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Amorphous Silica, a New Antioxidant Role for Rapid Critical-Sized Bone Defect Healing

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Abstract

Traumatic fractures cause structurally unstable sites due to severe bone loss. Such fractures generate a high yield of reactive oxygen species (ROS) that can lead to oxidative stress. Excessive and prolonged ROS activity impedes osteoblast differentiation, and instigates long healing times. Stimulation of antioxidants such as superoxide dismutase (SOD1), are crucial to reduce ROS, stimulate osteogenesis, and strengthen collagen and mineral formation. Yet, no current fixative devices have shown an ability to enhance collagen matrix formation through antioxidant expression. Here, we report plasma-enhanced chemical vapor deposition (PECVD)-based amorphous silicon oxynitride (Si(ON)_x) as a potential new fracture healing biomaterial that adheres well to the implant surface, releases Si⁺⁴ to enhance osteogenesis, and forms a surface hydroxyapatite for collagen mineral attachment. These materials provide a sustained release of Si⁺⁴ in physiological environment for extended times. The dissolution rate partially depends on the film chemistry and can be controlled by varying O/N ratio. The presence of Si⁺⁴ enhances SOD1, which stimulates other osteogenic markers downstream and leads to rapid mineral formation. *In-vivo* testing using a rat critical-sized calvarial defect model shows a more rapid bone-regeneration for these biomaterials as compared to control groups, that implies the clinical significance of the presented biomaterial.

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Keywords

Superoxide dismutase; bone formation; antioxidant; blood vessel formation; PECVD

1. Introduction

Bone fracture incidence from trauma, age-related fragility or disorders is estimated to rise 400% by 2050 as life expectancy increases. This contributes to ~\$8 billion in morbidity/mortality costs and ~800,000 procedures annually.^[1–3] Upon fracture, the site is structurally unstable and hypoxic due to severe bone loss and ischemia. Inflammation ensues after site re-vascularization and oxygen metabolites accumulate as reactive oxygen species (ROS: O_2^- , H_2O_2). During normal healing, osteoblasts express antioxidant enzymes (e.g., superoxide dismutase [SOD1]) to convert ROS into oxygen and water and induce osteoblast differentiation. This promotes collagen synthesis, cross-linking, strength, and osteogenic marker expression [osterix (OSX), osteocalcin (OCN), collagen (COL(I)), and lysyl oxidase (LOX)] for bone formation.

However, in the case of substantial bone defects, structural instability persists and the high ROS activity leads to oxidative stress, which inhibits osteogenesis and lowers bone regenerative capacity.^[4] Cells express antioxidant markers, including endogenous genes and exogenous enzymes that eradicate ROS in order to protect themselves against DNA damage.^[5] An imbalance between excessive ROS generation and insufficient antioxidant defense mechanism makes these fractures difficult to heal.^[6,7] ROS reduction requires enhanced enzymatic antioxidant production (e.g., SOD1, glutathione reductase, catalase).^[8,9] SOD1 is also important because its deficiency reduces LOX-induced collagen cross-linking, and bone strength by 25%.^[10] Patients suffering with such fractures given dietary antioxidants (Vitamin C/E) had higher SOD1 activity (1.5–3-fold), which down-regulated ROS (3-fold), increased OCN (3-fold),^[6,11] and showed the potential to cut health care costs by reducing hospital stays by 2 days.^[12] Thus, antioxidants for enhanced expression of SOD1 are critical to pursue normal bone healing in such fractures.

Despite their benefits, dietary antioxidants cannot heal fractures on their own and require structural support for bony union.^[13] Metal implants (e.g., titanium [Ti]) are used as structural materials because of their strength, however, they have long healing times, do not resorb, and have not shown any antioxidant effect.^[14] Previous attempts to coat the metal implants with bioglass show that ionic products like Si^{+4} from the dissolution of bioglass enhance osteogenic markers expression and provide high production of collagen by osteoblast cells.^[15,16] Similarly, strontium-doped bioactive glass coatings onto metal implants showed an improved anti-oxidative defense against ROS^[4] yet, bioactive glass coatings lack interfacial adhesion and face delamination issues due to thermal expansion mismatch.^[17] Bioglass with added MgO for improved thermal expansion matching, have shown a drawback to down-regulate important osteogenic markers associated with bone formation due to Mg^{2+} release.^[18] Thus, there is a great clinical need for novel biomedical device materials/designs that provide structural and antioxidant support to accelerate bone healing.

Alternatively, amorphous silica (SiOx) integration onto metal implants can provide structural support along with enhanced osteogenesis by inducing rapid hydroxyapatite (HA) formation needed for bone attachment. Moreover, SiOx network releases Si⁺⁴ that stimulates biomineral synthesis, thus, eliminates the need for drugs. Yet, sustaining Si⁺⁴ release, hastening surface HA formation, and understanding its osteoinductive role are needed to form SiOx-modified devices that support and improve fracture healing. In this study, we proposed PECVD-based nitrogen-incorporated amorphous silica (Si(ON)x) overlays, to modify biomedical devices for rapid fracture healing. We hypothesized that these devices would provide sustained Si⁺⁴ release to stimulate SOD1 expression and enhance osteogenesis while rapid formation of carbonated HA improves osseointegration and helps rapid fracture healing. Bio-inspired surface features at micro/nanometer scales are known to influence cellular behaviors such as cell adhesion, growth, migration and differentiation. [19–23] Micrograting structures (regular ridge and groove pattern) have gained maximum interest due to its simplicity.^[19] Moderately rough surfaces (1–2 µm) exhibit stronger bone responses than smoother or rougher surfaces.^[22] Recently marketed oral implants are moderately rough and allow bone ingrowth into minor surface irregularities, promoting osseointegration.^[22] Therefore, in this study, we will integrate micrograting patterns to make the surface moderately rough for enhanced cellular responses.

Thus, our central hypothesis was that Si(ON)x-modified devices would provide dual antioxidant and structural support for rapid bone regeneration and fracture healing. In order to study the antioxidant role of ionic Si, two treatment conditions (control, ionic Si) were prepared and tested with MC3T3 cell. Wild type and SOD1 knockdown cells (using siRNA) were tested with each treatment. Amorphous silica-based biomaterials were fabricated to develop an ionic Si delivery device. The amorphous silicon oxynitride Si(ON)x thin films were overlaid onto sample surfaces using plasma enhanced chemical vapor deposition (PECVD) process while 3-dimensional surface nano-features were formed by lithography and etching processes as described in our previous work.^[24] The samples were tested *in-vitro* to study cell-surface interactions and *in-vivo* evaluation of the optimized Si(ON)x chemistry was performed using a rat critical size calvarial defect model. Studies show that the implants are typically harvested 4 to 6 weeks postoperatively to analyze the bone healing effect.^[25–27] For that reason, we chose 5 weeks to look at the defect healing over the course of healing period. The results of this study demonstrated that Si(ON)x-modified devices provided sustained Si⁺⁴ release to stimulate osteogenesis and accelerated biomineralization via rapid bioactive HA formation. The ionic Si up-regulated SOD1 and several other osteogenic markers for wild type cells whereas SOD1 knockdown inhibits Si⁺⁴ enhancement of osteoblast differentiation. *In-vivo* testing of the optimized Si(ON)x chemistry showed rapid bone-regeneration in a rat critical size calvarial defect model whereas such a speedy bone healing was not observed in the absence of ionic Si.

2. RESULTS

Results of testing the effect of ionic Si on osteogenic differentiation are shown in Figure 1. Ionic Si was observed to enhance superoxide dismutase SOD1 (more than 3-fold) and several genes associated with osteogenesis including osterix (more than 2-fold), lysyl oxidase (more than 2.5-fold) and collagen type 1 (nearly 2.5-fold) for the wild type MC3T3 cells.

SOD1 knockdown cells were prepared by siRNA transfection over 6 days. Cell viability test verified that the transfecting vehicle did not impact cell viability, also evidenced by control siRNA and control treatments having similar effect on ECM formation (data not shown). As compared to wild type control treated cells, SOD1 knockdown cells (45% knockdown) were observed to decrease SOD1, osteonectin, lysyl oxidase and collagen type 1 expression and the presence of Si^{+4} didn't show any improvement in expression of these osteogenic markers for SOD1 knockdown cells. Results from optical microscopy of ECM samples (Figure 1B) also showed an absence of elongated collagen fiber matrices after 6 days for SOD1 knockdown cells. Whereas, the presence of ionic Si was observed to enhance the density of elongated collagen fibers in resultant extracellular matrices for wild type cells, evidenced by Picrosirius red stained tissue with yellow-orange elongated collagen fibers indicated by yellow dotted circles. Quantitative analysis confirms one order of magnitude higher collagen formation for wild type cells in the presence of ionic silicon (Figure 1C). Therefore, these results show that ionic Si enhanced osteogenic expression and elongated collagen matrix formation during osteoblast differentiation that was observed for wild type cells but not for SOD1 knockdown cells. It shows that the enhanced osteogenic gene expression and collagen matrix formation by ionic Si depends on SOD1 enhancement.

For ionic Si to be useful in applications of bone healing, delivery devices must be fabricated that can sustain the release of Si^{+4} to attached cells. Figure 2a shows a schematic diagram to illustrate the cross section view of the device fabrication process whereas Figure 2b shows the SEM micrograph for the actual device after fabrication. Plasma-enhanced chemical vapor deposition (PECVD) gave highly conformal, adherent and uniform thin film overlays onto ridge and groove patterned (microgratings) surfaces.^[24] PECVD-based amorphous silicon oxynitride ($\text{Si}(\text{ON})_x$) surfaces showed a sustained release of ionic silicon (Si^{+4}) when immersed in physiological environment. Figure 3a shows the ICP-OES data to demonstrate the release of Si^{+4} from $12 \text{ mm} \times 12 \text{ mm}$ sample sections immersed in 4 mL of α MEM and incubated for 0, 6, 12, 24, 48, 96, and 192 hours. According to manufacturer's specifications, α MEM contained little to no silicon ($<0.01 \text{ ppm}$). Initially in the soaking experiment, there was little silicon in solution, however, after 12 hours sample 1 ($n=1.80$) showed some evidence of dissolution. For the other $\text{Si}(\text{ON})_x$ chemistries studied, Si release was observed after 24 hours of dissolution. The data showed a sustained release of Si^{+4} over a period of 8 days and the degradation kinetics depending on the surface chemistry (incorporation of N). Comparison of surface topographies before and after *in-vitro* immersion (Figure 3b, 3c) exhibited the formation of hydroxyapatite (HA) on the surface, facilitated by the hydroxyl groups readily available on the surface by PECVD process as explained in our previous work.^[24] Table I lists coating thickness dissolved for each of the four $\text{Si}(\text{ON})_x$ sample chemistries as a function of time in dissolution. The starting coating thickness on all of these samples was 100 nm (with 3–5% variance based on manufacturer's specifications).

After 12 hours in solution, less than 5% of the coating dissolved for all chemistries except Sample 1. In this sample, about 35% of the coating was dissolved after 12 hours, and after 8 days (192 hours), approximately 20% of the coating still remained. For the other samples, release of Si was not observed until 24 hours in solution, where approximately 50% of the coating was left. However, after 8 days in solution, these coatings appeared to have

completely dissolved. The thickness values were calculated by using the ionic silicon (Si^{+4}) release data measured by ICP-OES analysis. The variance in the ICP measurements and the simplifying assumptions involved in thickness calculations could lead to inaccurate results with an estimated error of $\sim 5\%$. However, this data suggests that Si release kinetics is partially dependent on the coating chemistry, which can be altered and fine-tuned to get the desired Si^{+4} release whereas the coating thickness accounts for the length of release period.

Moreover, it was also desired to determine if there was an effect of the surface chemistry on osteogenic markers expression. Therefore, relative expression of several genes associated with osteoblast differentiation was studied using MC3T3 cells and the human periosteum cells. The results for MC3T3 cells (Figure 4) showed that expression of SOD1 was significantly increased at 6 hours after induction of differentiation (Figure 4a, 3 to 12 times relative to control glass cover slip group), and settled down to same level at 24 hours. The transcription factors Runx2 and Sp7, were chosen for measurement due to their vital role in stimulating osteoblast differentiation and mineralization. The transcription factors Runx2 and Sp7 showed the same trend as SOD1 gene expression, but their reduction was moderate compared to SOD1. The gene expression of OCN on wafer sample was remarkably increased at 6 hours, and continued until 48 hours after induction of differentiation. The gene expression of Col(I)- $\alpha 1$ up-regulated and down-regulated alternately at these time points. Similarly, human periosteum cells also showed (Figure 5) an enhanced expression of SOD1 on Si(ON)x surfaces at 24 hours after induction of differentiation. The up-regulated expression of SOD1 induced downstream expression of the osteoblast differentiation markers including Runx2 and collagen type 1, maximally enhanced at 48 hours time point.

In-vivo evaluation of these biomaterials was performed using a rat critical-sized calvarial defect model. The Si(ON)x test samples were implanted in the rat calvaria as shown in Figure 6. The implanted sample was extracted after allowing 5 weeks of recovery. The extracted bone-implant sample showed a strong fixation of the implant with the surrounding bone whereas micro-level computerized tomography (μCT) scan confirmed the presence of hard mineralized newly formed bone that filled almost all space around the Si(ON)x coated implant (Figure 7a). On the other hand, controls samples (substrate only) were found mobile and loosely connected to surrounding bone via soft tissue when extracted after 5 weeks. The micro-CT data revealed that no ample regeneration of new bone occurred to fill the interfacial gaps for control samples during the same recovery time (Figure 7b). Similarly, negative control (empty defect) showed no signs of sufficient healing and confirmed that the defect remained critical size and would not heal on its own (Figure 7c). The bone mineral density and the ratio of bone volume (BV) to total volume (TV) was determined using microCT data to give more quantitative insight into bone regeneration (Figure 7d). The results showed that Si(ON)x surfaces which were able to fill the interfacial gap with newly generated bone after 5 weeks of recovery had about 5 times higher BV/TV as compared to control samples. Higher BV/TV indicates larger volume of mineralized bone regenerated for the Si(ON)x surfaces. Similarly, histology analysis using Stevenel's Blue stain and Van Gieson Picro-Fuchsin counterstain also verified the formation of fully mineralized bone at the bone-implant interface for Si(ON)x test samples whereas the control samples showed less mineral formation with mostly collagenous fiber as shown in Figure 8. Histology data also indicated the presence of blood vessels in the newly generated bone and well-oriented

collagenous fibers with osteoblasts sitting along the bone edges, which designates the healing impact of the implanted material.

Raman spectroscopy data was analyzed to study the composition and the maturity (mineralization) of the newly formed bone after 5 weeks of recovery time. Intensity of PO_4^{3-} peak at 950cm^{-1} is proportional to mineralized content of the bone. Peaks at 955cm^{-1} and 957cm^{-1} indicate transient bone mineral phase from the immature bone. Peak at 853cm^{-1} and 872cm^{-1} indicate presence of collagen proline and hydroxyproline respectively. Peaks at 1242 and 1272cm^{-1} represent Amide III protein β sheet and protein α helix respectively. Peak at 1446cm^{-1} corresponds to protein CH_2 deformation and 1660cm^{-1} is the strongest Amide I peak with high polarisation sensitivity. Prominent shoulder at 1690cm^{-1} are indicative of immature cross links, β sheets and disordered secondary structure. The results (Figure 9) demonstrated that the surrounding bone (A), non-defected original bone, showed all the peaks expected to be present in a mature rat bone. The newly formed bone (B), at the boundaries of the initially created defect (adjoining surrounding bone), showed the peaks very similar to the surrounding bone and appeared fully mineralized indicated by the $\nu_1\text{PO}_4^{3-}$ peak intensity. The full width half maximum of $\nu_1\text{PO}_4^{3-}$ is proportional to c-axis length of crystal and hence proportional to maturity of the crystal. The content of mineralization decreased as we moved towards the center of the defect, indicated by drop in the intensity of $\nu_1\text{PO}_4^{3-}$ band indicating less phosphate present. At the far end of the newly formed bone towards Si(ON)x surface (C), we observed distinct peaks for collagen presence as well as Amide III and Amide I formation and CH_2 deformation. This indicates protein presence and some immature mineralized content. Whereas in the middle of the Si(ON)x sample (D), peaks for Amide III, Amide I and CH_2 deformation were seen but there was no collagen or phosphate-carbonate presence indicating no mineralization occurred. In addition a distinct peak showing Si-Si bond was recorded, which might have stemmed from the underlying silicon substrate.

The results from the XANES data (Figure 10) were analyzed to study the coordination chemistry of the newly grown bone as compared to the surrounding bone. The Ca K-edge data showed that Ca was present in large abundance on the newly formed bone surface and the overall dominant chemistry was similar to hydroxyapatite (HA). This was evident by the peaks specific to HA (Ca K-pre edge peak at 4039.1 eV , pre edge shoulder at 4044.4 eV , main peak b at 4048.27 eV and the post edge shoulder). The P L-edge data indicated that surrounding bone was more like stable hydroxyapatite whereas the new bone formed on Si(ON)x surface was also largely HA but was not mature as evidenced by the smaller peak d (phosphate specific peak at 146.7 eV) which was indicative of smaller phosphate content. The shoulder after peak c (137.8 eV) is characteristic of Ca-phosphates and arises from transitions from 2p of P to empty 3d orbitals of Ca. The presence of this shoulder clearly illustrates that the phosphates present on the surface are in the form of calcium phosphates/HA. Moreover, the new bone spectra had a weak post edge shoulder indicating that the Ca was not all coordinated in the form of HA and may have some soluble phosphates, that was also evident from the smaller peak a. Similarly, the P K-edge (Figure 10c) also confirmed the presence of HA on the surface, evident by the post edge peak b around 2155 eV , which is unique for HA and corresponds to transition of 1s P electron to 3d Ca orbital. The O K-edge data showed that the newly formed bone on the Si(ON)x surface

was a mixture of calcium carbonates and phosphates. Peak a (Figure 10d) represents carbonate ions substituted into bone mineral structure. It is shifted from CaCO_3 structure due to second nearest neighbor effects of mineral lattice on electron density.^[28] Peak between b and c match the higher energy peaks in phosphate-containing compounds suggesting beginning of formation of b-TCP or HA like structure that is relatively ordered and matrix is starting to mineralize whereas the peak d represents resonant feature. XANES data also verified that the composition of the newly formed bone was exactly the same as that of the surrounding bone. SEM micrographs (Figure 11) showed that the newly generated bone grew over the implant surface indicated by the dotted circle whereas arrow points out the implanted sample. EDS mapping showed the presence of Ca-P based bone formation over the implant coating surface. This was evident by the appearance of a small area that was not covered yet, indicated by excessive silicon presence. This reveals nearly complete bony union over the implant surface and indicates that rapid HA formation on the surface led to early collagen synthesis followed by mineralization to make the collagenous matrix into mature bone. Such rapid bone healing by Si(ON)_x surface demonstrated the osteoinductive nature of our material that induced osteogenesis leading to early formation of HA and biomineralization.

3. Discussion

In this study, we tested the hypothesis that ionic silicon and PECVD-based amorphous silica enhance osteogenesis via enhancement of superoxide dismutase 1 (SOD1), a known antioxidant. In the first part of this study, we found that ionic Si enhanced SOD1 expression and osteogenic markers (LOX, OSX, Col(I)) for wild type osteoprogenitor (MC3T3) cells. But such enhancement was not observed for SOD1 knockdown cells, indicating that SOD1 is required for Si^{+4} to enhance osteogenesis. This result is the first evidence showing this mechanism. SOD1 is dependent on the activity of local cations for its expression and activity. Since Si^{+4} is a cation with +4 valence state, it was hypothesized that ionic Si enhanced osteogenesis through this pathway. Previous evidence illustrating this effect was observed in plants where ionic Si was observed to enhance the immune response by plants under biotic or abiotic stresses such as drought, salinity, and nutrient toxicity or deficiency in which SOD1 was stimulated by the presence of Si^{+4} to maintain viability under these conditions.^[29,30]

PECVD-based amorphous silica thin film coatings were assessed for sustained delivery of Si^{+4} in physiological environments. We observed that amorphous silica with nitrogen incorporation Si(ON)_x gave continual release of Si^{+4} when immersed in cell-free *in-vitro* settings. The ionic release was controlled by the surface chemistry (O/N ratio) formulated by PECVD process under optimized conditions and the release period depended upon the thickness of the film. A 100 nm thick layer of Si(ON)_x gave a sustained release of Si^{+4} over 8 days of time period as shown in Table I above. Though more silicon was released than the theoretical maximum after 8 days immersion for sample chemistries 2 and 4 resulting in dissolved thickness higher than 100 nm, this was likely due to the simplifying assumption that the system consisted of a predominantly Si_3N_4 or SiO_2 system while in reality, there was ~10–20% excess Si in the coatings. Moreover, in order to estimate the percentage thickness of the coating dissolved, some simplifying assumptions were made. First of all, the

coatings were assumed to be chemically and structurally uniform in the direction normal to the surface. Secondly, since the exact densities of the coatings were unknown, a reasonable approximation was to use the density of similar low-temperature fabricated PECVD coatings. According to MIT's material property database, low-temperature PECVD deposited silicon nitride has a density of about 2.5 gcm^{-3} while similarly fabricated silicon dioxide has a density of about 2.3 gcm^{-3} .^[3] Since the Si(ON)x coatings composition were intermediate to these two extremes, these values were used to calculate the total amount of silicon that was able to dissolve as a function of time. Despite its limitations, this simplified model of dissolution coating thickness allowed for the observation of interesting trends in the data and relative release rates of Si^{+4} with varying O/N ratio in surface chemistries.

In-vitro cell culture studies using amorphous silica-based materials showed that these materials up-regulated the expression of SOD1, which induced downstream expression of Runx2 and collagen type 1. Though both the cell types (MC3T3 and human periosteum cells) showed enhanced expression of osteogenic markers on Si(ON)x surfaces but slight differences in time and expression levels were observed. It might have stemmed from the differences in their osteoblast differentiation kinetics including cell proliferation, cell migration–aggregation and cell differentiation. Osteogenic markers expression was maximally enhanced at an optimal Si(ON)x composition in which O/N was nearly unity (refractive index =1.58). The amorphous silica-based Si^{+4} delivery device showed even higher impact on osteogenesis enhancement via SOD1 expression as compared to ionic Si. This enhanced effect was likely owed to surface effects from the amorphous silica samples. These materials were patterned and thus had different surface energy than flat samples. Moreover, bio-inspired surface morphology amplified cell-surface interactions to have better cell-attachment, cell-growth and differentiation. Therefore, the cells appeared to adhere at a high density on the amorphous silica Si(ON)x surfaces as compared to control glass surfaces. These results indicate that PECVD process can be tuned to acquire amorphous silica-based biomaterial for an optimized release of Si^{+4} that enhances osteogenesis via SOD1 expression while surface morphology integrates bio-mechanical bonding to bio-chemical attachment of such coated implants. Cerium oxide coatings have also been previously tested with fibroblasts to study the antioxidant capacity of cerium oxide nanocrystals toward H_2O_2 -induced oxidative stress.^[31,32] However, these nanoparticles did not induce any effect on antioxidant enzymes or genes rather, they were shown to only electrochemically reduce hydrogen peroxide. Such reduction is not specific to the reduction of the key anion associated with oxidative stress, superoxide anion. The up-regulated effect of Si(ON)x and ionic Si shown in this work has the ability to target the expression of SOD1, the key antioxidant enzymes used by cells to reduce superoxide anion to water and oxygen.

In-vivo testing showed that the amorphous silicon oxynitride Si(ON)x overlay onto implant surfaces induced rapid regeneration of bone within rat calvarial critical-sized defects. The defect was nearly completely closed within 5 weeks (more than 50% of bone volume regenerated) whereas the control group showed no significant regeneration of bone over the same time period under exactly similar conditions. The empty defect showed no healing and confirmed that the defect was critical-sized defect and did require an external implant for healing. XANES data confirmed the presence of Ca coordinated with phosphates indicated by characteristic peaks for HA. It also illustrated that the newly formed bone matched the

coordination chemistry of the surrounding bone. P L-edge (Figure 10b) and P K-edge (Figure 10c) both confirmed the presence of HA on the newly formed bone surface but interesting information was observed when compared with the surrounding bone. P L-edge spectra indicated that the shoulder after peak c (137.8 eV) was not as significant for newly formed bone when compared to the surrounding bone. This indicates reduced bone maturity whereas P K-edge showed fully mature bone for both the newly formed and the surrounding bone. This might be due to different energy ranges and the penetration depth for the two beams. P L-edge (low energy beam) takes into account only the top 40–50 nm whereas P K-edge (high energy beam) data corresponds to a 3–4 μm penetration depth. This suggests that the top few tens of nanometer layers were not as mature as the surrounding bone but bottom layers were found to be fully mature. Though XANES provided valuable information on the coordination chemistry, it doesn't quantify the species present on the surface. This is where the Raman was employed to study, quantify and compare the content of phosphates and other species. The spectra showed interesting results on bone maturity with proportionally varying content of phosphates indicated by the $\nu_1\text{PO}_4^{3-}$ peak intensity. As mentioned above, the full width half maximum of $\nu_1\text{PO}_4^{3-}$ corresponds to c-axis length of crystal and hence relates to maturity of the crystal. Raman spectra revealed that the mineralized content ($\nu_1\text{PO}_4^{3-}$ peak intensity) was maximal at the interface of the surrounding bone and the newly grown bone and decreased as we moved towards the center of the defect. It also indicated the presence of collagen specific peaks, Amide III, Amide I formation and CH_2 deformation in the middle of the defect. Altogether, it validated the XANES data on the presence of phosphates and assessed the maturity of the newly grown bone by quantifying the mineralized content. It also illustrated the mechanism of bone regeneration for Si(ON)_x surface where early formation of HA promotes the collagen synthesis followed by biomineralization to generate mature bone.

In the immediate application of these materials, we have shown the effectiveness of these materials to enhance bone-healing *in-vivo*. Previously, we established that these overlay materials were well-adherent to metal surfaces like titanium (Ti), which is the typically used material for permanent/semi-permanent fixtures in fracture healing.^[24] Since ascorbic acid was used in the culture media and it is an antioxidant, so the role of Si is as a combinatorial antioxidant that works in tandem with other antioxidants to heal bone. The enhancement of the osteogenic pathway by ionic silicon via Si(ON)_x coated metal implants can be used to hasten the healing phase of bone formation. This can improve the efficacy of these implants in long-term to remain as a permanent fixture because the bone-SiOx-Ti/TiO₂ interface can remain as a composite interface. The mechanistic effect opens new roles into our understanding of cations and their impact on bone regeneration. This study shows the first evidence of the effect that cations have on linking osteogenic response to immune response. Although much evidence exists showing that amorphous silica and Si ions have a stimulatory effect on osteogenesis, this is the first evidence of showing how this takes place. Future work will expand upon this principle to determine what other aspects of healing can be utilized from this work.

4. Conclusions

In this study, we tested the hypothesis that ionic Si and PECVD amorphous silica-based biomaterials enhance osteogenesis via antioxidant expression. Ionic Si was tested with wild type and SOD1 knockdown osteoprogenitor cells (MC3T3). It showed enhanced expression of antioxidant and osteogenic markers in wild type cells but all markers were down-regulated in SOD1 knockdown cells. These results indicated the dependence of Si^{+4} on SOD1 to enhance osteogenesis in osteoprogenitor cells during osteogenesis. We devised PECVD-based amorphous silica for Si^{+4} release delivery and found similar results for these biomaterials using MC3T3 and human periosteum cells. The surface topographies of these test samples were patterned to influence the cellular behaviors. Favorable cell response to these surfaces gave an enhanced expression of osteogenic markers and a high density of cells attached to the surface as compared to control group. Maximal enhancement occurred at an amorphous silica surface with O/N ratio very close to unity. *In-vivo* evaluation of these samples in rat critical size calvarial defect model showed that these biomaterials stimulated the bone regeneration and filled the interfacial gap within 5 weeks which didn't occur for control group. XANES and Raman spectroscopy showed that the newly grown bone chemistry exactly matched the surrounding bone and the mineralized content was maximum at the interface of newly formed and the surrounding bone. This study therefore supports the conclusion that Si plays a combinatorial antioxidant role in enhancing osteogenesis and PECVD-based amorphous silica can potentially be used as an overlay onto metal implants for rapid bone healing. This study opens new avenues in how we interpret cellular function and how cells respond to their surrounding extracellular matrix cation environment.

5. Experimental Section

Study design

The first part of this study aimed to determine the effect of ionic Si on SOD1 and osteogenesis. Two treatment conditions (control, ionic Si) were prepared and tested with osteoblast progenitor cells (mouse calvarial 3T3, MC3T3). Control treatments were prepared by alpha-MEM supplemented with 10% FBS, 1% pen-strep and 50ppm ascorbic acid 2 phosphate whereas ionic Si treatment was prepared by adding 0.1 mM Na_2SiO_3 to the control treatment. Wild type and SOD1 knockdown cells (using siRNA) were tested with each treatment. The second part of this study aimed to devise and test an ionic Si delivery device for the up-regulation of SOD1 and osteogenesis. PECVD-based amorphous silicon oxynitride $\text{Si}(\text{ON})_x$ overlays with varying levels of O/N ratios were investigated for Si^{+4} release and osteogenic gene expressions *in-vitro*. The last part of this study contained *in-vivo* evaluation of these biomaterials for their bone-regeneration capability in a rat critical size calvarial defect model using control samples (substrate only group and no implant group).

5.1. Effect of ionic Si on SOD1 and osteogenesis

5.1.1. Cell culture—MC3T3 cells were cultured in 150 cm² flasks with alpha-minimum essential medium, α -MEM (Life Technologies Corp., Grand Island, NY, USA) containing 10% fetal bovine serum, FBS (VWR, Radnor, PA) and antibiotics (1% streptomycin/

penicillin) in a humidified atmosphere of 95% air and 5% CO₂ at 37° C and expanded as needed to obtain the required cells for each experiment. Cells of passage number 18 to 22 were used in this study.

5.1.2. SOD1 siRNA Transfection—The siRNA cocktails targeting the mouse gene SOD1 were purchased from Life Technologies - Thermo Fisher Scientific (Rochester, NY, USA). MC3T3-E1 cells were synchronized with α MEM, 1% FBS, and 1% pen-strep for 48 hours. Cells were then transfected by Lipofectamine (Invitrogen) using the transfection agent concentration of 6 nM for 10⁵ cells/well as per manufacturer's protocol. After 5–7 hours of transfection in a CO₂ incubator at 37° C containing growth media (without pen-strep), the cells were introduced to growth media for 24 hours followed by their respective treatments. Cells were lysed 72 hours after the treatments were introduced and analyzed for gene expression using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Histological analysis for collagen fiber bundle formation was performed 6 days after treatment.

5.1.3. qRT-PCR Analysis for Osteogenic Gene Expression Studies—For gene expression studies, cells were seeded at a density of 100,000 cells cm⁻² in 6-well plates. The cells were lysed using buffer RLT (guanidinium thiocyanate) as a lysis buffer (10 μ l β -Mercaptoethanol per 1 ml Buffer RLT). Then, the cells were sheared with rubber cell scraper (1.8 cm blade) under moderate pressure to lyse the cells on the surface and purified to collect mRNA (RNeasy Mini Kit, Qiagen, Valencia, CA, USA), converted to cDNA using qRT-PCR method (Reverse Transcription System, Promega, Madison, WI, USA) according to the manufacture's protocol. Absorbance measurements of mRNA and cDNA samples were performed using a micro-volume UV-Vis spectrophotometer (Nano Drop 2000c, Thermo Fisher Scientific Inc., Waltham, MA, USA). Using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal reference gene, relative quantification of gene expression was evaluated by the comparative cycle threshold (CT) method and fold change calculated using 2^{-CT} . Details regarding qRT-PCR were given in our previous work.^[33] Genes assayed included SOD1, LOX, OSX, and COL(I)- α 1.

5.1.4. Histology—Seeding of cells at 100,000/cm² was used for collagen matrix experiments. Cells were seeded onto autoclaved glass cover slips for 6 days and analyzed for collagen formation. Samples were fixed and stained using picrosirius staining using the previously described method^[33] and imaged using an optical microscope (Nikon Eclipse 80i) with a CCD camera and Image Pro software.

5.2. Fabrication of Si⁴⁺ Release Device and its Effect on SOD1 and Osteogenesis

5.2.1. Preparation of Nanofabricated Si(ON)x samples—The samples with amorphous silica based overlays were prepared using conventional photolithography followed by plasma enhanced chemical vapor deposition (PECVD) process. The fabrication process started with a single-side polished, p-type (100) orientation test grade silicon wafer (Nova Electronic Materials, Flower Mound, TX). Standard RCA cleaning was performed as explained previously.^[24,34,35] After cleaning of the wafer, Microposit HMDS primer was spin coated at 3000 rpm for 30 sec and baked on hot plate at 150 °C for 90 seconds before

spinning the photoresist. The primer was used as an adhesion promoter between the photoresist and the substrate surface. Then, the positive photoresist (Shipley S1813) was spin-coated at 4000 rpm for 30 sec to obtain a uniformly thick layer of photoresist (~1.35 μm). The wafer with primer and the photoresist was pre-baked on hot plate at 90 °C for 60 seconds and let it cool down for 1–2 minutes in ambient air before UV exposure. The wafer was then exposed to UV using EVG Aligner (I-line 365) at a dose of 139mJ/cm² in constant intensity mode and developed for 43 sec in MF-319 developer followed by hard bake (115 °C, 60 sec) to obtain the micrograting pattern on the surface of the wafer as shown in Figure 2.

After photolithography, the exposed Si surface was etched (200 nm etch depth) using deep reactive ion etching (DRIE) process in order to transfer the photoresist patterns into the underlying Si surface. TRION Deep Reactive Ion Etching System with CF₄ at 25 sccm gas flow rate, 25 mTorr pressure, 3000W ICP power and 100W RIE power etched Si at an etch rate of 862.5 Å/min. The photoresist was then removed completely by immersing the wafer in acetone and followed by piranha clean (3 H₂SO₄ : 1 H₂O₂) at 95 °C for 10 min. The wafer was rinsed in DI water and blow-dried under nitrogen.

The patterned wafer was used to deposit a uniform (non-uniformity < 1%) 100 nm thick oxide layer via PECVD process using TRION ORION II PECVD/LPCVD system. The oxidized wafer was then coated with 100 nm of amorphous silica oxynitride Si(ON)_x overlay. All coatings were fabricated at a substrate temperature of 400 °C, chamber pressure of 900 mTorr, and ICP power of 30W with a 13.56 MHz excitation frequency applied. Source gases included silane (SiH₄) diluted in argon (Ar) (15%/85%), nitrous oxide (N₂O), nitrogen (N₂), and ammonia (NH₃). The refractive indices and the thickness of the wafer coatings were measured using ellipsometry at a wavelength of 632.8 nm (LS300, Gaertner Scientific Corporation, Skokie, IL, USA), and results were confirmed through the use of a reflectometer (Ocean Optics NC-UV-VIS TF Reflectometer) and a scanning electron microscope (SEM, S-3000N, Hitachi High-Tech, Tokyo, Japan). The wafer was cut into 12 × 12 mm² sections for post-process characterization and *in-vitro* testing.

5.2.2. Si⁺⁴ Release Studies Using Inductively Coupled Plasma Optical Emission Spectra (ICP-OES) Characterization—ICP-OES analysis was performed at the Shimadzu Center for Advanced Analytical Chemistry using the Shimadzu ICPE-9000 ICP-OES system. 12 mm × 12 mm wafer sections were placed in 4 mL of α -MEM at 37°C and 5% CO₂ for 0, 6, 12, 24, 48, 96, and 192 hours. After soaking, 25x, 50x and 100x dilutions were used to investigate silicon content in the supernatant fluid. Aluminum was used as an internal standard, and the concentration measurements were taken from the α -MEM dissolution media in order to evaluate the surface degradation kinetics of the coatings. Single-element high purity ICP Al and Si standards (1000 $\mu\text{g/L}$) were purchased from Ultra Scientific. The variance in the ICP data for triplicate samples was used to determine the estimated thickness of the film dissolved in physiological conditions.

5.2.3. Cell Culture Studies for Si(ON)_x samples—Cells were seeded on the Si-wafer samples and glass cover slips (control surfaces) seated on the 6-well plate (BD, Franklin Lakes, NJ, USA). The glass cover slips and the test samples were sterilized (tissue

culture treated) exactly in the same process. A dry heat was applied using standard bacteriostat inside the biosafety cabinet and allowed to cool down for 1–2 minutes before seeding the cells on the surface. Triplicate samples were seeded with cells at the number of 100,000/well and these cells were synchronized (α -MEM, 1% FBS, 1% streptomycin/penicillin) for an additional 48 hours. Medium was then exchanged for growth media (α -MEM, 10% FBS, 1% streptomycin/penicillin) with 50ppm ascorbic acid-2-phosphate (AA2P, Sigma-Aldrich Corp., St. Louis, MO, USA) to induce differentiation. Cells were cultured for 6, 24, 48, and 72 hours before they were lysed for relative gene expression analysis.

The gene expressions of GAPDH, SOD1, OCN, RUNX2, Osterix (Sp7), and COL(I)- α 1 were studied. Data were normalized to GAPDH (internal reference gene) within each independent experiment and displayed as relative induction of control at corresponding time-point.

5.3. *In-vivo* Testing of Si(ON)x samples using Rat Calvarial Defect Model

5.3.1. Surgical and Postoperative Protocol—All procedures involving animals were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee at Baylor College of Dentistry, Texas A&M University. A critical size calvarial defect model was developed to assess the *in-vivo* bone healing efficiency of Si(ON)x coated materials as compared to control surfaces (substrate only group and no implant group). A total of 9 rats (3 for each test group) were used in this study and one sample per animal was implanted to have triplicate samples for evaluation. Adult male rats (about 430 g each) were selected to study the osteoinductive properties of the proposed material in relatively slower bone-healing subjects. The rat was initially weighed and placed into an induction chamber for anesthesia. The rat was anesthetized with 5% isoflurane in oxygen for about 2–3 minutes, then its head was shaved and held onto a stereotaxic frame. Figure 6 shows the step-by-step surgical procedure. The surgery started with an incision (about 1.5 cm) made by a scalpel blade. The periosteum layer was scratched down using the scalpel blade and the incision site was iteratively washed with sterile saline followed by the air blow to dry the exposed skull bone. A $3 \times 5 \text{ mm}^2$ Si(ON)x test sample (sterilized by standard bacteriostat) was placed at exact location where we wanted to implant it and marked the boundary with a marker on dry skull. The defect site was chosen to have maximum flat surface with minimum sutures involved. Then, the dental bur (#1 or #2) with about 0.5 mm diameter tip was used to precisely cut the bone along the drawn boundary to create the critical sized calvarial defect without nicking the dura. After creating the bone defect, the defect site was thoroughly washed with sterile saline followed by the air blow to get it dry. A small amount (~1–2 μ l) of gel glue was put on the bottom surface of the implant and placed upside down in the defect so that it is glued to the dura and doesn't move during healing (Figure 6e). Finally, monofilament taper point suture needle was used to suture the incision site making square knots and ensuring adequate space to allow skin healing. The incision site was cleaned thoroughly and a small volume (80–100 μ l) of painkiller/sedative medicine (nalbuphine) was injected intramuscularly using 1 ml syringe. The rat was observed for behavioral study to record any signs of pain/distress for the next few days and then allowed

5 weeks for healing before analyzing the effect of Si(ON)x based material on bone regeneration.

5.3.2. Micro-level computerized tomography (μ CT) Scan—The implants with the surrounding calvarial bone were extracted out after 5 weeks of recovery time and were analyzed using an X-ray microCT imaging system (μ CT 35, Scanco Medical, Basserdorf, Switzerland). The unit consists of an x-ray source directed towards a specimen holder that hosts bone-implant samples. Serial tomographic imaging at an energy level of 55 kV and intensity of 145 μ A for the samples was performed. 3D micrographs were generated to evaluate different sample surfaces for the fracture healing after the same recover time. The lower and upper threshold values used for this analysis were 280 and 1000 respectively. The microCT data was further analyzed to determine the bone mineral density and the ratio of bone volume (BV) to total volume (TV) using the built-in function of microCT imaging system.

5.3.3. X-ray Absorbance Near Edge Structure (XANES) Spectroscopy—XANES spectroscopy was performed at the Canadian Light Source at the University of Saskatchewan in Saskatoon, Saskatchewan, Canada. XANES is a very sensitive and advanced technique to probe the presence of atomic species and characterize the local coordination of individual elements, by using the fine structural features at the absorption edge.^[36] Since the bone mineral is mainly made of calcium phosphate apatite, calcium (Ca) and phosphorous (P) edges are mostly investigated for bone analysis to determine the nature and local coordination of Ca and P in bone.^[28,37,38] The Ca and the P K-edge spectra were obtained over the energy range of 2140–2190 eV and 4000–4130 eV, respectively using soft X-ray beam-line for the micro-characterization of materials (SXRMB) beam-line. The step size for Ca and P K-edge spectra were 0.3 and 0.25 eV respectively. Plane Grating Monochromator (PGM) beam-line was used to acquire the P L-edge spectra in the region of 130–155 eV. PGM operates at the low energy range between 5–250 eV and a step size of 0.1 eV. The oxygen (O) K-edge spectra was recorded for energy ranges between 525–560 eV using Spherical Grating Monochromator (SGM) beam-line that operates in the midrange energy of 250 – 2000 eV with a step size of 0.15 eV.

5.3.4. Raman Spectroscopy—Raman spectroscopy is a very convenient and valuable technique to analyze biological samples without need for staining, fixation or sample preparation but needs a relatively high-power laser beam to overcome the inherent low Raman scattering efficacy of biological molecules.^[39,40] Microspot Raman spectroscopy (DXR, Thermo Scientific) with 780 nm laser source and 100 mW power was used to characterize the extracted bone sample. Different regions (A, B, C, D) on the extracted sample were analyzed to evaluate the newly grown bone chemistry in comparison to the surrounding bone. The phosphates, amides and other significant peak intensities were used to gauge the mineralized content and the maturity of the bone. Details of the Raman procedure are provided in our earlier work.^[39]

5.3.5. Scanning Electron Microscopy (SEM) of Extracted Bone Samples—The extracted bone samples were completely dried and a thin layer of gold (50 nm) was coated

on the surface to make it conductive for electron microscopy. Samples were imaged using a scanning electron microscopy (SEM, JOEL USA Inc., Peabody, MA, USA) with secondary electron mode at 5–15 kV energy to get the finest results.

5.4. Statistical analysis

Data were presented as the mean $\pm$ standard error. One-way analysis of variance (ANOVA) was used for parametric analyses and $p < 0.05$ was used for statistical significance. Posthoc student T-test was used for between group comparisons and $p < 0.05$ was used to establish statistical significance.

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Figure 1.

(A). Effect of Si⁴⁺ on SOD1 and other osteogenic markers after 72 hours of treatment. Si⁴⁺ enhanced (a) SOD1 and osteoblast differentiation markers (b) LOX (c) OSX and (d) Col(I) for wild-type MC3T3-E1 cells whereas SOD1 knockdown cells using (siRNA) inhibited this enhancement. (ANOVA, *p<0.05, **p<0.01, ***p<0.001). (B). Si⁴⁺ promotes collagen matrix formation (dense, Picrosirius red stained tissue with yellow-orange elongated collagen fibers) vs. control treated cells where SOD1 knockdown cells inhibited the matrix formation and ionic silicon couldn't enhance the matrix formation. (C) Quantitative analysis

confirms one order of magnitude higher collagen formation for wild type cells in the presence of ionic silicon. It shows that enhancement of osteogenic markers and collagen matrix formation by Si^{+4} depends upon SOD1 enhancement.

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(a) Fabrication process

(b) Surface Topography

Figure 2. Fabrication of Si(ON)x overlays

(a) A schematic diagram to illustrate the fabrication process for PECVD amorphous silicon oxynitride overlays with Si wafer substrate. (b) SEM micrograph shows the surface topography of a representative sample after photolithography, etching and PECVD deposition of amorphous silica (feature size 2 μm).

Figure 3.

(a) Silicon release from Si(ON)_x sample chemistries immersed in αMEM as determined by ICP-OES analysis shows sustained release of Si^{+4} over a period of 8 days. Higher N content appears to slow down Si^{+4} release after 24 hours of *in-vitro* immersion. SEM micrographs show surface topographies for Si(ON)_x samples (b) before and (c) after *in-vitro* immersion.

Figure 4. Si(ON)_x surfaces showed an enhanced expression of (a) SOD1 (b) Runx2 (c) Sp7 (d) OCN and (e) Col(I)-α1 osteogenic markers for MC3T3 cells when compared to control surfaces. Maximal enhancement was observed for the surface with O/N ratio nearly equal to unity (n=1.58). Moreover, MC3T3 cells showed maximum enhancement to the Si(ON)_x surface at the very initial time point (6h).

Figure 5.

Human periosteum cells also showed up-regulated expression of (a) SOD1 which downstream enhanced the expression for other osteoblast differentiation markers including (b) Runx2 (c) Sp7 (d) OCN and (e) Col(I)-α1 on Si(ON)_x surfaces. Maximal enhancement for human periosteum cells was observed at 48 hours after induction of differentiation.

Figure 6.
Operative procedure to implant the test sample in a rat critical sized calvarial defect model

Figure 7. MicroCT micrographs showed that (a) Si(ON)x samples triggered the rapid bone-regeneration process and filled the gap nearly completely within 5 weeks whereas the control samples (b) and (c) didn't show much bone to fill the interfacial gap over the same time period. (d) Quantitative analysis shows larger volume of mineralized bone regenerated for the Si(ON)x surfaces, indicated by higher bone to volume ratio (5 folds) when compared to control surfaces. The Arrows A, B, C and D in part (a) indicate four different regions as we move from surrounding bone toward the middle of the implant. Figure 9 (below) shows Raman analysis of these four different regions.

Figure 8. Histology of extracted samples after 5 weeks of recovery demonstrate that (a) Si(ON)x surfaces showed fully mineralized bone at the bone implant interface whereas (b) the control samples showed less mineral formation with mostly collagenous fiber filling the interfacial gap.

Figure 9.

Comparison of mineralized content in the newly formed bone (A) Surrounding bone representing the non-defected original bone shows all the peaks expected to be present in a mature rat bone. (B) High intensity of $\nu_1\text{PO}_4^{3-}$ peak indicates the complete mineralization of the newly formed bone at the interface of new/surrounding bone. (C) A drop in $\nu_1\text{PO}_4^{3-}$ peak intensity and presence of distinct peaks for collagen, Amide III, Amide I and CH_2 deformation demonstrate the presence of collagenous matrix and some immature mineralized content whereas (D) no mineralized content is present in the center of the defect but amide peaks show the presence of collagen. The regions A,B,C and D are marked in Figure 7.

Figure 10.

X-ray absorbance near edge structure (XANES) spectroscopy comparative data for newly formed bone and the surrounding bone with respect to HA model compounds. Bone-implant test samples were probed to examine the presence of calcium and phosphorus. The data analysis reveals the presence of abundant Ca phosphates on the Si(ON)x coated implant surface. XANES also confirms that coordination chemistry for the newly grown bone exactly matches the surrounding bone.

Figure 11.

SEM micrographs (a) and (b) showed that the bone grows over the surface (indicated by the dotted circle) of the Si(ON)x test sample (pointed out by arrow) within 5 weeks of recovery time. (c) EDS analysis of extracted bone with Si(ON)x test sample shows that bone grows over the surface of the implant and mineralized content (Ca and P) is all over the surface. Excessive Si present at the implant surface that is exposed (not covered by newly generated bone yet) indicates that boney union is nearly complete. The sample was coated with gold so Au is uniform all over the surface.

Table ICoating thickness dissolved after immersion in α MEM (determined by Si release)

Sample	Thickness (nm) with an estimated error of $\pm 5\%$						
	0h	6h	12h	24h	48h	96h	192h
1 (n=1.80)	0	0.2	34.2	50.5	58.9	69.6	83
2 (n=1.68)	0	1.6	4.1	56.5	71.8	82.8	102.5
3 (n=1.58)	0	0.6	2.2	54.9	61.1	91.5	98.1
4 (n=1.45)	0	1.7	3.2	56.0	63.8	81.9	102.3