Bioactivity Studies with Organosilicate Gels

While organosilicate gels synthesized via sol-gel chemistry have shown interesting properties, their surface reactivity can be a detriment to biological applications. Many inorganic materials that are produced by sol-gel processing must undergo heat treatment to fully condense the gel network and burn out solvents and residual organics. While small amounts of residual reactants may not affect many non-biological applications, they can be extremely harmful in a sensitive biological environment. During our preliminary studies on organosilicate monoliths, an increase in cell proliferation was noted upon the incorporation of various salts. However, when these materials were placed in aqueous in vitro systems, any unreacted precursors would alter the pH of the surrounding medium, resulting in cytotoxicity.

Thus, the goal of this study is to synthesize fully condensed organosilicate nanoparticles through low-temperature sol-gel processing. The chemical composition of these nanoparticles will be modified so as to elicit a bioactive in vitro response similar to that of Bioglass®. The effects of various synthesis conditions on material properties and subsequent cellular response will be examined.

2.2. Experimental

2.2.1. Synthesis of Organosilicate Microspheres

As described earlier, increasing the pH promotes the polycondensation reactions and particle formation. Synthesis of monodisperse silica particles is a well-documented process that was initially developed by Stober et al. [9]. The sol-gel synthesis parameters used to control particle size include precursor concentration and pH. We used a basic catalyst of sodium hydroxide to produce organosilicate particles. A high water/alkoxide ratio was employed to ensure complete hydrolysis once the organosilane came into contact with the aqueous reaction medium.

Monodisperse organosilicate microparticles were initially synthesized using a method developed by researchers at the Shin-Etsu Chemical Company [10]. Methyltrimethoxysilane
(CH$_3$Si(OCH$_3$)$_3$, Gelest) was used as the organosilane precursor to synthesize micron-sized polymethylsilsequioxane (CH$_3$SiO$_{1.5}$) particles. It was slowly added dropwise to a solution of water and sodium hydroxide, and the particles were allowed to nucleate and grow. The derived particles could then be surface functionalized using silane precursors with the desired functional groups [10].

2.2.2. Synthesis of Organosilicate Nanoparticles

While micron-sized spherical particles were easily obtained by the above method, they tended to agglomerate and were difficult to disperse. To obtain dispersed nanoparticles, we dissolved Tween-80® (Sigma), an FDA-approved surfactant, in the aqueous solution before methyltrimethoxysilane addition. Tween-80® is a polyoxyethylene (20) sorbitan monooleate surfactant with a hydrophilic-lipophilic balance (HLB) of 15.0. This surfactant-stabilized suspension prevented particle growth and increased dispersion, resulting in much finer particle sizes.

The following salts were introduced to achieve bioactive glass compositions: calcium nitrate tetrahydrate (Ca(NO$_3$)$_2$·4H$_2$O, Fluka), magnesium nitrate hexahydrate (Mg(NO$_3$)$_2$·6H$_2$O, Fluka), sodium nitrate (NaNO$_3$, Sigma) and potassium nitrate (KNO$_3$, Sigma). The salt was added to the particle suspension after the particles were aged for 1 hr. After salt addition, the particles were allowed to age for ~ 24 hr before further processing.

2.2.3. Materials Characterization

Dynamic light scattering (DLS) experiments were performed at 25°C using a Lexel 95 Argon-ion laser and a Brookhaven high-precision photomultiplier at 90°. Data were acquired using a Brookhaven 9000AT correlator. The periods for acquisition of the autocorrelation function were 0.1 μs to 0.5 s, and the particle size distributions were obtained from non-negatively constrained least-squares (NNCLS) analysis of the data. Phase identification of organosilicate powders was performed by powder X-ray diffraction (XRD) using a Siemens D5000 θ-θ diffractometer (45 kV, 40 mA, Cu-Kα). Scanning electron microscopy (SEM) was performed on a JEOL 6320FV field emission high-resolution microscope, and transmission electron microscopy (TEM) was performed on a Philips EM 410 high-resolution microscope. Elemental analysis was performed by Desert Analytics, Inc. (Tuscon, AZ).
Surface characterization was performed with X-ray photoelectron spectroscopy (XPS) on a Kratos AXIS Ultra Imaging X-ray Photoelectron Spectrometer using a Mono (Al) anode. The binding energy range of 340-350 eV was analyzed to determine the amount of Ca species present on the surface. An additional sample of CaSiO$_3$ (Alfa Aesar) was run as a standard. Zeta potential measurements (Brookhaven ZetaPALS Zeta Potential Analyzer) were performed on 0.15 wt% nanoparticle suspensions in cell culture medium. Solution pH was measured using an Orion model 420A pH meter, and adjusted by the addition of KOH or HCl.

2.2.4. Cell Culture Experiments

Four different cell types were investigated for their response to organosilicate nanoparticles. The osteoblast cell culture experiments were performed with both an immortalized (MC3T3-E1) cell line and with primary osteoblasts obtained from neonatal mouse calvaria. Preliminary cell culture experiments were also performed on a cell line from connective tissue (C3H10T1/2 fibroblasts) and another cell line from non-connective tissue (HepG2 hepatocytes). The MC3T3-E1 cells obtained from ATCC were cultured in Dulbecco’s Modified Eagle medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (Hyclone) and 2% antibiotic-antimycotic (Invitrogen). Primary osteoblasts obtained from neonatal mouse calvaria (courtesy of Hao Wang, Brigham and Women’s Hospital, Boston, MA) were cultured similarly, except that minimum essential alpha medium (α-MEM, Invitrogen) was used instead of DMEM. The culture medium was also supplemented with 1% ascorbic acid (Sigma), as recommended by the protocol. C3H cells (ATCC) were cultured with Basal Eagle Medium (ATCC) supplemented with 10% fetal bovine serum (ATCC) and 1% antibiotic (ATCC). HepG2 cells (ATCC) were cultured with Minimum Essential Eagle Medium (ATCC) supplemented with 10% fetal bovine serum and 1% antibiotic.

The particles were ultrafiltered three times in serum-free cell culture medium using an Amicon Stirred Ultrafiltration Cell (Millipore) with a 50K polyethersulfone filtration membrane (Millipore) before suspending in complete cell culture medium.
2.2.4.1. Proliferation Studies

50-mm polystyrene petri dishes were seeded at an initial cell density of 80 cells/mm$^2$. The petri dishes were incubated at 37°C in 5% CO$_2$ and the cells were allowed to attach overnight. On day 1, the medium was aspirated and replaced with a stock solution of particle suspension and cell culture medium. Subsequent cell proliferation was measured by passaging the cells with 0.25% Trypsin-EDTA (Invitrogen), lysing the cells and performing a Cy-Quant DNA Assay (Molecular Probes). The proliferation of fibroblasts and hepatocytes was measured by staining the cells with trypan blue and counting the cells in a hemacytometer. Tissue-culture polystyrene (TCPS) was used as a positive control.

2.2.4.2. Uptake Studies

Samples for cellular uptake were prepared by seeding cells in 75-cm$^2$ tissue culture flasks and adding the particles after day 1 of cell attachment. The cell-particle samples were incubated for at least 24 hr to allow for maximum uptake. The cells were then washed once in phosphate buffered saline (PBS, Invitrogen), passaged, and resuspended in a 2% paraformaldehyde (Sigma) solution. They were allowed to incubate at room temperature for 2 hr in order to fix them in solution. The fixed cells were then stained with SYBR Green I (Molecular Probes), a fluorescent probe that would emit in the FITC region upon binding to silica nanoparticles. Fluorescent particle uptake was quantified through fluorescence-activated cell sorting (FACS) of the stained cell suspensions using a Facscan cytometer (Becton-Dickinson) equipped with an argon laser. At least 10,000 events were counted for each sample. The software package Cell Quest (Becton-Dickinson) was used to determine the events of interest from forward and side scatter parameters. The mean fluorescence intensity of the cells was obtained from the mean channel number of the fluorescence histograms of the gated population. Uptake values were calculated as shifts in the mean fluorescence of cell populations relative to the control cell population with no particles. Calibration curves for particle fluorescence were generated by measuring fluorescence vs. particle wt% in an Fmax microplate spectrofluorometer (Molecular Devices) using a 485-nm excitation/538-nm emission filter. The particle fluorescence data were analyzed using Softmax PRO (Molecular Devices), and all results were normalized to the fluorescence signal of fluorescent 10-nm Ca-SiO$_2$ particles.
For TEM studies, the cells were grown in Petri dishes and incubated with particles for at least 24 hr. These cells were fixed in a 2% paraformaldehyde solution for 1 hr. The fixed cells were then scraped, pelleted, and washed three times in sodium cacodylate (C$_2$H$_6$AsNaO$_2$·3H$_2$O, Electron Microscopy Sciences or EMS) buffer. The washed pellets were stained with 1% osmium tetroxide (OsO$_4$, EMS) solution and placed on ice in the dark for 1 hr. The stained pellets were incubated in uranyl acetate ((CH$_3$COO)$_2$UO$_2$·2H$_2$O, Sigma) overnight, washed in H$_2$O, and gradually dehydrated with 50%, 75%, 90%, 95% and 100% ethanol washes. The dehydrated pellets were incubated in propylene oxide (Sigma) solution overnight. They were embedded in Epon (EMS), and sectioned for microscopy.

2.3. Results and Discussion

2.3.1 Effect of Synthesis Conditions on Particle Size Distribution

2.3.1.1. Effect of Surfactant Addition

While the formation of microspheres could be obtained by increasing the water/alkoxide ratio and increasing the pH, monodisperse nanoparticles were difficult to achieve by just varying these two parameters, since particle agglomeration and growth would occur. To prevent further particle growth, a biocompatible surfactant was added as a dispersing agent. SEM and DLS indicated that the surfactant addition resulted in a substantial reduction in particle size from ~1.5 μm to < 10 nm (Fig. 2.1). It was found that the surfactant concentration did not affect the particle size. Thus, the surfactant concentration was kept at ~6 wt%, which was well above the critical micelle concentration of 0.01 wt%.

![Figure 2.1](image_url)  
**Figure 2.1.** SEM images of organosilicate nanoparticles synthesized (a) without surfactant (mean particle size = 1.5 μm) and (b) with surfactant (mean particle size = 7 nm).
2.3.1.2. Effects of Sol pH and Water/Alkoxide Ratio

The effects of both sol pH and alkoxide concentration on particle size are shown in Figure 2.2. For a given water/alkoxide ratio, increasing the pH resulted in smaller particle sizes. An increase in pH would increase the kinetics of the condensation reaction [11], and the presence of the surfactant would prevent any further growth of the condensed particle. An increased water/alkoxide ratio would promote nucleation versus particle growth, leading to finer particle sizes.

![Figure 2.2. Effects of pH and water/alkoxide ratio on the particle size distribution of organosilicate nanoparticles.](image)

2.3.1.3. Effect of Alkoxide Addition Rate

While the high sol pH greatly favors particle formation over gel formation, we found that the addition rate of the alkoxide precursor under basic pH conditions greatly affected the monodispersity of the particles produced. The results indicated that under certain conditions, increasing the alkoxide addition rate led to an increase in particle size as well as a broader particle size distribution. This phenomenon was most likely due to the competition between nucleation and particle growth; increasing the alkoxide addition rate favored particle growth since more alkoxide came rapidly into contact with the existing particles to promote growth before the new precipitates had time to nucleate. The effect of alkoxide addition rate was
more pronounced with finer particle sizes. To ensure a narrow particle size distribution, the alkoxide addition rate was kept at a slow rate of ~ 0.1 ml/s.

2.3.1.4. Effect of Salt Addition

In order to incorporate the bioactive components into the nanoparticle surface composition, we attempted various methods of salt addition. One method was to predissolve the salts in the surfactant-stabilized aqueous solution before addition of the alkoxide. This approach resulted in an extremely broad particle size distribution. Another method was to form the particles first and then immediately add them to an aqueous salt solution. This method also resulted in polydisperse particles. From these studies, we inferred that salt addition prior to sufficient particle growth would disturb the delicate balance between the hydrolysis and polycondensation reactions. Thus, the salt addition step was introduced only after sufficient aging of the particles (≥ 3 hr). This approach allowed the narrow particle size distribution to be maintained. Some particle agglomeration and broadening of the particle size distribution initially occurred due to the salt addition, which was to be expected [12]. However, after the dilution and sonication, the particles were effectively redispersed, and there was only a slight broadening of their size distribution. Figure 2.3 shows the particle size distributions for 10-nm particles before and after the addition of calcium nitrate.
2.3.2. Effect of Nanoparticles on Cellular Response

Bioactive silicate glasses containing various combinations of calcium, magnesium, and sodium oxides have been shown to stimulate cell proliferation [13-15]. To determine if similar cellular response could be achieved with nanoparticles of such salts, we have examined the effects of nanoparticle composition on cell proliferation (see Fig. 2.4). While MC3T3-E1 and primary cells exhibited similar trends, the osteoblast results discussed in this
chapter were associated with experiments done with primary cell cultures. This study showed that while certain compositions enhanced cell proliferation more than others, Ca-containing particles had the most positive effect on cell viability.

![Figure 2.4](image)

**Figure 2.4.** Effect of the surface composition of 10-nm organosilicate nanoparticles on osteoblast proliferation after 7 days. Nanoparticle loading: 0.15 wt%; nominal salt loading: 2.5 wt%. Values are mean ± standard error of the mean; n = 3.

After the initial screening study, the Ca-containing organosilicate particles (Ca-SiO$_2$) were investigated in detail as a function of particle size and Ca loading. Figure 2.5 shows that an increase in osteoblast proliferation was achieved with Ca-SiO$_2$ particles of ≤ 100 nm with low nominal calcium loadings.

![Figure 2.5](image)

**Figure 2.5.** Effect of nominal Ca loading and particle size of Ca-SiO$_2$ on osteoblast proliferation. Particle sizes of (■) 10 nm, (●) 50 nm, (▲) 100 nm and (♦) 200 nm are examined at a particle loading of 0.15 wt%. Values are mean ± standard error of the mean; n = 3.
2.3.3. Characterization of Ca-SiO$_2$ Nanoparticles

The composition of the Ca-SiO$_2$ nanoparticles was characterized by elemental analysis. All materials characterization was done after particle ultrafiltration in cell culture medium. Table 2.1 shows the actual calcium contents in the 10- and 50-nm particles. The results indicated that the upper limit for total calcium loading was $\sim$ 0.5 wt%.

<table>
<thead>
<tr>
<th>Nominal Ca Loading (wt%)</th>
<th>Actual Bulk Ca Loading (wt%)</th>
<th>10-nm Particles</th>
<th>50-nm Particles</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.50</td>
<td>0.13 ± 0.09</td>
<td>0.11 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>1.25</td>
<td>0.54 ± 0.14</td>
<td>0.57 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>2.50</td>
<td>0.51 ± 0.20</td>
<td>0.53 ± 0.18</td>
<td></td>
</tr>
</tbody>
</table>

The particles were amorphous by XRD analysis, which was to be expected since the effective Ca loading was very low. XPS was used to provide insight into the surface composition of Ca-SiO$_2$. Table 2.2 shows the surface Si/Ca ratios as measured by XPS. The effective surface Ca loadings calculated from XPS results were higher than the bulk Ca loadings shown in Table 2.1. This indicated that the Ca species were mainly located on the surface of the nanoparticles, which was to be expected since the Ca precursor was introduced to the organosilicate nanoparticles after the latter were formed and aged for $\geq$ 3 hr.

<table>
<thead>
<tr>
<th>Nominal Ca Loading (wt%)</th>
<th>Surface Si/Ca Atomic Ratio</th>
<th>Surface Ca Loading (wt%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>89.6</td>
<td>0.39</td>
</tr>
<tr>
<td>1.25</td>
<td>2.43</td>
<td>6.50</td>
</tr>
<tr>
<td>2.50</td>
<td>2.56</td>
<td>7.13</td>
</tr>
</tbody>
</table>

The XPS data also indicated that the phosphorous loading on the particles was $< 0.5$ wt%, suggesting that the formation of a calcium phosphate layer on the surface of the particles due to reaction with phosphate ions from cell culture medium was highly unlikely. While bioactive glasses would typically form a calcium phosphate layer on their surface, the time scale for that reaction to occur on the surface of these particles would be $\sim$ 2 weeks, as indicated by XPS (data not shown). This was in fair agreement with Kokubo et al. [16], who
reported that formation of a calcium phosphate layer on the surface of sodium silicate glasses took about 240 hr.

2.3.4. Effect of Ca-SiO$_2$ Nanoparticles on Cellular Uptake

The effect of Ca-SiO$_2$ nanoparticles on cellular uptake was also studied. TEM results indicated that 10-, 50- and 100-nm Ca-SiO$_2$ particles were taken up by cells (Fig 2.6). Multiple micrographs illustrated that cellular uptake was extremely selective; so that only particles with surface dopants of Ca, Mg and Na were ingested, while undoped SiO$_2$ particles were not. For Ca-SiO$_2$ nanoparticles, only particles of ≤ 100 nm were ingested, while for Mg- and Na-SiO$_2$ particles, only particles of ≤ 50 nm were ingested.

Nanoparticle uptake was quantified by the FACS assay so as to study the effects of various parameters on cellular uptake. Since particle uptake would reach a saturation after a certain particle loading (Fig. 2.7), all subsequent experiments were performed at a particle loading of 0.15 wt% to ensure that the experiments were conducted in this region of maximum uptake.

Figure 2.8 shows the effect of calcium loading on the cellular uptake of Ca-SiO$_2$ particles of various sizes. The FACS data supported the TEM findings, showing that organosilicate particles of ≤ 100 nm with nominal calcium loadings of ≥ 1 wt% were ingested by osteoblasts. Figure 2.9 shows that cell proliferation increased for Ca-SiO$_2$ nanoparticles with low nominal Ca loadings. This could be attributed to particle ingestion by cells. At higher nominal Ca loadings (> 10 wt%), however, cellular uptake (Fig. 2.8) did not correspond to cell proliferation (Fig. 2.9).
Figure 2.6. Transmission electron micrographs of primary osteoblasts that have ingested (a) 10-nm, (b) 50-nm and (c) 100-nm Ca-SiO$_2$ nanoparticles containing 2.5 wt% Ca. Particle loading: 0.15 wt%.
Figure 2.7. Effect of particle loading on the cellular uptake of (■) 10-nm, (♦) 50-nm and (▲) 100-nm Ca-SiO$_2$ particles. All particles had a nominal Ca loading of 2.5 wt%. Values are mean ± standard error of the mean; $n = 3$.

Figure 2.8. Effect of Ca loading and particle size on the cellular uptake of (■) 10-nm, (♦) 50-nm, (▲) 100-nm and (●) 200-nm Ca-SiO$_2$ particles. Particle loading: 0.15 wt%. Values are mean ± standard error of the mean; $n = 3$. 
Figure 2.9. Effect of Ca loading and particle size on the osteoblast proliferation for (■) 10-nm, (●) 50-nm and (▲) 100-nm Ca-SiO$_2$ nanoparticles. Particle loading: 0.15 wt%; n = 3.

The decrease in proliferation at higher nominal Ca loadings could be due to the greater degree of agglomeration caused by higher salt loadings. This agglomeration led to broader particle size distributions (see Fig. 2.10), so despite the fact that there were enough small particles at a particle loading of 0.15 wt% to result in maximum uptake, there were probably also some larger particles that did not enter the cell and simply depleted protein from the cell culture medium, resulting in a slight decrease in cell proliferation.
2.3.5. Intracellular vs. Extracellular Signaling

The increase in osteoblast proliferation might be because particle ingestion induced a paracrine response, prompting cells to proliferate faster [17]. In order to test this hypothesis of extracellular signaling, an experiment was performed with conditioned medium. First, the cells were cultured in the presence of nanoparticles (particle loading: 0.15 wt%) and allowed to proliferate. The conditioned medium was collected and then used to culture a fresh batch
of cells in the presence of nanoparticles. Presumably, if this conditioned medium contained signaling molecules released by the cells, the same molecules would also cause the fresh batch of cells to proliferate at an increased rate. However, as Fig. 2.11 indicates, cells cultured in the presence of the conditioned medium proliferated at a normal rate. This result suggested that the increase in proliferation was not associated with an extracellular mechanism, but rather with an intracellular mechanism.

![Graph showing cell density](image)

**Figure 2.11.** Osteoblast proliferation in the presence of fresh and conditioned cell culture media. Values are mean ± standard error of the mean; n = 3. *Nominal Ca loading: 2.5 wt%.

Once the particles entered the cells, some of them were surrounded by vesicles, possibly endosomal vesicles (see TEM images in Fig. 2.6). Endosomal pH is < 6, slightly less than that of the cytoplasm (pH = 7.4) [18]. Under these slightly acidic conditions, the once insoluble calcium on the surface of the particles might become slightly soluble and Ca$^{2+}$ could be released. XPS analysis before and after acidification indicated that the surface calcium loading was 2.43 wt% at pH = 5.5 for 50-nm particles, which was dramatically lower than the Ca loading at pH = 7.4 (7.13 wt%) (see Table 2.2).

Ca$^{2+}$ is ubiquitous in various cellular processes from contraction to cell proliferation (see Fig. 2.12). Ca$^{2+}$ serves as an intracellular messenger in many signaling pathways, and cellular responses to changes in Ca$^{2+}$ levels depend on both the cell type and the nature of
stimulation [19]. An intracellular increase in Ca\(^{2+}\) due to the presence of the particles may lead to a cascade of events involved in a mitogenic pathway.

![Figure 2.12. Basic mechanisms of calcium signaling [20].](image)

**2.3.6. Effect of Ca-SiO\(_2\) Nanoparticles on Other Cell Types**

While the increase in cell proliferation due to ingestion of Ca-SiO\(_2\) nanoparticles was evident in osteoblasts, this phenomenon did not extend to other cell types. Cell proliferation in fibroblasts was fairly unaffected by the presence of Ca-SiO\(_2\) nanoparticles, as shown in Fig. 2.13. On the other hand, the presence of Ca-SiO\(_2\) nanoparticles led to decreased proliferation of hepatocytes, which might be due to a rise in Ca\(^{2+}\), since an increase in Ca\(^{2+}\) above a certain threshold could lead to cell death. This threshold might vary depending on the cell type. Since Ca\(^{2+}\) played a significant role in mineralization and bone formation, the threshold for Ca\(^{2+}\) concentrations could be higher in osteoblasts than in hepatocytes [20,21]. This might allow us to target Ca-SiO\(_2\) nanoparticles for gene delivery to osteoblast cells selectively.
2.3.7. Effect of Other Nanoparticle Compositions on Osteoblast Uptake and Proliferation

A slight increase in osteoblast proliferation was noted with Mg-SiO₂ particles, but not with Na-SiO₂ particles (Fig. 2.14). There has been some prior experimental evidence that osteoblasts would respond to cations other than calcium [22-23]. Maillard et al. have suggested that this “cation-sensing mechanism” might participate in the regulation of the skeletal apposition of calcium circulating in the plasma [24].

Compared to Ca-SiO₂ particles, Mg-SiO₂ and Na-SiO₂ particles gave rise to lower osteoblast proliferation and uptake. We noted that in all three systems, finer particle sizes led to higher cell density and cellular uptake. Mg-SiO₂ and Na-SiO₂ particles of < 100 nm and < 50 nm, respectively, were taken up by osteoblasts (see Fig. 2.15). The presence of Mg, Na and Ca all resulted in a slight decrease in the negative surface charge of the organosilicate nanoparticles (Fig. 2.16). This decrease might have facilitated particle attachment to the cell membrane and subsequent endocytosis, if the particles were small enough in dimension.
Figure 2.14. Effect of (■) Ca-SiO$_2$, (●) Mg-SiO$_2$ and (▲) Na-SiO$_2$ nanoparticle size on the proliferation of osteoblasts. Particle loading: 0.15 wt%; nominal salt loading: 2.5 wt%. Values are mean ± standard error of the mean; n = 3. Note: cell density in the absence of nanoparticles is ~ 230 cells/mm$^2$.

Figure 2.15. Effect of (■) Ca-SiO$_2$, (●) Mg-SiO$_2$ and (▲) Na-SiO$_2$ nanoparticle size on the uptake of osteoblasts. Particle loading: 0.15 wt%; nominal salt loading: 2.5 wt%. Values are mean ± standard error of the mean; n = 3.
Figure 2.16. Zeta potential vs. pH for nanoparticle suspensions of (●) Ca-SiO$_2$, (▲) Mg-SiO$_2$, (■) Na-SiO$_2$ and (□) SiO$_2$ in complete cell culture medium. Particle loading: 0.15 wt%; nominal salt loading: 2.5 wt%.

2.3.8. Calcium Transporters in the Osteoblast

While the slight decrease in the negative particle surface charge due to the presence of Ca$^{2+}$ might facilitate particle attachment and subsequent endocytosis, the increase in surface charge alone would not explain the enhanced uptake of the Ca-SiO$_2$ nanoparticles. If surface charge was the only mechanism, then uptake for Mg-SiO$_2$ and Ca-SiO$_2$ nanoparticles would be similar since they displayed similar zeta potentials at pH = 7.4 (Fig. 2.16). While much research has been done on the transport systems of cells from various tissues such as the nervous system, cardiac and skeletal muscle, and epithelia [25], information on ion pumps, transporters and channels in bone cells still remains limited compared to other tissues. This lack of information on bone cells is largely because the methods for isolation and culture of bone cells have only been established recently [26], whereas the other tissues mentioned earlier can be manipulated with more ease. Recent research done by groups such as Francis et al. has just begun to elucidate some of the transport mechanisms involved in bone cells [21]. There is a particularly high density of Ca-dependent transporters involved in osteoblasts (Fig. 2.17), suggesting the relative importance of calcium in osteoblast mineralization and subsequent bone formation. The presence of Ca-dependent transporters indicates that
mechanisms other than non-specific endocytosis may be involved in the transport of Ca-SiO$_2$ particles across the plasma membrane. While the specific mechanism of cellular uptake is not explored in detail in this thesis, the enhanced uptake of these Ca-SiO$_2$ nanoparticles would be very useful towards the delivery of DNA to osteoblasts.

Figure 2.17. Transport systems in osteoblasts [21].

2.4. Summary

We have synthesized monodisperse organosilicate particles of various sizes and compositions via alkoxide sol-gel processing. The effects of these nanoparticles on osteoblast response were investigated, and Ca-SiO$_2$ nanoparticles were found to be taken up by osteoblasts and to stimulate osteoblast proliferation. The increase in proliferation was found to be osteoblast-specific and did not extend to fibroblasts or hepatocytes. The significant uptake of Ca-SiO$_2$ nanoparticles by osteoblasts and the resulting enhancement in osteoblast
proliferation suggest that these nanoparticles are well-suited for gene delivery applications to bone cells.

2.5. References

Chapter 3 – Osteoblast Protein Expression in Response to Organosilicate Nanoparticles

3.1. Introduction

While cellular proliferation is a fundamental indicator of material performance in cell culture systems, the expressions of cellular proteins and other biochemical markers are also important parameters by which to evaluate material biocompatibility [1]. Once osteoblast cells have ingested nanoparticles, it is essential that these cells retain their normal metabolic function in order to synthesize new bone. This metabolic function can be evaluated by quantifying the osteoblast expression of proteins that are involved in the bone formation process. In addition, the expression levels of certain cytokines, or inflammatory markers, can be quantified to assess any potential trauma that the cells might experience due to nanoparticle uptake. The expression of certain cytokines often leads to bone resorption and fibrous tissue formation [2], so expression of such cytokines should be minimal.

The goal of this chapter will be to study the osteoblast expression of a wide variety of proteins in response to different nanoparticles in order to further elucidate the cell-nanoparticle interactions in our gene delivery system. The proteins of relevance in our study will range from those involved in bone mineralization to those expressed during inflammatory response.

3.1.1. Proteins Involved in Bone Formation

The first subset of proteins we examined is specific to the osteoblast and pertinent to new bone formation. Bone-specific alkaline phosphatase (ALP) is a tetrameric glycoprotein that is found on the surface of osteoblasts [3]. The function of the ALP enzyme is not clearly understood, although it has been shown to be a biochemical marker of bone turnover. ALP expression is also considered to be a strong indicator of osteoblastic phenotype [4-6]. We will measure the ALP expression of osteoblasts in response to different nanoparticles to ensure that the osteoblastic phenotype is retained after particle ingestion.

Another bone-specific protein studied is osteocalcin. Osteocalcin is a vitamin K-dependent protein produced by osteoblasts. This protein contains residues that are thought to be involved in calcium ion and hydroxyapatite binding [7]. While the specific role of osteocalcin in vivo is not known, its affinity for bone mineral constituents implies a role in...
bone formation [7]. Osteopontin is another protein expressed by matrix-producing osteoblasts. It is an extracellular glycosylated bone phosphoprotein, which binds calcium and interacts with the vitronectin receptor that is involved in cell adhesion [8]. In addition to osteopontin production, we will also study the osteoblast expression of fibronectin. Fibronectin is a glycoprotein with various binding sites that allow it to act as an integrating protein in the extracellular matrix of osteoblasts [9].

3.1.2. Cytokine Expression

Cytokines are a large array of proteins that are secreted by a cell for the purpose of altering either its own functions (autocrine effect) or those of adjacent cells (paracrine effect) [10]. The cytokine network is extremely important in the regulation of inflammatory and immune response. Different cytokines may have multiple biological activities. We examined the expression of a variety of cytokines, including interleukins such as IL-2, IL-4 and IL-6, as well as Tumor Necrosis Factor (TNF-α). Since consistently elevated levels of various cytokines would be indicative of a potential inflammatory response in vivo, expression of these cytokines should be minimal for a biocompatible gene delivery system. Inflammation has been known to be induced when crystalline silica-based particles are inhaled [11-13]. In contrast, amorphous silica has widespread use in the food and pharmaceutical industries due to its non-toxicity and bioinertness [14-16]. For this reason, our amorphous organosilicate nanoparticles are expected to elicit minimal inflammatory response, if any.

3.2. Experimental

Cell culture experiments were performed on primary osteoblasts and C3H10T1/2 fibroblasts. Cells were cultured according to established protocols (see Section 2.2) [17]. All absorbance measurements were read in a VersaMax microplate spectrophotometer (Molecular Devices), and the absorbance data were analyzed using Softmax PRO software (Molecular Devices).

For ALP activity measurements, the protocol of Sampath et al. was followed [18]. Cells were seeded in 24-well culture plates at a density of 80 cells/mm² and incubated overnight. On day 1, the nanoparticle suspensions were added at a concentration of 0.15 wt%. After one week, the cells were passaged and lysed. 50 μL of cell lysates were assayed for enzymatic
activity in 96-well plates with \( p \)-nitrophenyl phosphatase (p-NP, Sigma) as a substrate. After 60 min of incubation at 37°C, the reaction was stopped, and the absorbance was measured at 405 nm with \( p \)-nitrophenol as a standard. ALP activity was expressed as nanomoles of \( p \)-NP produced by 1 \( \mu g \) of protein/hr. The protein concentration of the samples was determined by a total protein assay (Pierce).

ALP staining was done by seeding cells in 8-well chamber slides and incubating overnight. On day 1, nanoparticle suspensions were added at a concentration of 0.15 wt%. After one week, the cells were fixed in 2% paraformaldehyde and stained for ALP using a Fast Red Violet Liquid Substrate Kit (Sigma). The stained cells were viewed using an optical light microscope (Nikon).

Osteocalcin measurements were obtained using an Enzyme-Linked Immunosorbent Assay (ELISA) kit for mouse osteocalcin (Biomedical Technologies, Inc.) For protein measurements, cells were seeded in 75-cm\(^2\) culture flasks and incubated overnight. On day 1, nanoparticle suspensions were added at a concentration of 0.15 wt%. After one week, the medium was collected for testing. This assay used a sandwich-ELISA format. 25 \( \mu L \) of samples were added to a 96-well plate coated with antibody. The plate was then incubated for 2.5 hr at 37°C. The medium was then aspirated and the wells were washed three times. Another antibody was added and allowed to incubate for 30 min at room temperature. After washing, 100 \( \mu L \) of substrate were added to the samples and allowed to react. After 15 min of reaction, 100 \( \mu L \) of stop solution were added and the absorbance was measured at 450 nm. For osteopontin measurements, a very similar procedure was performed using a mouse osteopontin ELISA kit obtained from Assay Designs, Inc. This assay also involved a sandwich-ELISA experiment, with different incubation times as specified by the protocol.

Fibronectin expression was measured using a human fibronectin ELISA kit (Quantimatrix). Rather than using a sandwich-ELISA format like the osteocalcin and osteopontin kits, this assay uses a competitive inhibition ELISA. Samples were diluted and preincubated with a polyclonal rat antibody to human fibronectin, which binds with any fibronectin present in the samples. 100-\( \mu L \) sample mixtures were then transferred to a human fibronectin coated plate. After incubating at room temperature for 1 hr, the wells were washed four times with wash buffer. 100 \( \mu L \) of a Goat anit-Rabbit IgG-HRP Conjugate were then added to each well and incubated at room temperature for 30 min. The wells were
washed before the addition of 100 μL of substrate to each well. After the reaction was complete, 100 μL of stop solution were added to each well and the absorbance was measured at 450 nm.

For the cytokine measurements, sandwich-ELISA kits (Ebioscience) were used to detect all interleukin levels. The general procedure was as follows: a 96-well plate was coated with 100 μL/well of capture antibody. The plate was then sealed and incubated overnight at 4°C. The wells were then aspirated and washed three times, and then blocked with 200 μL/well of assay diluent. The blocked wells were incubated at room temperature for 1 hr. After incubation, the wells were washed and then 100 μL of sample were added to each well. Binding of any free cytokine to the capture antibody was allowed to occur over the next 2 hr at room temperature. After binding, the wells were washed, 100 μL/well of detection antibody were added, and the plate was incubated for 1 hr. The wells were then washed and 100 μL/well of a second antibody were added and allowed to incubate for 30 min at room temperature. Finally, the wells were washed, 100 μL/well of substrate solution were added, 50 μL/well of stop solution were introduced, and the absorbance of the plate was read at 450 nm.

3.3. Results and Discussion

3.3.1. ALP Activity Measurements

The effect of various nanoparticles on ALP expression was measured. As with the proliferation and uptake experiments in the previous chapter [17], all protein expression experiments were done at a nanoparticle loading of 0.15 wt% to ensure maximum uptake. All control samples were cells grown under normal conditions without any particles. Fig. 3.1 shows that the ALP activity of primary osteoblasts incubated with nanoparticles of different compositions remained fairly constant. In other words, the phenotype of osteoblasts was retained in the presence of these nanoparticles.
Figure 3.1. Effect of nanoparticle composition on the ALP activity of osteoblasts. Particle size: 50 nm; nominal salt loading: 2.5 wt%. Values are expressed as nanomoles of p-NP produced by 1 µg of total protein during 1 hr. Values are mean ± standard error of the mean; n = 3.

To study the effect of the Ca-SiO$_2$ system in more detail, ALP activity was also measured in the presence of Ca-SiO$_2$ nanoparticles of different sizes (Fig. 3.2). The phenotype of osteoblasts was retained regardless of the Ca-SiO$_2$ particle size.

Figure 3.2. Effect of Ca-SiO$_2$ particle size on the ALP activity of osteoblasts. Nominal Ca loading: 2.5 wt%. Values are expressed as nanomoles of p-NP produced by 1 µg of total protein during 1 hr. Values are mean ± standard error of the mean; n = 3.
These measurements also indicated that osteoblastic phenotype was retained regardless of particle uptake, which was different for different nanoparticle composition and size. This consistency of ALP expression after particle ingestion was a significant finding, since it was imperative that these osteoblasts retained their phenotype in order to continue their function of synthesizing healthy bone. Figure 3.3 optically depicts the retention of osteoblastic phenotype in the presence of Ca-SiO$_2$ nanoparticles. The cells stained positive for ALP after nanoparticle ingestion.

![Image of osteoblasts](image)

Figure 3.3. Optical micrographs of osteoblasts (a) before and (b) after incubation with 50-nm Ca-SiO$_2$ nanoparticles. The darker regions indicate ALP expression.

### 3.3.2. Osteocalcin Expression Measurements

Figure 3.4 shows the effect of nanoparticle composition on osteocalcin expression. All samples continued to secrete osteocalcin in the presence of nanoparticles, with a noticeable increase in osteocalcin expression in the Ca-SiO$_2$ samples. A rise in osteocalcin expression is often associated with a mature osteoblastic phenotype and the onset of mineralization [19], both of which are desirable for bone regeneration. The effect of calcium loading for Ca-SiO$_2$ nanoparticles on osteocalcin expression was not significant (data not shown), but there was a slight effect of particle size, with 50-nm particles showing higher osteocalcin levels (Fig. 3.5). Like the ALP activity findings, these results were encouraging since they showed the retention of osteoblastic phenotype and normal osteoblastic activity in the presence of organosilicate-based nanoparticles.
Figure 3.4. Effect of nanoparticle composition on the osteocalcin expression of osteoblasts. Particle size: 50 nm; nominal salt loading: 2.5 wt%. Values are expressed as proteins produced by $10^6$ cells. Values are mean ± standard error of the mean; $n = 3$.

Figure 3.5. Effect of Ca-SiO$_2$ particle size on the osteocalcin expression of osteoblasts. Nominal Ca loading: 2.5 wt%. Values are expressed as proteins produced by $10^6$ cells. Values are mean ± standard error of the mean; $n = 3$.

3.3.3. Osteopontin Expression Measurements

The effect of nanoparticle composition on osteopontin expression is shown in Fig. 3.6. The experiments indicated that the ingestion of nanoparticles did not result in any significant change in osteopontin production. Osteopontin expression was also unaffected by Ca-SiO$_2$ particle size (Fig. 3.7). These results suggested that the doped SiO$_2$ nanoparticle-based gene
delivery system would allow for normal levels of osteopontin production and potential bone formation.

Figure 3.6. Effect of nanoparticle composition on the osteopontin expression of osteoblasts. Particle size: 50 nm; nominal salt loading: 2.5 wt%. Values are expressed as proteins produced by $10^6$ cells. Values are mean ± standard error of the mean; n = 3.

Figure 3.7. Effect of Ca-SiO$_2$ particle size on the osteopontin expression of osteoblasts. Nominal Ca loading: 2.5 wt%. Values are expressed as proteins produced by $10^6$ cells. Values are mean ± standard error of the mean; n = 3.
3.3.4. Fibronectin Expression Measurements

Fibronectin expression also remained fairly constant in the presence of nanoparticles of different compositions (Fig. 3.8), as well as in the presence of Ca-SiO₂ nanoparticles of different sizes (Fig. 3.9). This result was not surprising, since fibronectin production has been known to often depend on the biomaterial surface [20-21], and all the cells in these experiments were grown on the same polystyrene surface. The consistency of fibronectin production was also an encouraging finding that suggested that the osteoblasts would continue to adhere and migrate normally in vivo even after the ingestion of nanoparticles.

![Graph showing fibronectin levels](image)

Figure 3.8. Effect of nanoparticle composition on the fibronectin production of osteoblasts. Particle size: 50 nm; nominal salt loading: 2.5 wt%. Values are expressed as proteins produced by 10⁶ cells. Values are mean ± standard error of the mean; n = 3.
Figure 3.9. Effect of Ca-SiO$_2$ particle size on the fibronectin production of osteoblasts. Nominal Ca loading: 2.5 wt%. Values are expressed as proteins produced by $10^6$ cells. Values are mean ± standard error of the mean; $n = 3$.

3.3.5. **Cytokine Production**

In order to further elucidate the biocompatibility of the nanoparticles, the expression levels of various cytokines were studied. Consistently elevated cytokine levels *in vitro* would indicate the potential of *in vivo* trauma. A number of groups have documented inflammatory response to small particles in various cell lines and through different routes of administration [22-25], particularly through inhalation. However, we expected that both the bioactive composition of these nanoparticles and their administration directly to the site of connective tissue would minimize any inflammatory response.

In addition to the effects of particle composition and mode of administration, the effects of parameters such as particle size, shape and crystallinity on inflammatory response have also been examined [23, 26]. Specifically, some groups have shown that while smaller particle sizes could result in more cytokine secretion, this effect was secondary to particle morphology. For example, Lacquierrier *et al.* found that needle-like hydroxyapatite particles resulted in more cytokine secretion than spherical particles [23]. This was because needle-like particles caused substantial mechanical damage to the cell membrane during uptake [23]. Our organosilicate particles were spherical in morphology, which would minimize such damage. In addition, any inflammation observed with amorphous particles was often
temporary [27-29], whereas crystalline particles have been shown to elicit a more severe and persistent inflammatory response.

3.3.5.1. Interleukin Levels

The effect of various parameters on the presence of a number of different interleukins was investigated. Figure 3.10 shows the effect of nanoparticle composition on the osteoblast production of IL-2, IL-4 and IL-6. These results indicated that while production of IL-2 remained low, elevated levels of IL-4 and IL-6 were evident in the presence of certain nanoparticles. Specifically, the presence of undoped SiO$_2$ nanoparticles gave rise to the most dramatic effect on IL-4 and IL-6 production.

![Figure 3.10. Effect of nanoparticle composition on the interleukin levels of osteoblasts. Particle size: 50 nm; nominal salt loading: 2.5 wt%. Values are expressed as proteins produced by 10$^6$ cells. Values are mean ± standard error of the mean; n = 3.](image)

While SiO$_2$ particles did not enter cells, their presence in the culture medium could have resulted in the adsorption of proteins onto their surface, leading to protein depletion from the medium itself. Cell culture medium was supplemented with a variety of growth factors and proteins to nourish the cells under culture. If a foreign material in the medium caused a deficiency of such proteins, cell trauma and possibly elevated cytokine production might occur. Fig. 3.11 shows the protein depletion from cell culture medium due to the presence of
nanoparticles of various compositions. This experiment was done without any cells in order to measure only the effect of protein adsorption on the particle surface. Protein depletion was calculated by measuring the total protein in the cell culture medium before and after a week-long incubation with nanoparticles. Doped SiO$_2$ particles essentially resulted in similar depletion of protein over a week-long period as compared to the undoped SiO$_2$ particles (Fig. 3.11). However, when these particles were incubated with cells, some of the doped SiO$_2$ nanoparticles were ingested by cells within the first 24 hr, and would not contribute to further protein depletion from the medium. Consequently, only the excess particles that have not been ingested would cause protein depletion that actually affected the cells over the course of one week. This depletion of nutrients could explain the substantial increase in IL-4 and IL-6 production due to the undoped SiO$_2$ nanoparticles.

![Figure 3.11](image-url) Figure 3.11. Effect of nanoparticle composition on the protein depletion from cell culture medium. Particle size: 50 nm; nominal salt loading: 2.5 wt%. Values are expressed as protein depleted after one week of incubation with nanoparticles. Values are mean ± standard error of the mean; n = 3.

Ingestion of nanoparticles has been shown to stimulate cytokine levels in a variety of cell types, which would explain the elevated IL-6 levels associated with Ca-SiO$_2$, Mg-SiO$_2$, and Na-SiO$_2$ nanoparticles. Many groups have reported the downregulation of osteoblastic phenotypic marker expression under the influence of various cytokines [6, 30]. However, while excess IL-6 production was observed due to nanoparticle ingestion (Fig. 3.10), downregulation of osteoblastic phenotype was not noted (Figs. 3.1-3.3). The fact that the
slightly elevated IL-6 levels were not significant enough to result in a loss of osteoblastic phenotype was a very positive finding, since it suggested that osteoblasts would continue to function normally after particle ingestion.

The effect of Ca-SiO$_2$ particle size on osteoblast IL-6 production is shown in Fig. 3.12. Smaller particle sizes resulted in greater IL-6 production. This effect was most likely due to the increased cellular uptake of smaller particles, as reported in Chapter 2 [17], since inflammatory response due to particle ingestion was often dependent on particle dosage [26]. While an elevated level of IL-6 was observed due to Ca-SiO$_2$ nanoparticle ingestion, the osteoblasts continued to express phenotypic markers at control levels (Figs. 3.2 and 3.5). In addition, there was enough evidence in the literature to indicate that this cytokine response might not have a permanently damaging effect \textit{in vivo}, since mild inflammatory response due to amorphous materials was often temporary [27-29]. The retention of osteoblastic phenotype despite the elevated IL-6 expression supported the use of Ca-SiO$_2$ nanoparticles as osteoblast gene delivery vehicles.

![Figure 3.12. Effect of Ca-SiO$_2$ particle size on the IL-6 levels of osteoblasts. Nominal Ca loading: 2.5 wt%. Values are expressed as proteins produced by $10^6$ cells. Values are mean ± standard error of the mean; n = 3.](image)

Fibroblasts were also assayed for interleukin expression. As with osteoblasts, the presence of Ca-SiO$_2$ nanoparticles led to slightly elevated IL-6 levels in fibroblasts (Fig. 3.13).
Figure 3.13. Effect of Ca-SiO$_2$ nanoparticle size on the IL-6 levels of fibroblasts. Nominal Ca loading: 2.5 wt%. Values are expressed as proteins produced by $10^6$ cells. Values are mean ± standard error of the mean; n = 3.

3.3.5.2. TNF-α Levels

In addition to our detailed study of the interleukin expression levels, we have also quantified the cellular levels of TNF-α in response to various nanoparticles. TNF-α has been cited to play an integral role in bone resorption; it has also been implicated to stimulate the production of other pro-inflammatory cytokines [6,30]. Figure 3.14 shows the TNF-α levels for osteoblasts cultured with nanoparticles of various compositions. These results indicated that there was no substantial induction of TNF-α due the ingestion of organosilicate nanoparticles. The expression of TNF-α also remained unaffected by variations in calcium loading and particle size (Fig. 3.15).

Fibroblasts were also evaluated for TNF-α induction, as shown in Fig. 3.16. The results indicated that various compositions of nanoparticles did not substantially stimulate TNF-α secretion by fibroblasts. These data suggested that any inflammatory response caused by nanoparticles administered to the connective tissue would be minimal. The fact that the Ca-SiO$_2$ nanoparticles caused virtually no increase in TNF-α expression was a very positive result. Minimization of this particular cytokine in orthopedic procedures is particularly important, since a rise in TNF-α often stimulates the secretion of other cytokines.
Figure 3.14. Effect of nanoparticle composition on the TNF-α levels of osteoblasts. Particle size: 50 nm; nominal salt loading: 2.5 wt%. Values are expressed as proteins produced by $10^6$ cells. Values are mean ± standard error of the mean; n = 3.

Figure 3.15. Effect of Ca-SiO$_2$ particle size on the TNF-α levels of osteoblasts. Nominal Ca loading: 2.5 wt%. Values are expressed as proteins produced by $10^6$ cells. Values are mean ± standard error of the mean; n = 3.
3.4. Summary

We have examined osteoblast protein expression in response to cellular uptake of doped organosilicate nanoparticles. Osteoblastic phenotype was retained after nanoparticle ingestion, as evidenced by the normal expression of alkaline phosphatase, osteocalcin, osteopontin and fibronectin. A detailed study of cytokine production showed that while IL-2, IL-4 and TNF-α levels were not substantially affected by particle uptake, there was an increase in IL-6 production in both osteoblasts and fibroblasts. This increase, however, did not affect osteoblastic phenotype, induce cell toxicity, or stimulate production of the other cytokines. The non-toxicity and preservation of osteoblastic phenotype after particle uptake were very positive in vitro results that provided strong support for the further examination of these nanoparticles as gene delivery vehicles for osteoblasts.
3.5. References


4.1. Introduction

Osteogenic proteins such as bone morphogenetic proteins (BMPs) have received attention as promising therapies for fracture repair [1]. The pioneering work in this area was done by Urist and coworkers who first identified BMP activity in tissue extracts [2]. However, it has been almost 40 years since that discovery and there is still no effective recombinant protein therapy available for bone repair. The reasons for this slow progress lie in the multiple challenges associated with protein delivery. These challenges include the development of a delivery system that can maintain the structural integrity and functional activity of the protein in vivo, and deliver the therapeutic dosage of the protein required to induce bone formation. The tissue engineering of bone involves the incorporation of such proteins into cell-seeded three-dimensional scaffolds to restore tissue function. While this approach has had some success, maintenance of cell mass at the fracture site is difficult, and the mass transfer limitations associated with large and complex fractures continue to be a problem [3].

To overcome these challenges, researchers have recently begun to consider gene therapy for fracture repair [4-5]. Rather than delivering osteogenic proteins to the fracture site, delivering the genes that encode for these proteins would allow cells to produce such proteins at therapeutic times and levels. Fang et al. have shown that direct transfer of naked DNA via a polymer matrix can induce bone formation [4]. However, naked DNA transfection is often associated with low transfection efficiencies. In most cases, DNA requires a delivery vehicle or vector to aid its entry into the cell. While the development of non-viral vectors for gene therapy has been an active area of research, non-viral vectors are plagued by cytotoxicity and lower transfection efficiencies than their viral counterparts. The development of non-viral vectors that can transfect bone cells at higher efficiencies than naked DNA without inducing cytotoxicity would allow for authentic growth factor production and cell maintenance at the fracture site, thus accelerating fracture healing.

Our previous work has shown that surface-doped organosilicate nanoparticles were able to enter the osteoblast cell membrane without inducing cytotoxicity [6-7]. We have quantified the effects of particle size and composition on cellular uptake, cell proliferation and
cellular protein expression. In particular, Ca-SiO₂ nanoparticles were found to stimulate cell proliferation and maximize cellular uptake, making them ideal candidates for intracellular delivery vehicles. In this chapter, we will explore the potential of these nanoparticles as non-viral vectors to deliver plasmid DNA to osteoblasts. We will investigate how properties such as particle size and composition affect nanoparticle-DNA complex formation. This understanding is then used to develop a DNA delivery system that transfects osteoblasts without inducing cytotoxicity.

4.2. Experimental

4.2.1. Synthesis of Nanoparticle-DNA Complexes

The plasmid gWiz™ GFP (5757 bp) encoding the green fluorescent protein (GFP) gene was obtained from Aldevron, Inc. This plasmid contains the GFP gene under the control of a modified promoter from the cytomegalovirus (CMV) immediate early (IE) gene. The ready-to-use reporter plasmid was obtained as a 1.0 mg/ml stock solution in aqueous Tris-HCl/EDTA buffer. The GFP produced has an excitation peak at 470-480 nm and an emission peak at 510 nm.

For Ca-SiO₂ nanoparticle-DNA complex synthesis, Ca(NO₃)₂·4H₂O (Fluka) was added to plasmid DNA diluted in a buffer solution and quickly vortexed. Ca loading was varied through the addition of different dilutions of a 250 mM Ca(NO₃)₂·4H₂O stock solution to the DNA solution. SiO₂ nanoparticles (synthesized according to protocol in Section 2.2 [6]) were ultrafiltered three times in buffer in an Amicon Ultrafiltration Cell (Millipore) using a 50K PES membrane (Millipore). The nanoparticles were slowly added to the Ca-DNA solution, vortexed and allowed to form complexes for 30 min at 25°C.

NH₃⁺-SiO₂ nanoparticles were synthesized via sol-gel surface functionalization of SiO₂ nanoparticles. SiO₂ nanoparticles were prepared as described in Section 2.2 [6] and aged in reaction solution. After 3 hr, 3-aminopropyltrimethoxysilane ((CH₃O)₃Si(CH₂)₃NH₂ or aminoTMOS, Gelest) was slowly added to the particle suspension at an aminoTMOS to methyltrimethoxysilane (Gelest) molar ratio of 0.2:1. The resulting particles were aged for 3 hr before ultrafiltration. For complex formation, the NH₃⁺-SiO₂ nanoparticles were added to a plasmid DNA solution and mixed for 30 min.
The cationic lipid lipofectamine 2000 (Sigma) was used as a positive control. Lipofectamine was diluted in a buffer solution, added to a plasmid DNA solution, and mixed for 30 min to form lipofectamine-DNA complexes.

4.2.2. Physicochemical Characterization of Vector-DNA Complexes

Complex size was measured using dynamic light scattering (DLS) at 25°C using a Lexel 95 Argon-ion laser and a Brookhaven high-precision photomultiplier at 90°. Data were acquired using a Brookhaven 9000AT correlator. The periods for acquisition of the autocorrelation function were 0.1 µs to 0.5 s, and complex size distributions were obtained from non-negatively constrained least-squares (NNCLS) analysis of the data. Zeta potential measurements were performed on 0.15 wt% nanoparticle suspensions in cell culture medium using a ZetaPALS Zeta Potential Analyzer (Brookhaven).

Solution pH was measured using an Orion model 420A pH meter, and adjusted by the addition of KOH or HCl. Agarose gel electrophoresis was used to determine the ability of the nanoparticles to bind DNA. It was performed in a 1% (w/v) gel, ethidium bromide induced for visualization for 3 hr at 60 V. DNA binding efficiency was measured by centrifuging the complexes at 4000 g and measuring the amount of unbound DNA in the supernatant using the PicoGreen® DNA Quantitation Kit (Molecular Probes).

Atomic force microscopy (AFM) samples were prepared by incubating synthesized complexes on freshly cleaved mica discs obtained from Ted Pella, Inc. (Redding, CA) for 20 min. After sample adsorption, the discs were washed three times with 200 µL of deionized water to remove any unbound sample. AFM was performed on a Multimode AFM (Digital Instruments/Veeco, Santa Barbara, CA) controlled by a Nanoscope IV Controller (Digital Instruments/Veeco). Topographic and phase images were collected in the tapping mode in air.

4.2.3. Transfection Experiments

Primary osteoblasts, MC3T3 osteoblasts, HepG2 hepatocytes, and C3H10T1/2 fibroblasts were cultured according to established protocols (see Section 2.2) [6]. Cells were grown in 8-well chamber slides for microscopy experiments, and 75-cm² flasks for transfection efficiency experiments. Vector-DNA complexes were prepared as described above. Various compositions of complexes were added to nearly confluent cell layers 2-3
days after cell seeding. After addition of the complexes, cells were incubated at 37°C in 5% CO₂ atmosphere. After 3 hr, the complexes were aspirated and replaced with culture medium.

After 48 hr, cells were assayed for GFP expression both qualitatively through fluorescence microscopy and quantitatively through fluorescence-activated cell sorting (FACS). For microscopy, the cells grown in chamber slides were fixed with 2% paraformaldehyde. The slides were then viewed under a Zeiss Axiovert 200 fluorescence microscope equipped with a SPOT digital camera. FACS experiments were performed using a Facscan cytometer (Becton-Dickinson) equipped with an argon laser. At least 10,000 events were counted for each sample. The software package Cell Quest (Becton-Dickinson) was used to determine the events of interest from forward and side scatter parameters. The mean fluorescence intensity of the cells was obtained from the mean channel number of the fluorescence histograms of the gated population. For transfection efficiency measurements, total fluorescence intensity was plotted versus the GFP fluorescence intensity on a log scale and cell auto-fluorescence was eliminated. Total GFP expression was quantified from a shift in the mean fluorescence of cell populations relative to the control cell population with no DNA.

4.3. Results and Discussion

4.3.1. Synthesis and Physicochemical Characterization of Ca-SiO₂-DNA Complexes

4.3.1.1. Effect of Precursor Addition Sequence

Addition of DNA to the preformed Ca-SiO₂ nanoparticles did not result in complex formation, due to charge repulsion between the negatively charged DNA molecules and the negative surface charge of the Ca-SiO₂ nanoparticles. In order to form Ca-SiO₂-DNA complexes, the DNA was premixed with the Ca(NO₃)₂ 4H₂O solution and then mixed with the SiO₂ nanoparticles. This synthesis method is modified from the concept used to form calcium phosphate-DNA complexes [5], in which the DNA is premixed with one of the precursors and complex formation is initiated by the addition of the other precursor to form a precipitate.

While the Ca-DNA solution addition to the nanoparticle suspension resulted in some complex formation, this sequence of addition led to a broader complex size distribution compared to the slow addition of nanoparticles to the Ca-DNA solution (Fig. 4.1). Plasmid DNA molecules alone exhibited a broad DLS distribution with a mean of ~ 350 nm.
Figure 4.1. Complex size distributions of complexes formed by addition of (a) Ca-DNA solution to 50-nm SiO$_2$ nanoparticles and (b) 50-nm SiO$_2$ nanoparticles to Ca-DNA solution. Nominal Ca loading: 1.25 wt%; DNA loading: 24 µg/ml.

The data indicated that the Ca-DNA disrupted the colloidal suspension of SiO$_2$ more than the addition of the stable SiO$_2$ suspension to the Ca-DNA mixture. Other groups have
also reported the preparation of nanoparticle-DNA complexes with narrow size distributions through the addition of nanoparticles to DNA [8].

4.3.1.2. Effects of Nanoparticle Size and Composition on DNA Binding Affinity

In order to confirm that DNA was complexed with the particles, agarose gel electrophoresis was performed. This technique uses a matrix of highly purified agar to separate macromolecules based on charge and size. Electrophoretic immobilization of the DNA results in gel retardation of the complexes, implying binding of the DNA to the nanoparticle surface [9,10]. Figure 4.2 shows gel electrophoresis results for complexes formed with 50-nm SiO$_2$ particles of various Ca loadings. The results indicated that no complex formation occurred with Ca-free SiO$_2$ nanoparticles, since the electrophoretic mobility of the DNA was almost identical to that of the free plasmid. This was most likely due to the lack of charge attraction between SiO$_2$ and DNA molecules. However, the DNA appeared to form complexes with the SiO$_2$ nanoparticles of increased Ca loadings, as indicated by the DNA retention.

![Figure 4.2. Agarose gel electrophoresis of nanoparticle-DNA complexes prepared with 50-nm SiO$_2$ with nominal Ca loadings of 1 wt% Ca (Lane 1), 0.5 wt% Ca (Lane 2) and 0 wt% Ca (Lane 3). Lane 4 represents free DNA without complex formation, and Lane 5 is the DNA marker. Particle loading: 0.15 wt%; DNA loading: 240 μg/ml.](image)

The extent of DNA binding was measured using co-sedimentation analysis by ultracentrifuging the complexes and measuring the amount of bound DNA through fluorescence. Figure 4.3 indicates that as Ca loading increased, the amount of bound DNA
Figure 4.4. Effects of particle size and Ca loading on the size distribution of Ca-SiO$_2$-DNA complexes. Particles of (■) 10 nm, (●) 50 nm and (▲) 100 nm are examined at a particle loading of 0.15 wt% and a DNA loading of 24 μg/ml. Values are mean ± standard error of the mean; n = 3.

Figure 4.5. AFM image of pure plasmid DNA.
also increased. This was to be expected, since the addition of Ca promoted complex formation. The further addition of Ca led to agglomeration between complexes, and these large aggregates bound even more DNA. The data also indicated that as nanoparticle size increased, bound DNA increased. The colloidal suspensions of larger particles were less stable than those of smaller particles, and the addition of DNA to the larger particles would lead to greater aggregate formation, binding more DNA.

![Graph showing DNA binding affinity with particle size and Ca loading](figure4_3.png)

**Figure 4.3.** Effects of particle size and Ca loading on the extent of DNA binding in Ca-SiO$_2$-DNA complexes. Particles of (■) 10 nm, (♦) 50 nm and (▲) 100 nm are examined at a particle loading of 0.15 wt% and a DNA loading of 24 µg/ml.

### 4.3.1.3. Effects of Particle Size and Ca Loading on Complex Size

While the DNA binding affinity was shown to increase with increasing particle size and Ca loading, the complex size analysis showed that above certain parameters, extremely large complexes were formed (see Fig. 4.4). Addition of Ca and DNA to the SiO$_2$ nanoparticle suspension resulted in an increased complex size and in a broadening of the complex size distribution. The broad complex size distribution was caused by the structural range of complexes that were formed when the nanoparticles were mixed with Ca and DNA. This phenomenon was illustrated by AFM (see Figs. 4.5-4.6). The image of pure DNA (Fig. 4.5) depicted the various plasmid conformations that often led to the polydispersity of DNA complexes (Fig. 4.6).
It is interesting to note that while 10- and 50-nm Ca-SiO$_2$ particles possessed discretely different size distributions when prepared as colloidal suspensions, the addition of DNA not only led to a broadening of their size distributions, but also resulted in substantial overlap of their size distributions (Fig. 4.4). For example, at a Ca loading of 1.5 wt%, the complex size distributions for 10- and 50-nm particles were almost identical, indicating that the addition of Ca and DNA to the 10-nm SiO$_2$ particle suspension resulted in a significant degree of agglomeration. This agglomeration might be due to the energetically favorable reduction in surface free energy that occurred from the decrease in surface area [11]. However, while the 10- and 50-nm particles formed complexes of similar sizes, the 100-nm particles led to much larger complexes (see Fig. 4.4), indicating that the 100-nm particle suspension was less stable from agglomeration. Many groups have shown this agglomeration trend in pure colloidal silica, whereby as particle size increased, the critical salt concentration required for aggregation decreased [12]. The complex size data agreed with the DNA binding data, suggesting that agglomeration caused increases in both complex size and DNA binding.

4.3.2. Synthesis and Physicochemical Characterization of NH$_3^+$-SiO$_2$-DNA Complexes

Although we were able to successfully form Ca-SiO$_2$-DNA complexes, we wanted to investigate if we could further reduce complex size in order to deliver more DNA into the cell cytoplasm. The ability to control complex size is extremely important, since smaller complexes have been shown to enhance cellular uptake [13-15].
SiO₂ nanoparticles has also displayed the significant effect of particle size on cellular uptake [6]. Researchers have shown that the amount of DNA delivered to the cell is an important determinant of transfection efficiency [16]. Since stronger electrostatic interactions between the nanoparticles and plasmid DNA would likely result in greater DNA condensation and smaller complex sizes, we have functionalized the SiO₂ nanoparticles with –NH₃⁺ surface groups.

Surface functionalization of silica particles has been studied extensively using various organosilane surface modifiers [17]. NH₃⁺-SiO₂ nanoparticles were synthesized via sol-gel functionalization of SiO₂ nanoparticles using aminoTMOS. To determine if the aminoTMOS precursors were indeed reacting with the surface of SiO₂ particles and not condensing with other aminoTMOS, the particle size distribution was analyzed after the aminoTMOS precursors had been introduced. The retention of a single normal particle size distribution (not shown) confirmed that aminoTMOS only reacted with the SiO₂ surface.

The zeta potential of the particles indicated that unlike SiO₂ and Ca-SiO₂ nanoparticles, the NH₃⁺-SiO₂ nanoparticles were positively charged at neutral pH (Table 4.1). This positive surface charge might allow for greater DNA condensation. This was confirmed by Fig. 4.7, which shows that for similarly sized SiO₂-based nanoparticles, the NH₃⁺-SiO₂-DNA complexes were smaller than the Ca-SiO₂-DNA complexes.

Table 4.1. Zeta potential values of SiO₂, Ca-SiO₂ and NH₃⁺-SiO₂ nanoparticles.

<table>
<thead>
<tr>
<th>Particle Composition</th>
<th>Zeta Potential at pH = 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>SiO₂</td>
<td>-30.4</td>
</tr>
<tr>
<td>Ca-SiO₂*</td>
<td>-15.8</td>
</tr>
<tr>
<td>NH₃⁺-SiO₂</td>
<td>21.3</td>
</tr>
</tbody>
</table>

*Nominal Ca loading for Ca-SiO₂ nanoparticles: 2.5 wt%.
Figure 4.7. The effect of particle size on the DNA binding of (○) NH₃⁺-SiO₂ and (□) Ca-SiO₂ nanoparticles and the size distributions of (●) NH₃⁺-SiO₂-DNA and (■) Ca-SiO₂-DNA complexes. Nominal Ca loading: 1.5 wt% for 10-nm and 50-nm Ca-SiO₂ particles, 1.0 wt% for 100-nm Ca-SiO₂ particles.

The extent of DNA complexed with the NH₃⁺-SiO₂ nanoparticles was measured using the DNA binding assay described earlier. Figure 4.7 indicated that the NH₃⁺-SiO₂ nanoparticles bound more DNA than the Ca-SiO₂ nanoparticles.

4.3.3. Transfection Studies

4.3.3.1. Effect of Nanoparticle Composition on Transfection Efficiency

Since the NH₃⁺-SiO₂-DNA complexes bound more DNA and formed smaller complexes than the Ca-SiO₂-DNA complexes, they might be able to deliver more DNA to the cells. However, Fig. 4.8 indicates that the transfection efficiencies of NH₃⁺-SiO₂-DNA were very low in both osteoblasts and hepatocytes. The transfection efficiency of Ca-SiO₂-DNA in osteoblasts was much higher than that of NH₃⁺-SiO₂-DNA.
These data suggested that factors other than cellular uptake and DNA delivery were controlling the ultimate transfection efficiency. There are many intracellular barriers to gene delivery, including release of the DNA inside the cell and entry of DNA into the nucleus. In some cases, gene expression can occur in the cytoplasm [18-19]. However, for the gWiz™ GFP plasmid used in our experiments, GFP production would only occur if the plasmid has entered the nucleus. While some groups have reported nuclear entry of entire vector-DNA complexes [20-21], our previous microscopy data showed that organosilicate nanoparticles did not enter the nucleus [6]. Thus, the major barrier preventing gene expression in the case of the NH₃⁺-SiO₂-DNA complexes was most likely the intracellular release of DNA, since the DNA would have to make its way to the nucleus in order for GFP to be expressed. Schaffer et al. have stressed the importance of “vector unpackaging” [22] as a major barrier to transfection. In our NH₃⁺-SiO₂-DNA system, while stronger electrostatic interactions resulted in smaller complex sizes, the increased DNA binding strength might have hindered DNA release from the complexes, thus preventing the entry of DNA into the nucleus.

Some groups have reported successful transfection of other cell lines with silica [23] and gold [24] nanoparticles functionalized with quaternary ammonium groups. The alkyl spacer length between the nanoparticle surface and the ammonium group has been suggested
to play a role in DNA condensation and subsequent release, with a longer chain length allowing for steric hindrances to prevent the DNA from binding too tightly. While these approaches have had some success, they continue to exhibit some cytotoxicity.

Our Ca-SiO\textsubscript{2}-DNA complexes exhibited lower transfection efficiencies in osteoblasts and hepatocytes than lipofectamine-DNA complexes (Fig. 4.8). However, the former demonstrated excellent selectivity towards transfecting osteoblasts versus hepatocytes compared to lipofectamine-DNA complexes. This was most likely due to the surface Ca content in the Ca-SiO\textsubscript{2}-DNA complexes. This selectivity could be very useful in the systemic administration of these complexes to a fracture site.

### 4.3.3.2. Effects of Particle Size and Ca Loading on Transfection Efficiency

Since the Ca-SiO\textsubscript{2}-DNA complexes exhibited successful transfection in osteoblasts, the effects of particle size and Ca loading in this system on transfection efficiency in osteoblasts were investigated in detail (see Fig. 4.9). Figure 4.10 provides a visual depiction of some of the transfected cells.

![Figure 4.9. Effect of Ca loading on the osteoblast transfection efficiency of Ca-SiO\textsubscript{2}-DNA complexes formed with (■) 10-nm, (●) 50-nm and (▲) 100-nm particles. Particle loading: 0.15 wt%. Values are mean ± standard error of the mean; n = 3.

The data indicated that Ca-SiO\textsubscript{2}-DNA complexes formed with 10-nm and 50-nm Ca-SiO\textsubscript{2} particles resulted in successful transfection. Complexes formed with 100-nm particles
resulted in almost no transfection. The inability of these complexes to transfect osteoblasts could be attributed to their effective complex size. Figure 4.4. shows that the complexes formed with 100-nm Ca-SiO₂ nanoparticles were extremely large, with a mean complex size of > 450 nm. These large aggregates have major difficulty in entering the cell to deliver DNA to the nucleus.

Fig. 4.10. Fluorescence micrographs of osteoblasts transfected with Ca-SiO₂-DNA complexes formed with 50-nm Ca-SiO₂ particles with a nominal Ca loading of (a) 0.50 wt%, (b) 0.75 wt%, (c) 1.00 wt% and (d) 1.25 wt%.

Figure 4.9 also shows that the transfection efficiencies exhibited by complexes formed with 10-nm and 50-nm Ca-SiO₂ particles were fairly similar. This similarity was somewhat surprising, since Section 2.3.4 indicated that the cellular uptake of 10-nm Ca-SiO₂ particles was significantly higher than that of 50-nm Ca-SiO₂ particles [6]. Because of this difference in uptake, one would expect to see significantly higher transfection efficiencies with the
complexes formed with 10-nm Ca-SiO₂ particles as compared to those formed with 50-nm Ca-SiO₂ particles. However, the sizes of the Ca-SiO₂-DNA complexes formed with these two different Ca-SiO₂ particle sizes were quite similar (see Figure 4.4). This similarity in complex sizes led to the similarity in their overall transfection efficiencies. An optimal Ca content of 1.25 wt% led to transfection efficiencies of 14.7% and 17.4% in osteoblasts for the complexes formed with 10-nm and 50-nm Ca-SiO₂ particles, respectively. This optimal Ca loading gave rise to one of the smallest average complex sizes formed with 10-nm and 50-nm Ca-SiO₂ particles (Fig. 4.4).

In order to further examine the correlation between nanoparticle uptake and nanoparticle-DNA transfection efficiency, the effects of particle size and Ca loading on uptake and transfection efficiency were investigated (Fig. 4.11). The data showed that a higher nominal Ca loading was required for optimal transfection efficiency vs. optimal uptake. These results suggested that in the case of the nanoparticles alone, Ca merely modified the surface composition (not the particle size) to enhance cellular uptake. On the other hand, in nanoparticle-DNA complexes, Ca played an additional role of reducing the complex size. Since Ca interacted with the anionic groups of DNA in order to condense the large DNA molecules, more Ca was required to achieve optimal transfection.

Figure 4.11. Effect of Ca loading on the uptake of (□) 10-nm and (○) 50-nm Ca-SiO₂ nanoparticles and the osteoblast transfection efficiency of complexes formed with (■) 10-nm and (●) 50-nm Ca-SiO₂ particles. Particle loading: 0.15 wt%; DNA loading: 24 μg/ml; n = 3.
4.3.3.3. Transfection Efficiencies of Ca-SiO₂-DNA Complexes in Other Cell Types

Besides osteoblasts, other connective and non-connective tissue cell types were also examined in transfection studies. Figure 4.12 shows the transfection efficiencies for osteoblasts, fibroblasts and hepatocytes. Lipofectamine, the cationic lipid used as a positive control, transfected all cells due to non-specific interactions. While Ca-SiO₂-DNA complexes exhibited lower transfection efficiencies in all cell lines as compared to lipofectamine, they selectively transfected osteoblasts and fibroblasts versus hepatocytes. This indicated that the Ca-SiO₂-DNA complexes were selective toward cells of the connective tissue. Such selectivity would be extremely useful towards a clinical application for bone, which is comprised of both osteoblasts and fibroblasts. The selectivity of the Ca-SiO₂-DNA complexes for bone cells would be especially valuable for gene delivery to a fracture site.

![Figure 4.12](image)

Figure 4.12. Transfection efficiencies in osteoblasts, fibroblasts and hepatocytes with (□) lipofectamine-DNA, and Ca-SiO₂-DNA complexes formed with (□) 10-nm and (■) 50-nm Ca-SiO₂ particles. DNA loading: 24 µg/ml; vector loading: 0.15 wt%. Values are mean ± standard error of the mean; n = 3.

4.3.3.4. Cellular Proliferation after Transfection

Despite its significant transfection efficiency, lipofectamine’s toxicity would prevent its clinical application [25-26]. In contrast, the Ca-SiO₂ nanoparticles entered cells without exhibiting any toxicity [6-7]. In this section, the potential toxicity of Ca-SiO₂-DNA complexes was evaluated by conducting proliferation studies in conjunction with transfection
experiments. Figure 4.13 shows the effects of particle size and Ca loading on both cellular proliferation and transfection efficiency.

The data indicated that for a given particle size, the Ca loading required for optimal cell proliferation was lower than that required for optimal transfection. Optimal proliferation required a smaller Ca loading, which corresponded well with that required for optimal cellular uptake (see Fig. 4.11). As discussed earlier, Ca played the additional role of condensing DNA in nanoparticle-DNA complexes, so a higher Ca loading was needed for optimal transfection. We also note that greater cell proliferation was achieved with complexes formed with 50-nm Ca-SiO$_2$ particles than with 10-nm Ca-SiO$_2$ particles. This was because for the same nominal Ca loading, the sizes of Ca-SiO$_2$-DNA complexes formed with 10-nm and 50-nm Ca-SiO$_2$ particles were similar (see Fig. 4.4). This indicated that in addition to uptake of the complexes, it was likely that a greater number of free 10-nm Ca-SiO$_2$ particles (that were not bound to DNA) were ingested relative to free 50-nm Ca-SiO$_2$ particles. This increased ingestion of free 10-nm particles as opposed to free 50-nm particles might have effectively resulted in a higher intracellular rise in Ca$^{2+}$ concentration for the former. This rise in Ca$^{2+}$ concentration for the cells that were transfected with complexes formed with 10-nm Ca-SiO$_2$ particles might have been above the threshold of optimal Ca loading required to stimulate cell proliferation.

Figure 4.13. Effect of Ca loading on the cellular proliferation (closed symbols) and the transfection efficiency (open symbols) of osteoblasts transfected with Ca-SiO$_2$-DNA complexes formed with (■) 10-nm and (●) 50-nm Ca-SiO$_2$ particles.
4.3.3.5. Total GFP Expression due to Transfection

While transfection efficiency is a very useful parameter for evaluating gene delivery systems, the most therapeutically relevant parameter for our application is the amount of protein produced due to transfection. Transfection efficiency is given by \((\text{the number of transfected cells})/(\text{the number of viable cells})\). A transfected cell could be transfected with one or more copies of a plasmid, and as a result, each transfected cell could produce a different amount of the expressed protein [27]. In the case of our model system, the protein of interest was GFP. In a system of interest to fracture repair, the protein of relevance would be a growth factor such as bone morphogenetic protein (BMP).

![Figure 4.14](image)

Figure 4.14. Effect of Ca loading on the GFP expression of osteoblasts transfected with Ca-SiO\(_2\)-DNA complexes formed with (□)10-nm and (■) 50-nm particles. GFP expression of osteoblasts transfected with lipofectamine-DNA is indicated by (□).

The total GFP expression was calculated by FACS as the total shift in the mean fluorescence distribution of the transfected cell population compared to the control cell population with no DNA. Figure 4.14 indicated that the combined effect of transfection efficiency and cellular proliferation for some Ca-SiO\(_2\)-DNA complexes contributed to a total GFP expression that approached that of cells transfected with lipofectamine. The fact that certain Ca-SiO\(_2\)-DNA complexes could cause osteoblasts to produce such high levels of GFP...
without any toxicity \textit{in vitro} suggested that they would be extremely useful as \textit{in vivo} gene delivery systems.

4.4. Summary

We have used our previous understanding of cell-nanoparticle interactions to develop a gene delivery system based on Ca-SiO\textsubscript{2}-DNA complexes. A model system was constructed using plasmid DNA encoding for GFP. Ca-SiO\textsubscript{2} parameters such as particle size, Ca loading and surface charge were examined for their effects on DNA binding affinity and complex size. Specifically, 10-nm and 50-nm Ca-SiO\textsubscript{2} particles led to the successful transfection of osteoblasts without any toxicity. The resulting Ca-SiO\textsubscript{2}-DNA complexes were able to transfect connective tissue cells selectively versus hepatocytes, unlike lipofectamine-DNA complexes. The optimal Ca-SiO\textsubscript{2}-DNA complexes induced GFP expression levels comparable to that of lipofectamine-DNA complexes. The ability of Ca-SiO\textsubscript{2} nanoparticles to transfect osteoblasts efficiently and selectively without any cytotoxicity effects makes a very compelling case for their use as gene delivery vehicles for bone regeneration.
4.5. References


Chapter 5 – Recommendations for Future Work

In this thesis, organosilicate nanoparticles were developed as gene delivery vehicles for bone cells. Fundamental cell-nanoparticle interactions were studied in vitro to help determine which material properties had the most significant effects on cellular behavior. Specifically, the effects of particle size and composition on osteoblast uptake, proliferation and protein expression were investigated. While the cell culture experiments performed on three different cell types yielded useful information, future research should use these in vitro results to formulate various in vivo studies.

The cellular proliferation and protein expression studies indicated that the nanoparticles were not toxic to osteoblasts, allowed the cells to retain their osteoblastic phenotype, and did not induce any significant inflammatory response. To confirm these results in vivo, the nanoparticles should be administered to an animal bone defect site, and histological and biochemical characterization should be performed. The increased osteoblast proliferation and the phenotype retention that were evident in vitro would presumably lead to increased bone formation and increased production of bone phenotypic markers in vivo. Also, the retention of healthy cell morphology and the absence of macrophage cells at the defect site would suggest minimal inflammatory response.

The in vitro results also indicated that osteoblast uptake was maximized with Ca-SiO$_2$ nanoparticles of ≤ 100 nm. For in vivo uptake studies, the histology sections should be visualized for any nanoparticle ingestion by cells to see if the trends that were evident in cell culture would correspond to an animal model. While in vitro uptake was quantified precisely through a fluorescence-based assay, in vivo uptake would most likely have to be performed more qualitatively using analytical microscopy techniques.

The understanding gained by studying the effect of nanoparticle parameters on cellular behavior was used to design a model gene delivery system for bone cells. While the plasmid used in our model system encoded for green fluorescent protein (GFP), future research should use a plasmid that encodes for a bone morphogenetic protein (BMP) that would aid bone cell growth and differentiation. BMP-2 and BMP-7 have been shown to induce significant osteogenic activity, so a plasmid that encodes for one of these proteins would be ideal. The extension of our model system to such a plasmid should be fairly straightforward, since the portion of the plasmid that contains the gene of relevance is extremely small. Thus, it is very
likely that a plasmid that encodes for BMP-2 would interact with the nanoparticles similar to the GFP-encoding plasmid. *In vivo* studies should be performed on the Ca-SiO$_2$-DNA complexes by injecting them into a defect site and assaying for BMP production and bone formation. Histology should also be performed to assess the targeting ability of these complexes for cells of the connective tissue by visualizing nanoparticle uptake through analytical microscopy techniques.

The *in vitro* results obtained in this thesis are very useful in that they quantified cell-material interactions in detail. While animal studies are often limited by sample size and number, this was not the case for the cell culture experiments. While the clinical potential of these nanoparticles can only be verified and assessed through *in vivo* studies, the extensive *in vitro* characterization performed in this thesis would set the framework and understanding for the design of an appropriate *in vivo* model.
Chapter 6 – Conclusions

We have designed bioactive organosilicate nanoparticles for gene delivery to bone cells. Monodisperse organosilicate nanoparticles were synthesized via surfactant-stabilized sol-gel processing. The composition of these nanoparticles was modified through the addition of various surface dopants, and particle size was controlled via sol pH and alkoxide concentration. Ca-SiO$_2$ nanoparticles of ≤ 100 nm were found to maximize osteoblast proliferation and cellular uptake.

The effects of particle size and composition on cellular protein expression were also characterized. Osteoblastic phenotype was retained in the presence of various organosilicate nanoparticles, and cytokine expression was found to be minimal. These results provided strong support for the further investigation of these nanoparticles as gene delivery vehicles for osteoblasts.

The nanoparticles were complexed with plasmid DNA encoding for green fluorescent protein (GFP). The effects of nanoparticle size, composition and surface charge on complex formation were studied. These findings were used to design a Ca-SiO$_2$-DNA gene delivery system that transfected osteoblasts and fibroblasts selectively versus hepatocytes. The ability of these nanoparticles to transfec osteoblasts selectively without inducing any *in vitro* toxicity makes them very attractive for gene delivery applications aimed at bone regeneration.