**Nanostructured Silicate Substituted Calcium Phosphate (NanoSiCaPs) Nanoparticles –
Efficient Calcium Phosphate based Non-Viral Gene Delivery Systems**

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Abstract

Nano-structured ceramic particles, particularly, nano-particles of calcium phosphate (CaP) remain an attractive option among the various types of non-viral gene delivery vectors studied because of their safety, biocompatibility, biodegradability, and ease of handling as well as their adsorptive capacity for DNA. We have accordingly developed an enhanced version of nano-structured calcium phosphates (NanoCaPs), by substituting known amounts of silicate for phosphate in the hydroxyapatite (HA) lattice (NanoSiCaPs). Results indicate that in addition to the excellent transfection levels exhibited by un-substituted NanoCaPs alone *in vitro*, an additional 20-50% increase in transfection is observed for NanoCaPs containing 8.3-50 mol% silicate aptly called NanoSiCaPs, owing to its rapid dissolution properties enabling nanoparticles escaping the lysosomal degradation. However, high silicate substitution (> 50 mol%) resulted in a drastic decline in transfection as the synthesized NanoCaPs deviated far from the characteristic hydroxyapatite phase formed as evidenced by the materials characterization results.

Keywords: Calcium phosphate (CaP), Nanoparticles, Gene delivery, Plasmid DNA, Hydroxyapatite (HA)

1. Introduction

Gene therapy holds significant promise for treating a variety of diseases from genetic disorders, infections arising from cancer and can also be considered to hold tremendous potential for augmenting scaffold functions in the field of regenerative medicine. Although non-viral vectors currently are generally accepted to be less efficient in terms of transfection compared to viral vectors, they have nevertheless emerged as promising and safer alternatives to viral systems due to the following reasons: capability of high reproducibility, low cost, amenable for large scale production and potential non-immunogenicity. Among all the various non-viral gene delivery systems currently under investigation [1-12], calcium phosphate (CaP) mediated gene transfection is considered to be an attractive option for *in-vitro* transfection of a wide variety of mammalian cells with little or no toxicity [13]. Although CaP based systems have been thoroughly studied due to the excellent biocompatibility and bioresorbability, they are still not accepted as the ideal choice for gene delivery because of the known difficulties associated with endosomal escape, and the partial protection of DNA from nuclease degradation that automatically results in lower levels of gene expression [14].

The inherent propensity of CaP particles to aggregate rapidly with time in dispersing media is also another determining factor for achieving efficient gene delivery [15]. Rejman et al. have reported that particles <100 nm are readily endocytosed by cells[16]. The expected uncontrolled growth and aggregation of CaP particles however, expectedly results in lower levels of gene expression. There are nevertheless, reports on stabilizing the growth of CaP particles by coating with lipids[17], as well as adsorption of chitosan on growing CaP aggregates to prevent particle aggregation while providing overall stability to the gene delivery vector[18]. There are also reports in the literature describing cationic substitution of magnesium and strontium for calcium and

anionic substitution of carbonate and fluoride on the hydroxyl or phosphate sites in the calcium phosphate (CaP) based delivery systems contributing to enhanced transfection efficiency[19-24]. This increase in transfection efficiency due to the substitution with cations or anions is attributed to the possible reduction in particle aggregation and faster dissolution of the endocytosed particles. This motivation for ionic substitution of CaPs further arises from the fact that the natural bone mineral is composed of not only calcium and phosphate moieties, but also carbonate, sodium, magnesium, potassium, zinc, barium, copper, aluminum, iron, fluoride, chloride, and silicon ions[25, 26]. Recent work in the literature has also shown that incorporation of silicon into hydroxyapatite (HA) results in changes to the properties of HA such as the lattice parameter, morphology, crystallinity, surface chemistry, kinetics of dissolution, etc. [26-29]. This therefore sets the stage for an ideal opportunity to explore silicon substituted CaPs as a potential gene delivery vector and explore further whether these properties of modified HA would prove beneficial particularly, for non-viral gene delivery.

There are numerous extracellular and intracellular barriers to a gene delivery vector before the gene is successfully transcribed into a functional protein. One of the most crucial intracellular barrier for CaP based vectors is escape from endosomes and release of the functional plasmid DNA in the cytosol[30]. Upon entry into the cells, the nanoparticles undergo acid-triggered dissolution (pH 6.0-6.5, early endosomal pH) that results in a massive proton influx creating an osmotic pressure across the endosomal membrane, followed by its rupture and finally release of plasmid DNA from the vesicles in the cytosol for nuclear translocation[14, 30]. DNA that escapes endosomes at an early stage, escaping lysosomal degradation, successfully make their way to the nucleus through the nuclear pore complex or get distributed during cell division. Since escaping endosomes at an early stage is crucial for the CaP based vectors, putatively, this makes it even

more pertinent to fine tune the dissolution characteristics of nanostructured CaPs (NanoCaPs) by incorporation of various cations and anions, as reported in the literature[20, 21, 23, 24, 31]. Despite the fact that silicon substituted HA is considered to be a bioactive bone substitute due to its enhanced solubility [27, 28], no attempt to date, to the best of our knowledge, has been made to evaluate the potential of silicon substituted CaPs (NanoSiCaPs) as a gene delivery vector.

In a previous study, our group has already reported a novel simple synthesis approach that generates nanocrystalline HA particles (NanoCaPs) approximately 50-100 nm in size under physiological conditions that were efficient at both binding and condensing the pDNA[32]. Furthermore, the results of transfection indicate their superior response compared to other commercially available non-viral transfection agents[33]. The current work aims to demonstrate further improvement resulting in dramatic enhancement of the transgene expression by the inclusion of a critical concentration of silicate species into the NanoCaPs system to generate silicate substituted NanoCaPs that is aptly termed as NanoSiCaPs. As alluded above, there are no studies to the best of our knowledge addressing the influence of silicon substitution on the gene transfection efficiency of the nanostructured CaPs.

The aim of the current work presented here is therefore to exploit the aforementioned properties of silicate substituted HA (SiHA) to improve the gene delivery prowess, by incorporating different levels of silicate anions into the nanostructured CaPs (NanoCaP) to accordingly form the NanoSiCaPs system. In order to distinguish between the calcium phosphate used in other applications, the use of CaPs as gene delivery vectors will henceforth be referred to as NanoCaPs while calcium phosphate in the bulk will be referred to as HA throughout the text to avoid any confusion. Different analytical techniques were further utilized to understand the role of silicate substitution on the structure, morphology, dissolution properties and phase change of the

substituted HA phase. The impact of silicate substitution on the transfection efficiency of NanoCaPs was further assessed using flow cytometry while also assessing the binding capability of Si-NanoCaPs (Nano-SiCaPs) compared to un-substituted NanoCaPs. Results of all these studies are presented and discussed in this manuscript.

2. Materials and methods

2.1 Materials

Calcium chloride ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), tri-sodium phosphate do-decahydrate ($\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$), sodium chloride (NaCl), Bis-Tris, and dextrose were purchased from Fisher Scientific (Pittsburgh, PA). Potassium chloride (KCl) was purchased from Sigma Aldrich (St. Louis, MO). Sodium hydroxide (NaOH) was purchased from Mallinckrodt Baker Inc. (Phillipsburg, NJ). Sodium metasilicate ($\text{Na}_2\text{SiO}_3 \cdot 5\text{H}_2\text{O}$) was purchased from Alfa Aesar (Ward Hill, MA). HEPES and ethidium bromide (EtBr) were obtained from EMD Chemicals (Gibbstown, NJ). Reporter plasmid, gWizTM GFP (Green Fluorescent Protein), were purchased from Aldevron LLC (Fargo, ND). All of the reagents were used as received without further modification or purification.

2.2 Sample preparation

2.2.1 Synthesis of as-prepared bulk HA and SiHA

The precursors used for synthesizing as-prepared HA were $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$, and NaOH , based on the following chemical reaction which has also been reported by us earlier[34]:

The molar ratio of Ca/P was fixed at 1.67, and correspondingly, stoichiometric amounts of OH were added to maintain the HA phase and composition. To summarize the synthesis, 5.8 g of

CaCl₂·2H₂O was dissolved in 100 ml of double-distilled water (ddH₂O) with a conductivity of 18.2 Ω/cm to form solution A. 9.12 g of Na₃PO₄·12H₂O and 0.36 g of NaOH were dissolved in another 100 ml of ddH₂O to form solution B. Solution A was then added into solution B drop by drop under constant stirring at room temperature. The solution mixture quickly turns turbid, and the formation of a white precipitate is instantly observed. The white precipitate was collected using a Beckman J2-MC centrifuge (Beckman Coulter Inc, Fullerton, CA) at 2000 rpm and the supernatant was accordingly discarded. The white precipitate was then re-suspended into ddH₂O to rinse away the NaCl salt, obtained as a byproduct. The washing and centrifugation steps were repeated at least 3 times to ensure the complete removal of NaCl. The white precipitate obtained was then air dried in an oven (Isotemp, Fisher Scientific, Pittsburgh, PA) at 70°C for 72 hours and ground to a fine powder before conducting any further materials characterization.

Silicate substituted HA, or SiHA referred henceforth, was also synthesized in a similar fashion. Various amounts of silicate (Si) were substituted into HA based on the molar ratio of Ca/ (P+Si) (SiHA). The precursors used for synthesizing SiHA were once again, CaCl₂·2H₂O, Na₃PO₄·12H₂O, NaOH, while Na₂SiO₃·5H₂O served as the Si precursor for generating SiHA. The ratios of Ca/ (P+Si) were fixed at 1.67, and correspondingly, stoichiometric amounts of OH⁻ were also added to maintain the HA composition. The proposed chemical reactions for SiHA is:

Where x = number of moles of substituted silicate and the percentage of molar substitution is determined by the site substitution of PO₄³⁻ (% mol substitution = $\frac{x}{6} * 100$). Note that all of the reactions followed for synthesizing SiHA only took into account the physical site substitution of

SiO_4^{4-} ions into any of the available PO_4^{3-} or OH^- sites, while any potential charge imbalances that may arise due to the substitutions of anions with the different ionic charges of trivalent or pentavalent P and tetravalent Si were disregarded throughout this study. Table 1 summarizes the amount (mol.) and weight (wt.) of Ca, P and Si precursors used for generating each of the SiHA powder prepared with x equal to 0, 0.5, 1, 2, 3, 4, or 5 hereafter designated HA, 8.3SiHA, 16.6SiHA, 33.3SiHA, 50SiHA, 66.6SiHA and 83.3SiHA, respectively. Herein, we follow the convention for representing the silicate substitution in mol %, and not on a weight percent (wt.%) basis.

Table 1. Precursors composition of calcium, phosphorus and silicon for synthesis of HA and SiHA.

Substitution	x= 0	x=0.5	x=1	x=2	x=3	x=4	x=5
% mol	0	8.3	16.6	33.3	50	66.6	83.3
% weight	0	1.4	2.8	5.8	8.7	11.6	14.6
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (mol)	0.4	0.4	0.4	0.4	0.4	0.4	0.4
$\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ (mol)	0.240	0.220	0.200	0.160	0.120	0.080	0.040
$\text{Na}_2\text{SiO}_3 \cdot 5\text{H}_2\text{O}$ (mol)	0	0.019	0.039	0.079	0.12	0.159	0.199
NaOH (mol)	0.09	0.09	0.09	0.09	0.09	0.09	0.09
Ca/(P+Si)	1.66	1.66	1.66	1.66	1.66	1.66	1.66
Ca/P	1.66	1.81	2	2.5	3.33	5	10

2.2.2 Synthesis of pDNA loaded NanoCaPs and Si-substituted NanoCaPs

The synthesis of the pDNA loaded NanoCaPs and Si- substituted NanoCaPs (NanoSiCaPs) is very much dependent on the Ca/P ratios of the corresponding Ca and P precursors. In order to maintain the nano-scale size range of the substituted and un-substituted calcium phosphate (CaP) particles,

the ratios of Ca/P and Ca/(P+Si) were set at 130 instead of the 1.67 ratio normally used for synthesizing HA in the bulk. The ratio of 130 was selected based on the Ca/P ratio that resulted in formation of nanostructured HA, while also displaying the most optimal transfection levels from our previously published report[32]. Herein, the reported nanostructured HA particles are designated as NanoCaPs-pDNA for un substituted samples and correspondingly, following the nomenclature mentioned above, NanoSiCaPs-pDNA for silicate substituted samples with different amounts of silicate substitution being 8.3, 16.6, 33.3, 50, 66.6 and 83.3 NanoSiCaPs (% mol) as shown in Table 1. The synthesis of un-substituted NanoCaPs-pDNA and NanoSiCaPs-pDNA complexes were adapted from the synthesis of HA and SiHA described above. Briefly, 15.53 μ l of 2M CaCl_2 and 6.35 μ l of plasmid DNA (1mg/ml, gWiz GFP) were diluted in 103.2 μ l of ddH₂O to form the solution A (125 μ l total). An equal volume (125 μ l) of solution B containing HEPES-buffered solution (280 mM NaCl, 10 mM KCl, 12 mM dextrose, and 50 mMHEPES) and 1.5 mM Na_3PO_4 was also prepared. The HEPES-buffered solution was intended to maintain the pH of the precursors at 7.5. The precursors of NanoSiCaPs contained the same solution A, and the solution B used for NanoSiCaPs also contained the HEPES-buffered solution and a mixture of 1.5 mM of ($\text{Na}_3\text{PO}_4 + \text{Na}_2\text{SiO}_3$) for silicate, respectively with the appropriate adjustments needed for the addition of the silicate ion. The molar ratios of *Ca/P and Ca/(P+Si)* as mentioned above, was also maintained at 130 for all the samples synthesized. The un-substituted NanoCaPs-pDNA complexes and NanoSiCaPs-pDNA complexes were then synthesized using a Harvard Apparatus PHD 2000 Infuse/Withdraw syringe pump (Holliston, MA) to introduce and mix the two solutions in a controlled drop wise fashion to generate the desired homogeneous dispersion of the NanoCaPs and NanoSiCaPs in solution similar to our published reports. Hence, solution A was added drop by drop into solution B at an addition rate of 13.4 μ l/s while solution B was vortexed at 500 rpm. The

addition rate was also selected following our previously published report [32]. Accordingly, the as-synthesized NanoCaPs-pDNA complexes and NanoSiCaPs-pDNA complexes in suspension were used for in-vitro transfection, particle size characterization and pDNA binding experiments.

2.3 Materials characterization

X-ray Diffraction (XRD) patterns for both the as-prepared bulk HA and SiHA powders (8.3, 16.6, 33.3, 50, 66.6 and 83.3SiHA) were collected using a Philips X'Pert PRO X-ray diffraction system (PAN Analytical, Westborough, MA) with $\text{CuK}\alpha$ radiation ($\lambda = 1.5418\text{\AA}$). The diffractometer was operated at 45 kV and 40 mA and scanned over the 2θ range of $20\text{--}60^\circ$. The step size was 0.03 and the time per step was 50.17 s for each X-ray run. The XRD patterns were generated using the Philips X'PertData Collector and analyzed using the Philips X'PertHighscore Plus software. All the XRD patterns were verified and compared to existing library of references from the Joint Committee on Powder Diffraction Standards (JCPDS).

Fourier-transform Infrared Spectroscopy (FTIR) was used to identify the various functional groups present in the as-prepared HA and different SiHA powders. FTIR spectra of the as-prepared HA and silicate substituted SiHA samples were collected using the Nicolet 6700 spectrophotometer (Thermo Electron Corporation) using a diamond ATR (Smart Orbit) Scanning electron microscopy (SEM) was used to observe the morphology of both, the as-prepared HA and SiHA samples. Powdered samples of as-prepared HA and 8.3SiHA, 50SiHA and 83.3SiHA were accordingly mounted onto double-sided carbon tapes and sputter coated with palladium using a Cressington sputter coater 108A (Cressington Scientific Instruments, Ltd, Watford, UK). The SEM images were taken on a Philips XL30 field emission gun SEM (Philips-XL30 FEG, Philips) and Energy Dispersive X-ray Analysis (EDAX) results were obtained using the built-in EDAX system (EDX

Genesis, EDAX Inc.). To measure the dissolution properties of HA and SiHA samples, ion release test was performed in Bis-Tris buffer at pH 7.2, pH 6.5 and pH 5.8, respectively. Bulk synthesized samples were used for the dissolution study, wherein 100mg of as-prepared HA, 8.3 SiHA, 50 SiHA and 83.3 SiHA was dispersed in 150ml of buffer at different pH and incubated at 37°C for 24 hours. 10ml of the Bis-Tris Buffer was taken for elemental analysis to be used for estimating the calcium ion concentration (n=3). Immediately after taking each aliquot, the samples were centrifuged at 7000rpm for 20 minutes to ensure separation of any residual solids. After centrifugation, the supernatant was carefully aspirated and stored at 4°C until elemental analysis was conducted. Spectroscopic elemental analysis were performed through inductively coupled plasma optical emission spectroscopy (ICP-OES, iCAP duo 6500 Thermo Fisher). The calcium ion concentrations were correspondingly measured for different samples using 0.2, 0.5, 1, 5, 10, 50 and 100ppm standards.

2.4 In vitro transfection studies in mammalian cell line

All cells were cultured in 75 cm² flasks in a humidified incubator at 37°C in 5% CO₂. NIH3T3 cells were cultured with DMEM supplemented with 10% FBS and 1% P/S; and MDA-MB-231 cells were cultured with EMEM supplemented with 10% FBS, 1% sodium pyruvate and 1% P/S. Cells were maintained to a sub-confluence state and were passaged at least twice a week. For the transient transfection experiments, the cells were seeded in 12-well plates at cell-density of about 1.0×10^5 cells per well. The gWiz GFP was complexed with either NanoCaPs or silicate substituted NanoCaPs, i.e. NanoSiCaPs as described above in Section 2.2, and immediately transferred drop by drop into each well (n=3). The volume of the NanoCaPs-pDNA complexes or NanoSiCaPs-pDNA complexes added into each well was maintained at 81.5µl. The amount of total pDNA complexed with each sample was 6.35µg, equivalent to 2.11µg per well. The NanoCaPs were

incubated with the cells for 24 hours after which transfection efficiency was measured by counting the GFP positive cells using the Accuri C6 Flow Cytometer.

2.5 Flow cytometry of GFP transfected cells

Cells were allowed to incubate with NanoCaPs and NanoSiCaPs for 24 hours after which they were extracted from the well plates using 0.5% trypsin-EDTA solution. The cells were re-suspended in 400 μ l PBS (pH of 7.4) and transferred to 15 mm tubes for flow cytometry. On the Accuri C6 (Accuri Cytometers, Ann Arbor, MI) flow cytometer, the FLA-1 filter set corresponds to the GFP wavelengths. The cells were selected using a forward v/s side scatter plot from a sample of GFP negative cells serving as the control by using accordingly, cells transfected with void NanoCaPs (without plasmid DNA). This also gave us a filter for correcting the auto fluorescence. After filtering out the auto fluorescence, the ratio of GFP-positive to total number of cells was then obtained for each well (total of 5000 cells were counted for each sample). This ratio was subsequently used as a measure for calculating the transfection efficiency for the respective transfection agent.

2.6 Plasmid DNA binding assay

The extent of pDNA binding for particles synthesized was determined via Nanodrop 2000 spectrophotometer (Thermo Fisher). 250 μ l solutions of un-substituted NanoCaPs and NanoSiCaPs, loaded with pDNA as well as void, were prepared for each sample (n=3). Once synthesized, all the solutions were then centrifuged at 4°C for 10 minutes at 12,000g using a Mikro 200R ultracentrifuge (Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany). Following centrifugation, the amount of unbound, non-precipitated DNA remaining in the supernatant of each solution was then quantified by measuring the optical density (OD) at 260 nm using the NanoCaPs

without pDNA as a blank. It was also verified that none of the supernatants absorbed at 320nm, since this would indicate that either not all of the precipitate was removed by centrifugation or that further precipitation occurred after centrifugation. The binding efficiencies were then determined using the following equation:

$$E\% = ([pDNA]_o - [pDNA]_f) / [pDNA]_o \times 100 \quad (3)$$

Where $[pDNA]_o$ represented the concentration of pDNA added to the reaction mixture and $[pDNA]_f$ represented the concentration of the non-precipitated pDNA remaining in the supernatant.

2.7 Transmission electron microscopy (TEM) of the synthesized NanoCaPs-pDNA and NanoSiCaPs-pDNA complexes

The NanoCaPs-pDNA complexes were prepared according to the aforementioned synthesis protocol (Section 2.2). One drop, approximately 5 μ l, of un-substituted NanoCaPs-pDNA, 8.3, 50 and 83.3 NanoSiCaPs-pDNA was then immediately added onto a carbon coated copper grid (Electron Microscopy Sciences, Hatfield, PA) and allowed to equilibrate for 3 min. The grids were then stained with 2% ACS grade uranyl acetate (Sigma Aldrich, St. Louis, MO), rinsed in 95% ethanol and allowed to air dry. Images were subsequently taken using a JEOL 1011 transmission electron microscope (TEM) (Tokyo, Japan) set to an accelerating voltage of 200 kV.

2.8 Statistical analysis

Statistical calculations were performed using the IBM SPSS Statistics 20 statistical analysis program. The data was analyzed to test for significant ($p < 0.05$) mean differences on dissolution properties, pDNA binding values and in vitro transfection efficiencies for both NIH3T3 and MDA-MB-231 cells across the various substituted nanoparticles using the one-way analyses of variance

(ANOVA). Post hoc tests for pair-wise differences and identification of homogeneous subgroups were also all performed using the Tukey HSD procedures and discussed in the results section for the relevant groups.

3. Results

3.1. Materials Characterization

Fig. 1. XRD patterns of standard HA, as-prepared HA and 8.3SiHA. Peaks were indexed based on the JCPDS #01-074-0565.

Fig. 2. XRD patterns of as-prepared HA and SiHA with different silicon concentrations. XRD peaks of 16.6SiHA and 83.3SiHA were indexed based on JCPDS # 01-74-0565 and JCPDS # 00-029-0331, respectively.

XRD analysis of samples prepared in the bulk was performed to identify the crystalline phases present and also to verify whether the substituted silicate species were incorporated into the HA lattice structure preserving the single phase HA structure or phase separate out as a distinct and detached phase. The XRD patterns of HA, 8.3SiHA and 16.6SiHA contained peaks that matched

with the standard diffraction pattern of HA (JCPDS 01-074-0565), with major peaks centered at $2\theta = 26.0^\circ$ (hkl = 002 plane), 32.1° (112), 33.1° (300), and 34° (202) (Fig. 1). XRD pattern of the as-prepared HA and the different silicate containing HA are shown in Fig. 2. No other phase was detected for all these samples although samples exhibit broad diffraction lines, which indicates low crystallinity and nano-crystalline state of the synthesized materials. With further increase in substitution to 33.3mol%, the XRD pattern becomes completely amorphous with no characteristics peaks of HA. The pattern for 50 SiHA, 66.6SiHA and 83.3SiHA however, in addition to the semi-amorphous phase, displayed characteristics of calcium silicate hydrate phases (see supplementary figure). Although the crystalline XRD peaks for 66.6SiHA and 83.3SiHA matches with the 1.4-nm crystallite size of the tobermorite phase ($\text{Ca}_5\text{Si}_6\text{O}_{16}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$) of calcium silicate hydrate[35], no further attempts were however, made in this study to characterize the compositions and structures of the tobermorite and the amorphous phases. The XRD patterns thus indicate that for the amount of silicon, Si mol. % < 33.3 (or $0 < x < 1$), the crystalline phase of the precipitates is HA and silicon can be incorporated into the apatite structure or amorphous phase or in both likely in the form of silicate species.

Fig. 3. FTIR spectra of standard HA, as-prepared HA and 8.3SiHA.

cm^{-1} and 599 cm^{-1} , $\nu_3(\text{PO}_4)$ bands at 1017 cm^{-1} and 1089 cm^{-1} , $\nu_1(\text{PO}_4)$ band at 962 cm^{-1} , OH^- stretching band at 3571 cm^{-1} and OH^- bending band at 631 cm^{-1} [25, 34]. Other bands corresponding to HA were also observed, including bands for absorbed water at 1639 cm^{-1} and 3385 cm^{-1} , and ν_3 bands for carbonate CO_3^{2-} at 1421 cm^{-1} and 1459 cm^{-1} , and ν_2 bands for CO_3^{2-} at 875 cm^{-1} that could be attributed to the incorporation of atmospheric CO_2 into the sample.

The presence of both absorbed water and CO_3^{2-} has

FTIR was therefore performed to identify the molecular groups present in the samples, which in conjunction with XRD, can be used to identify the crystalline and amorphous phases present in the samples. Fig. 3 shows the FTIR spectra for pure HA, as-prepared HA and 8.3SiHA. The FTIR spectrum of the as-prepared HA and 8.3SiHA exhibited bands characteristic of pure HA, including $\nu_4(\text{PO}_4)$ bands at 559

Fig. 4. FTIR spectra of standard HA and SiHA with different silicon concentrations.

also been previously reported [28, 34, 36]. Few small additional bands not apparent in samples below 33.3 mol% substitution, were observed for 50SiHA, 66.6SiHA and 83.3SiHA (Fig. 4) at $\sim 980\text{ cm}^{-1}$ which are attributed to Si-O stretching vibrations and group of bands near 500 cm^{-1} that become apparent with increasing silicate concentration are an indication of calcium silicate hydrate phases[37], present as an amorphous phase as indicated by XRD results discussed above. Detection of these molecular vibrations via FTIR irrespective of the amorphous or crystalline state is a clear indicator that all of the silicates for low levels of substitution exhibited the HA phase.

SEM was then used to observe the morphology of the as-prepared HA and SiHA powders. Due to the semi-amorphous nature of the as-prepared samples, most of the samples appeared to comprise hard aggregates (Fig. 5) showing no preferred or characteristic features of facets or platelet formation typical of the apatitic structures. Hence, it was difficult to detect or distinguish any particular morphology of the particles. Visually, therefore there was also no secondary phase clearly observed in the SiHA samples.

EDAX spectra collected on the samples considered for SEM analysis were used to verify the

Fig. 5. SEM of as prepared HA and SiHA samples: a) as prepared HA, b) 8.3SiHA, c) 50SiHA and d) 83.3SiHA.

Figure 6 depicts the EDAX spectra for 8.3SiHA, 50SiHA and 83.3SiHA, respectively. In addition to Ca, P, O peaks, a silicon (Si) peak was also detected in each of these samples. As anticipated, the 8.3SiHA sample exhibited a larger P peak than Si peak, while the 83.3SiHA sample showed the opposite, reflecting the initial amounts of P and Si in the precursor. The Ca/P molar ratio (1.57) of the as prepared HA powder was lower than the expected value of 1.67 based on the concentration of the starting precursors. Moreover, the ratio of Ca/(P+Si) decreases with increase in silicate substitution most likely due the formation of structural defects, substitution of other cations and

elemental composition of the samples. The EDAX spectrum obtained for the as-prepared HA powders showed peaks belonging to calcium (Ca) and phosphorus (P) indicating presence of both elements, as well as small peaks for oxygen (O) (Fig. 6). The presence of Ca, P, and O confirmed that the sample contained calcium phosphate.

Fig. 6. EDAX spectra as prepared HA and SiHA samples: a) as prepared HA, b) 8.3SiHA, c) 50SiHA and d) 83.3SiHA.

anions, and the formation of secondary phases that have been observed in the XRD and FTIR discussed above.

Dissolution characteristics of the as-prepared samples were evaluated in order to understand the effect of silicate substitution on HA dissolution at different pH. Accordingly, the as-prepared HA and SiHA samples were dispersed in Bis-Tris buffer and aliquots were taken at 24 hour time points. ICP-OES was then used to determine the calcium ion concentrations in the buffers at pH 7.2, 6.5 and 5.8, respectively. ICP-OES measurements

Figure 7: ICP Dissolution of HA and SiHA in Bis Tris buffer showing released ion concentration of calcium. n=3 samples were used for each buffer condition (P<0.05). The data were reported as mean $\pm$ SD.

show that the silicate substituted samples released much higher levels of calcium than the as-prepared HA, and the dissolution profile of samples increased with higher amounts of silicate (Fig. 7). It should also be noted that the dissolution increases with decreasing pH, as all the samples released higher amounts of calcium ion in the increasing order of pH $5.8 > 6.5 > 7.2$ (P<0.05). This dissolution profile for the various silicate substitutions is clearly an important factor that will contribute to the *in vitro* transfection of these silicate substituted compositions that will be discussed in the following.

3.2. In vitro transfection results

The flow cytometry results of GFP transfected MDA-MB-231 and NIH3T3 cells by NanoCaPs-pDNA complexes and NanoSiCaPs complexes are shown in Fig. 8. GFP Fluorescent results at 24 hours post-transfection showed that both cell lines transfected with NanoCaPs, 8.3 NanoSiCaPs, and 16.6 NanoSiCaPs exhibited the highest number of green-fluorescing cells. On the other hand, presence of very few green fluoresced cells with 66.6 NanoSiCaPs and 83.3 NanoSiCaPs are observed indicating that very poor transfection is observed for these large silicate substitutions. For NIH3T3cells (Figure 8) in particular, the transfection levels displayed a volcano trend as the anion substitution increased from 0 mol% (un-substituted NanoCaPs) up to

The flow cytometry results of GFP transfected MDA-MB-231 and NIH3T3 cells by NanoCaPs-pDNA complexes and NanoSiCaPs complexes are shown in Fig. 8. GFP Fluorescent results at 24 hours post-transfection showed that both cell lines transfected with NanoCaPs, 8.3 NanoSiCaPs, and 16.6 NanoSiCaPs exhibited the highest number of green-fluorescing cells. On the other hand, presence of very few green fluoresced cells with 66.6 NanoSiCaPs and 83.3 NanoSiCaPs are observed indicating that very poor transfection is observed for these large silicate substitutions. For NIH3T3 cells (Figure 8) in particular, the transfection levels displayed a volcano trend as the anion substitution increased from 0 mol% (un-substituted NanoCaPs) up to 83.3 mol% (83.3

Fig. 8. Flow cytometry analysis (at 24hours) of GFP transfected MDA-MB-231 and NIH-3T3 cells by NanoCaPs-pDNA and NanoSiCaPs-pDNA complexes with different silicon concentrations. The control used for the flow analysis included GFP negative cells using cells transfected with void NanoCaPs (without pDNA). Transfection experiments were run in duplicates for each sample and repeated three times, $n=6$ ($P<0.05$). The data were reported as mean $\pm$ SD. Post hoc analysis of the transfection profile of NanoSiCaPs with NanoCaPs showed statistical significance ($P<0.05$), indicated by the asterisk (*).

NanoSiCaPs) with an optimum transfection observed for NanoCaPs corresponding to 8.3, 16.6, 33.3 and 50 Si (mol%) substitutions. The transfection results for both the cell lines showed a considerable increase in transfection levels for 8.3, 16.6 and 33.3 NanoSiCaPs compared to the un-substituted NanoCaPs (P value<0.05). For samples with 66.6 and 83.3 Si (mol%) substitutions, the transfection levels however, began to drop by nearly 10-fold, to the point that the transfection levels practically became insignificant with more than 50% silicate substitution indicating the strong influence of incorporation of silicate ions into the HA structure on pDNA transfection.

3.3. Plasmid DNA binding analysis

DNA loading efficiencies of NanoCaPs and NanoSiCaPs are shown in Fig. 9. 8.3 NanoSiCaPs were found to be capable of packaging 81% of total pDNA, similar to the control sample of un-substituted NanoCaPs (~82% of total pDNA), statistically not significant.

However, as the amount of silicate substitution increased, the corresponding loading efficiency was observed to decline. In fact, for 66.6 and 83.3 NanoSiCaPs, only as much

as 10-30% of pDNA was observed to be loaded onto the particles while most of the initial pDNA was observed to remain in the supernatant. This drastic decline in the pDNA loading efficiency in

Fig. 9. pDNA binding efficiency of NanoCaPs-pDNA and NanoSiCaPs-pDNA complexes with different silicon concentrations. Samples were analysed in duplicates and repeated three times, $n=6$ ($P<0.05$). The data were reported as mean $\pm$ SD. Post hoc analysis of pDNA binding efficiency of NanoCaPs, 8.3 NanoSiCaPs with 66.6 and 83.3 NanoSiCaPs were statistically significant ($P<0.05$), indicated by asterisk (*).

these samples therefore contributed to the poor transfection levels since the majority of the initial pDNA was unable to be bound to the NanoSiCaPs gene delivery carriers due to the likely presence of multiple phases as discussed above as well as the probable instability of these phases in the solid state causing the pDNA to remain largely in solution.

3.4. Electron microscopy Analysis of NanoCaPs-pDNA and NanoSiCaPs-pDNA complexes

TEM was used to qualitatively assess the particle size and morphology of the un-substituted NanoCaPs and NanoSiCaPs (8.3, 50 and 83.3 mol% Si substitutions). As shown in Fig. 10, un-substituted NanoCaPs and NanoSiCaPs appear to be spherical in nature. However, for the un-substituted NanoCaPs, all of the particles observed appear agglomerated and with the increase in substitution, the particles appear to be smaller and less agglomerated

Fig. 10. TEM images of nanoparticles: (a) un-substituted NanoCaPs, (b) 8.3 NanoSiCaPs, (c) 50 NanoSiCaPs and (d) 83.3 NanoSiCaPs.

demonstrating the influence of Si substitution. The particle size estimate for un-substituted NanoCaPs is in the range of 80-100nm where as for the NanoSiCaPs, the particle size is in the smaller size range of 50-100nm size.

4. Discussion

There are many potential implications for substituting metallic or non-metallic ionic species into HA, since the substitutions are likely to cause complex structural changes at the unit-cell level[38]

. Possible consequences of such ionic substitutions include changes in the physical and chemical properties of HA such as lattice parameter, particle morphology, crystallinity, thermal stability, surface chemistry, overall phase solubility, as well as kinetics of dissolution[24, 27, 31, 39]. Silicon has been reported to be incorporated as a substituent into HA, tri-calcium phosphate (TCP) [40] and bone cements [41], even though HA continues to remain the most popular choice for generating substituted apatites. It has also been demonstrated that the substitution of silicon into HA increases the *in vivo* bioactivity of HA by enhancing its dissolution and that the dissolution rate is enhanced by the presence of site deficiencies and crystallographic imperfections within the HA structure created by the different ionic substitutions[29, 42, 43]. The fact however, still remains that silicon substitution into HA creates a vast range of improvements over pure HA in the field of bioactive bone substitutes. This proven conclusion indicates that it is also possible to achieve similar effects by incorporating silicon into the already published and demonstrated efficacy of NanoCaPs as gene delivery vectors. Therefore, the aim of the current study was to investigate the critical concentration of silicate ions that could be incorporated into the HA lattice without inducing a phase change and consequently, evaluate the gene delivery potential of the silicate substituted HA which is aptly termed as NanoSiCaPs.

Unlike conventional methods involving hydrothermal treatments to force the incorporation of ionic substituents into the HA lattice at extreme temperatures, this study focused on a simple aqueous precipitation approach at room temperature, under ambient pressure and more importantly, physiological conditions. From the XRD and FTIR data it is clear that the as-prepared HA and SiHA are indeed semi-amorphous, and nanocrystalline in nature, but nevertheless, display the presence of the HA phase only at low concentrations of silicate. With increase in silicate substitution, the XRD patterns become completely amorphous for 33.3SiHA, and upon further

increase in the silicate concentration in addition to the semi amorphous phase, there is also the presence of a secondary phase of calcium silicate hydrate having a structure of 1.4 nm crystallite size phase of tobermorite (see supplementary).

Based on the published literatures the exact amount of silicate ion incorporation is not well established. However, some recent literature clearly suggest that low levels of silicon, in the range of $0 < \text{Si wt. \%} < 3.51$, substitution is possible into the HA lattice [27]. Although the synthesis conditions used in our study is very different from the work reported earlier, the agreement nevertheless is a validation of the solubility of low silicate concentrations into the HA lattice with varying starting precursors, and the formation of single phase HA structure with no evidence of any crystalline secondary phase.

In addition, FTIR data reveals that other than very slight wavenumber shifts in some of the band positions (Fig 3), the 8.3SiHA spectrum very closely matches that of the un-substituted HA. It is also evident that as the Si substitution increased beyond 33.3 mol %, the intensities of the PO_4 bands become less prominent, which correspond to decreasing phosphate and increasing silicate incorporation into the SiHA. In addition to the changes in the PO_4 bands intensities, the OH bands at $3571 \text{ (stretching) cm}^{-1}$ and 631 cm^{-1} (bending) also decreased in intensity for the 8.3SiHA sample and disappeared altogether for samples with 33.3 (mol%) Si substitution and beyond. This may be due to two possible phenomena occurring. First, increasing the Si substitution could lead to the OH^- groups being substituted by the SiO_4^{4-} , or second, loss of OH^- groups in order to maintain the overall charge balance[44, 45]. Moreover, the FTIR spectra of the as-dried samples present the characteristic pattern of partially carbonated hydrated hydroxyapatites, as extensively discussed elsewhere [46]. It is also clear that all of the silicates for low levels ($\leq 16.6 \text{ mol \%}$ or $\leq 2.8 \text{ wt. \%}$) of substitution exhibited the partially hydrated carbonated HA phase. The incorporation of

atmospheric CO₂ into the HA structure during the synthesis is also well known which results in the formation of carbonated substituted hydroxyapatite or dahllite[34]. According to literature, the presence of carbonate ion within the as-precipitated apatite phase is also known to inhibit the conversion of the amorphous precipitate to the crystalline phase [47, 48], thus being responsible for the low crystallinity of the as-precipitated samples as evidenced by the XRD results. The influence of time-temperature history on the as-prepared powders were however, not included in this study as the main focus was primarily on the synthesis of silicate-substituted nanoparticles for in-vitro gene transfection under ambient conditions.

Due to the semi amorphous nature of the as-prepared samples it was difficult to detect or distinguish any particular preferential morphology of the particles. As the particles were agglomerated, presence of any distinct secondary phase morphology for 50SiHA and 83.3SiHA was not unequivocally visible (Fig 5). However, EDAX spectra collected from the SEM images revealed the elemental composition of the samples (Fig. 6). As anticipated, the 8.3SiHA sample exhibited a larger P peak than Si peak, while the 83.3SiHA sample showed the opposite, corresponding to the initial nominal precursor amount of P and Si.

It should also be noted that the obtained ratio of Ca/P of 1.57 by EDAX in the as prepared HA is very similar to the value reported earlier by our group using ICP-OES[49]. The non-stoichiometry of the as prepared HA powder by simple precipitation is very common and is known to mostly depend on the influence of various process parameters[49, 50] and also due to the formation of defects such as vacancies in the crystal lattice[51, 52], and also the substitution of diverse ions such as carbonate, sodium and chloride in the lattice structure[53]. Therefore, the slight decrease in (Ca/P+Si) ratio for 8.3SiHA (Fig. 6b) is not surprising and most likely due the formation of vacancies or defects and other ion substitutions into the HA structure. However, a large decrease

in (Ca/P+Si) ratio for the 50SiHA and 83.3SiHA is most likely due the presence of a secondary phase in addition to the vacancies or substitution of by other ions in the HA structure. The XRD and FTIR results (Figs. 2 and 4) have confirmed the formation secondary phases of 1.4-nm crystallite sized tobermorite phase ($\text{Ca}_5\text{Si}_6\text{O}_{16}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$) and other amorphous calcium silicate hydrate phase. The Ca/Si ratio in tobermorite is 0.83 and therefore, it expected that the formation of tobermorite will reduce the overall (Ca/P+Si) ratio. This also implies that some part of the calcium salt remained unreacted in the solution and did not precipitate due to the lack of critical concentration of anions necessary for reaching the required solubility product to initiate the precipitation of the compound.

The in vitro gene delivery potential of NanoCaPs and NanoSiCaPs was evaluated using pDNA encoding GFP on a hard to transfect, NIH-3T3 fibroblast cell line and MDA-MB-231, a breast cancer cell line (Fig. 8). At higher levels of silicate substitution, the chemical composition of the nanoparticles appeared to have a major impact on the gene delivery results. As evidenced from the XRD and FTIR results, formation of different phases at high levels of silicate substitution and their varying ability to bind pDNA together could be attributed for the decline in the observed gene delivery transfection results for these samples. The drastic decline in DNA loading efficiency in these samples therefore resulted in the poor transfection levels since the majority of the initial DNA was unable to be bound to the NanoSiCaPs gene delivery carriers due to the likely presence of multiple phases with poor pDNA binding affinity as discussed above. Further, the differences in the negative charge caused by the incorporation of higher amounts of silicate could have led to increased repulsion between the silicate and the negatively-charged DNA, as well as the increase in the site defects and vacancies within the structure of NanoSiCaPs caused by higher silicate substitution. All of these aspects could contribute to the decreased capacity for the NanoSiCaPs of

higher silicate content to efficiently bind DNA. Due to these reasons, increasing the silicate substitutions to a large degree primarily contributed to causing a reduction in the observed transfection efficiency.

On the other hand, 8.3 NanoSiCaPs that maintained the HA structure indicated a two-fold increase in the transfection efficiency for NIH3T3 cells (Fig. 8). Previous studies have indicated that incorporation of silicate ions into the HA lattice would distort and destabilize the structure, therefore making it energetically favorable for substituted HA to dissolve faster than the as prepared HA phase [28]. This higher solubility and unstable characteristic of Si substituted HA could play a crucial role in the rapid dissolution of the endocytosed nanoparticles and thus facilitate escape of the lysosomal degradation, preserving the DNA in its pathway to the nucleus. The faster dissolution will lead to rapid release of ions in the endosomes and these ions will in turn increase the osmotic pressure thereby rupturing the endosome membrane enabling release of their contents in the cytosol.

In order to validate this reasoning, dissolution characteristics for other anion and cation substituted calcium phosphate gene delivery systems have been investigated[21, 24, 31]. Dissolution of the as prepared HA and SiHA samples in Bis-Tris buffer (Fig. 7) clearly showed that the silicate substituted samples underwent more dissolution as compared to HA. ICP data also clearly indicated that higher amounts of calcium ions are released from the SiHA samples in the buffer system having a physiological pH-7.2, early endosome pH-6.5 and late endosome pH-5.8 (Fig. 7). ICP analysis of 50SiHA and 83.3SiHA at pH-7.2 also shows that at higher concentrations of silicate, the prepared SiHA samples are unstable at physiological pH and undergo rapid dissolution, thereby further explaining the low transfection and pDNA binding capabilities of these samples. This result also clearly indicates that Si gets incorporated into the HA lattice at low levels

of substitution, which thereby influences the dissolution characteristics of the as prepared samples. The presence of Si in the as prepared samples is also extensively explained by Hayakawa et al, wherein the authors have shown that as prepared SiHA consists mainly of partially amorphous hydrated and carbonated calcium phosphate incorporating polymeric silicate specie[28]. It is indeed the presence of Si in the system that leads to accelerated degradation of apatite particles in line with our observations reported here. TEM analysis of the complexes also revealed that the particles tend to agglomerate less with silicate substitution (Fig. 10). However, the primary particle size of NanoCaPs-pDNA and NanoSiCaPs-pDNA seem to be in the same range of 50-100nm, although the NanoSiCaPs complexes consist of smaller aggregates. This trend was also observed at multiple locations on the TEM grid for NanoCaPs and 8.3 NanoSiCaPs. On the other hand, 50 and 83.3 NanoSiCaPs-pDNA complexes had nearly no aggregate formation to an extent that it was difficult to image any complexes for 83.3 NanoSiCaPs-pDNA. This can also be attributed to the poor stability of these samples under physiological pH as evidenced in the dissolution study (Fig. 7), which can explain the presence of fewer particles for these samples in the TEM analysis. These results are in accordance with previous reports describing cationic substitution of magnesium and anionic substitution of fluoride ions, wherein substitution of ions in trace amounts resulted in enhanced transfection owing to faster dissolution and reduced particle aggregation of CaP based vectors[20, 21]. However, the synthesis procedure, nature of pDNA and cells transfected in the previous reports differ from the current study. A general comparison can albeit be drawn, that small amounts of ionic substitution does have a positive influence on the transfection efficiency of CaP based vectors. Therefore, combination of rapid dissolution and smaller agglomerate formation of the NanoSiCaPs-pDNA complexes combined with the maintenance of the HA structure result in efficient pDNA binding and condensation. In summary, these aspects altogether thus render 8.3

mol% Si substituted NanoSiCaPs a superior gene delivery vector compared to pure and unsubstituted NanoCaPs.

5. Conclusions

In this study different silicate substituted nano-particulates of calcium phosphates were prepared and characterized using XRD, FTIR, SEM and TEM. Silicate substitution below a certain concentration ($x \leq 16.6$ mol %) retains the single phase of partially hydrated and carbonated hydroxyapatite structure. Upon increasing the silicate incorporation ($16.6 \text{ mol \%} \leq x \leq 33.3 \text{ mol \%}$), the nanoparticles of hydroxyapatite phase of calcium phosphate changes to an amorphous form and upon further increase in substitution ($x \geq 33.3 \text{ mol \%}$) leads to formation of nanoparticles containing hydrated calcium silicate phases. pDNA transfection results indicate that 20-50 % increase in transfection was observed for 8.3-50 mol % silicate containing samples compared to NanoCaPs. However, further silicate substitution ($>60 \text{ mol \%}$) resulted in considerable reduction in transfections. Instability of the silicate substituted NanoCaPs and the optimal concentration of Si responsible for maximal binding of pDNA result in the 8.3 mol% Si substituted NanoSiCaPs demonstrating optimal transfection. From these results it can thus be concluded that suitable concentration of substitution of phosphates by silicates in nanostructured calcium phosphates forming NanoSiCaPs thus significantly improve the *in-vitro* pDNA transfection suggesting NanoSiCaPs to be promising gene delivery systems.

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