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Nanostructured silicate substituted calcium phosphate (NanoSiCaPs) nanoparticles — Efficient calcium phosphate based non-viral gene delivery systems

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ABSTRACT

Nanostructured ceramic particles, particularly, nanoparticles 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 nanostructured 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.

1. Introduction

Gene therapy holds significant promise for treating a variety of diseases from genetic disorders to infections arising from cancer and holds tremendous potential for augmenting scaffold mediated gene delivery 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, increase in binding efficiency due to higher charge density of the substitute ion, 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,

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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 platform to explore silicon substituted CaPs as a potential gene delivery vector and investigate 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 ($CaCl_2 \cdot 2H_2O$), tri-sodium phosphate dodecahydrate ($Na_3PO_4 \cdot 12H_2O$), 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 ($Na_2SiO_3 \cdot 5H_2O$) was purchased from Alfa Aesar (Ward Hill, MA). HEPES and ethidium bromide (EtBr) were obtained from EMD Chemicals (Gibbstown, NJ). Reporter plasmid, gWiz™ 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 $CaCl_2 \cdot 2H_2O$, $Na_3PO_4 \cdot 12H_2O$, and NaOH, based on the following chemical reaction which has also been reported by us earlier [34]:

$$10CaCl_2 + 6Na_3PO_4 + 2NaOH \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 20NaCl. \quad (1)$$

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 $CaCl_2 \cdot 2H_2O$ was dissolved in 100 ml of double-distilled water (ddH_2O) with a conductivity of 18.2 Ω/cm to form solution A. 9.12 g of $Na_3PO_4 \cdot 12H_2O$ and 0.36 g of NaOH were dissolved in another 100 ml of ddH_2O 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 ddH_2O 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 h 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, $CaCl_2 \cdot 2H_2O$, $Na_3PO_4 \cdot 12H_2O$, NaOH, while $Na_2SiO_3 \cdot 5H_2O$ 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:

$$10CaCl_2 + (6-x)Na_3PO_4 + xNa_2SiO_3 + (2-x)NaOH + x/2O_2 \rightarrow Ca_{10}(PO_4)_{6-x}(SiO_4)_x(OH)_{2-x} + (20-2x)NaCl + xCl_2 \quad (2)$$

where x = number of moles of substituted silicate and the percentage of molar substitution is determined by the site substitution of PO_4^{3-} (% 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 as 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.