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Combinatorial effect of Si^{4+} , Ca^{2+} , and Mg^{2+} released from bioactive glasses on osteoblast osteocalcin expression and biomineralization

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ABSTRACT

Osteocalcin (OCN) expression is an essential osteogenic marker of successful bone regeneration therapies. This study hypothesizes that Si^{4+} and Ca^{2+} combinatorial released by bioactive glass enhance osteoblast biomineralization through up-regulation of OCN expression; and Mg^{2+} release delays such enhancement. Osteoblasts (MC3T3-E1) were treated with ionic products of bioactive glass dissolution (6P53-b experimental bioactive glass and 45S5 commercial BioglassTM). Results showed that gene expressions, including OCN and its up-stream transcription factors (Runx2, ATF4, MSX1, SP7/OSX), growth factors and signaling proteins (BMP2, BMP6, SMAD3), were enhanced in both 45S5 and 6P53-b glass conditioned mediums (GCMs). This up-regulation led to enhanced mineral formation by 45S5 glass conditioned mediums ([GCM], Si^{4+} + Ca^{2+}) after 20 days, and by 45S5 GCM and 6P53-b GCM (Si^{4+} + Ca^{2+} + Mg^{2+}) after 30 days. In examining the extra-cellular matrix generated by cells when exposed to each GCM, it was found that 45S5 GCM had slightly elevated levels of mineral content within ECM as compared to 6P53-b GCM after 30 days while control treatments exhibited no mineral content. The formation of well-defined mineralized nodules (distinct PO_4^{3-} [960 cm^{-1}] and CO_3^{2-} [1072 cm^{-1}] peaks from Raman Spectra) was observed for each GCM as the soluble glass content increased. In examining the individual and combined ion effects between Si^{4+} , Ca^{2+} , and Mg^{2+} , it was found Mg^{2+} down-regulates OCN expression. Thus, ions released from both 45S5 and 6P53-b bioactive glasses up-regulate OCN expression and biomineralization while 6P53-b GCM Mg^{2+} release down-regulated OCN expression and delayed osteoblast biomineralization. These results indicate that Si^{4+} , Ca^{2+} , and Mg^{2+} combinatorially regulate osteoblast OCN expression and biomineralization.

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1. Introduction

Stimulation of bone healing through enhanced osteogenesis is important in the treatment of osseous defects due to dental-related diseases. During bone healing, osteoblast progenitors (e.g., human periodontal ligament fibroblasts, bone marrow stromal cells) follow a prescribed timeline of gene expression and bone matrix development. At the later stages of bone matrix formation, osteocalcin (OCN) is expressed. OCN binds extracellular matrix calcium to the developing bone matrix [1], and high serum levels are correlated with high bone mineral density [2,3]. An in vitro model showed OCN

expression occurred within the ECM after 18 days [4], while endogenous expression occurred as early as 3 days after osteogenic induction [5]. Preceding or concurrent to OCN expression, other transcription factors, growth factors, and signaling proteins are expressed during osteoblast progenitor differentiation into the mineralizing phenotype. Therefore, bone regeneration strategies that up-regulate the expression of genes that promote enhanced OCN expression and osteoblast biomineralization are greatly desired.

Current dental regeneration therapies include biologically-based (autologous, allogeneic bone grafts) or synthetic (polymers, hydroxyapatite, titanium) treatments. However, each of these treatments has its own individual limitations. For example, biologically-based grafts can potentially compromise the patient's donor site or introduce disease to the patient [6–8], while synthetic materials either lack sufficient strength, resorb too slowly, or do not bond directly to bone [9]. Moreover, these therapies can be rendered ineffective if they cannot treat the root cause of the patient's lack of generating new bone.

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As an alternative, bioactive glasses are being used as a bone healing strategy [10–12]. The currently FDA approved glass for these applications is known as Perioglass™ (Mo-Sci, Rolla, MO) and is based on the chemistry of Hench's Bioglass™ (45S5, 45 wt.% SiO₂, 24.5 wt.% Na₂O, 24.5 wt.% CaO, 6 wt.% P₂O₅). Although 45S5 has shown bone healing success in non-load bearing defects, it cannot be used in load-bearing applications (e.g., femoral, humeral, craniofacial bone replacements), which require structurally supporting, load bearing implants.

Saiz, and Tomsia and colleagues [13–16] modified the composition of Bioglass™ for titanium implant coating applications by partially substituting CaO with MgO and Na₂O with K₂O (i.e., 6P53-b, 52.7 wt.% SiO₂, 10.3 wt.% Na₂O, 2.8 wt.% K₂O, 18.0 wt.% CaO, 10.2 wt.% MgO, 6.0 wt.% P₂O₅). These materials survive the coating process and form a chemical bond at the metal–glass interface. The advantages of these materials are that they can potentially be used in structural implants as a coating as well as defect filling implant applications. However, the particle concentration of this new family of bioactive glasses has not been elucidated to examine the potential enhancement of biomineralization in bone defect filling applications.

The osteogenic behavior between these glass materials could potentially differ because of their differing total ionic dissolution. Both 6P53-b and 45S5 glasses have an initial diffusion-controlled ion release mechanism that eventually becomes surface-reaction limited [17,18]. Both materials release Na⁺, Si⁴⁺, Ca²⁺, and PO₄³⁻, while bioactive coating glasses release additional K⁺ and Mg²⁺. After the initial rapid ion exchange in which Na⁺ and K⁺ are released from 6P53-b, it was found that dissolution led to elevated levels (0.4 mM) of Si⁴⁺, Ca²⁺, and Mg²⁺ in conditioned mediums [19]. Si⁴⁺ and Ca²⁺ have been shown to enhance osteoblast markers such as OCN [19].

Yet, release of Mg²⁺ from 6P53-b may have varied effects on osteogenesis. Mg²⁺ is important for bone remodeling and is present within bone matrix. However, high Mg²⁺ concentrations (>0.1–1 mM) caused reduced osteogenic marker expression and reduced osteoblast differentiation [20]. Such an effect by Mg²⁺ on OCN expression and subsequent biomineralization has not yet been studied and is explored in this work both individually and combinatorially as described previously [21].

The effect these ions have on osteoblast differentiation and mineralization will be studied using the osteoblast progenitors MC3T3-E1 subclone 4, which is a well-characterized cell line that lacks heterogeneity in phenotype that exist with primary cultures during differentiation. In addition, ion dissolution products and individual and combined ion treatments are tested at different dosages in the absence of glass surfaces to better determine their specific impact on OCN expression and biomineralization.

Therefore, it is hypothesized that Si⁴⁺ and Ca²⁺ combinatorial release by bioactive glass enhances osteoblast biomineralization through enhanced OCN expression, and Mg²⁺ delays such enhancement. The aims of this study are (1) to determine the dose and temporal effect of ions released from bioactive coating glass and commercial Bioglass™ on OCN expression and biomineralization and (2) to determine if Mg²⁺ down-regulates osteocalcin expression either individually or in combination with other ions that are released from bioactive glasses.

2. Materials and methods

2.1. Study design

Pertinent to the aims outlined above, bioactive glasses (45S5, 6P53-b) were prepared as a glass conditioned medium (GCM). Basal medium for MC3T3-E1 differentiation was used as a control. Temporal- and dose-dependent effects of GCM and control treatments on MC3T3-E1 OCN expression and biomineral synthesis were studied over the course of 30 days. mRNA lysates were assayed for gene expression using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR, using glyceraldehyde phosphate (GAPDH) as an

internal reference gene). Osteocalcin (OCN) expression from medium extracts and protein lysates was assayed using enzyme linked immunosorbent assay (ELISA). Visualization of biomineral formation was accomplished using histology (Alizarin red S stain), scanning electron microscopy (SEM), EDS, and Raman spectroscopy. Individual and combined ion effects of Ca²⁺, Si⁴⁺, and Mg²⁺ were studied to delineate the specific individual and combined ion effects on osteoblast osteocalcin expression. Experiments were conducted with triplicate sampling and all experiments were repeated.

2.2. GCM preparation

Experimental bioactive coating glass (6P53-b, SEM-COM, Toledo, OH) and commercial Bioglass™ (45S5, Mo-Sci, East Rolla, and MO) were used in this study. These melt-derived glasses were ball milled and sieved into a microparticulate form (100–300 µm average particle diameters). 45S5 and 6P53-b glass samples were removed and ultrasonicated in acetone and 2-propanol, for subsequent drying overnight in a desiccator. The samples were then soaked in α-MEM (Invitrogen, Carlsbad, CA) at a volume to surface area ratio of 3 mL cm⁻² within a capped centrifuge tube and stored in an incubator (37 °C, 5% CO₂, 100% RH) for 72 h. The loading concentration was 0.8, 1.6, and 2.4 g/glass per 50.0 mL. The medium was then collected as an ion extract, and sterile filtered (0.2 µm). Each ion extract was then supplemented (by volume) with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (pen–strep) to make GCM.

Individual ion treatments were prepared by dissolving 0.4 mM Na₂SiO₃, CaCl₂·2H₂O or MgCl₂·2H₂O and supplemented as described above. Combined ion treatments were prepared by combining 0.8 mM of each individual ion treatment at a 1:1 ratio of Si⁴⁺:Mg²⁺, Ca²⁺:Mg²⁺, or Si⁴⁺:Ca²⁺ and then supplemented as described above. (Note: the inorganic sources for each ion were added at these specified concentrations such that their dissolved ion concentrations matched previously determined ion concentrations from bioactive glass dissolution [19]).

2.3. Cell culture

MC3T3-E1 subclone 4 (ATCC, Manassus, VA) cells were seeded (50,000 cells cm⁻²) into 6-well plates. The plates were incubated overnight to allow for attachment and proliferation through their doubling time (38 h). Cells were then immersed in synchronization mediums (α-MEM, 1% FBS, 1% pen–strep) for 48 h. The synchronization mediums were removed and 45S5 and 6P53-b GCMs and basal mediums were added. All treatments were further supplemented with AA (50 ppm) and β-GP (10 mM) to induce differentiation. A parallel experiment was conducted in which cells were cultured on glass cover slips (not bioactive glasses) for ECM visualization. Visualization, gene expression, and protein expression assays were carried out over a 30 day time period. Individual and combined ion experiments were arranged in a similar manner, however, β-GP was not added and the time period of the experiments was fixed at 6 days. To determine the dose dependence of these materials on osteocalcin and biomineral production, cells were cultured for 30 days in which protein lysates were measured for osteocalcin content and cells on cover slips were imaged biomineral formation.

2.4. qRT-PCR

In quantifying levels of gene expression, qRT-PCR was used. Cells cultured in each treatment were lysed at the time points specified above to collect mRNA (RNeasy Mini Kit, Qiagen, Valencia, CA) and converted to cDNA using reverse transcriptase (Reverse Transcription System, Promega, Madison, WI). cDNA samples were all diluted to a concentration of 100 ng µl⁻¹ prior to PCR amplification. Reactions

were executed using a real-time PCR machine (ABI7900, Applied Biosystems Inc., Foster City, CA).

PCR amplification was evaluated using relative quantification by comparison to an internal housekeeping gene (glyceraldehyde 3-phosphate dehydrogenase or GAPDH). PCR amplification curves were collected using the SDS thermal cycler software package and regressed for their threshold cycle (C_T) using the sigmoidal curve fitting method [22] (SigmaPlot software package (Systat Software Inc., San Jose, CA)). Relative quantification of gene expression was evaluated using the $\Delta\Delta C_T$ method (as described previously [21]). Genes assayed in this work included osteocalcin (OCN) and its upstream transcription factors (core binding factor a1 (cbfa1/Runx2), activating transcription factor 4 (ATF4), Msh homeobox 1 (MSX1), osterix (SP7/OSX)), growth factors and signaling proteins (bone morphogenetic proteins 2 and 6 (BMP2, BMP6), mothers against decapentaplegic homologues 3 (SMAD3)).

2.5. ELISA

Protein expression was assayed from days 20–30. After mediums were removed and collected, cells were lysed to determine the relative expression of osteocalcin in both GCM and control cells. At the desired time point, cells were extracted from culture by removing mediums from cell layers, washing twice in PBS, and lysing cells (Cel-Lytic M, Sigma Inc., St. Louis, MO). Lysed cells were assayed for expressed osteocalcin using an enzyme-linked immunosorbent assay (ELISA, Biomedical Technologies, Inc., Boston, MA). Medium conditions were also assayed for unbound extracellular osteocalcin expression using ELISA.

2.6. Alizarin Red staining

Cells-on-cover slip samples were visualized for mineralized tissue formation using Alizarin Red staining (Sigma Inc., St. Louis, MO). This Alizarin Red stain forms insoluble orange-red lakes, identifying points of insoluble cations that are incorporated within mineralized tissue. At the above time points, samples were moved to a new 6-well plate, washed twice in PBS, and fixed with 10% formalin for 30–60 min. The samples were stained with Alizarin Red for 30 s to 5 min and washed with deionized water. Vigorous shaking removed any excess water from the samples, and the specimens underwent sequential alcohol dehydration (70–100% ethanol) for 2 min total. The samples were imaged by standard optical microscopy.

2.7. Raman/SEM imaging

Dehydrated samples from day 28 were mapped using microspot Raman spectroscopy (DXR, Thermo Scientific, 780 nm, 100 mW, 50 μ m slit, 10 $\times$ objective, 10 s exposure). Thirty two spectra per location were recorded between 400 and 2000 cm^{-1} . The ratio of the area of the carbonate 1072 cm^{-1} and phosphate 960 cm^{-1} bands of each averaged spectra was then recorded to compare mineral development. The samples were then analyzed using SEM imaging (Hitachi S-3000N, 10 keV, 15 mm WD, high vacuum) with energy dispersive spectroscopy (EDS), (EDAX TSL, 19 keV, 15 mm WD).

2.8. Statistics

Statistical comparison of gene expression results was analyzed using GraphPad Prism 4. Two-way analysis of variance (ANOVA) followed by Bonferroni testing was used for an overall comparison of the independent factors, time and treatment, with the dependent factor, gene expression. For individual and combined ion studies, one-way ANOVA and Tukey's analysis were used for ion and control comparisons. Statistical comparisons shown are relative to corresponding control group and between GCM groups (indicated by

bracket lines). A confidence level of $p < 0.05$ was used to report statistical significance. All data analyses were calculated from triplicate sampling per experiment.

3. Results

3.1. The effect of bioactive glass ion treatments on transcription factor expression

The transcription factors Runx2, ATF4, MSX1, and SP7/OSX (Fig. 1a, b, c, and d, respectively), were chosen for examination due to their involvement in osteoblastic differentiation and mineralization. The results showed a moderate enhancement of ATF4 (2.5-fold, Fig. 1b) and SP7 (2.5-fold, Fig. 1d) gene expression by 45S5 GCM on day 6, while significant enhancement was seen by both 45S5 and 6P53-b GCMs on Runx2 (3.5- and 3-fold, respectively; Fig. 1a) and MSX1 (3.5- and 5-fold, respectively; Fig. 1c). 45S5 GCM also significantly increased Runx2 expression (7-fold, Fig. 1a) on day 14. Significant differences in transcription factor gene expression were also observed between the GCMs. For example, 45S5 GCM induced moderately greater expression of Runx2, ATF4, and SP7 in MC3T3 cells compared to 6P53-b GCM; whereas, 6P53-b GCM resulted in greater expression of MSX1.

3.2. The effect of bioactive glass ion treatments on growth factors and signaling proteins

In our study, both 45S5 and 6P53-b GCMs resulted in an enhancement in the levels of growth factor and signaling protein (BMP2, BMP6, SMAD3) gene expression. For instance, BMP2 (Fig. 2a) was significantly enhanced (7.5-fold) in osteoblasts treated with 6P53-b GCM at day 6. The expression of BMP6 (Fig. 2b) was markedly enhanced by 45S5 GCM on day 6 (2-fold) and day 10 (21-fold). SMAD3 was found to be significantly enhanced by approximately 22-fold on day 2 and 7-fold on day 20 for 45S5 GCM treated cells (Fig. 2c). For 6P53-b GCM treated cells, SMAD3 gene expression was enhanced approximately 2-fold on day 6 (Fig. 2c). BMP2 and SMAD3 gene expressions were also statistically different between the GCMs, where 45S5 GCM resulted in greater gene expression levels in MC3T3 cells. The BMP2 gene, on the other hand, was expressed substantially more by MC3T3 cells treated with 6P53-b GCM compared to 45S5 GCM.

3.3. The effect of bioactive glass ion treatments on osteocalcin

Endogenous osteocalcin expression showed significant enhancement in 45S5 GCM treated cells on days 2 and 6 (12- and 46-fold, respectively; Fig. 3a) while 6P53-b GCM treated cells induced increased expression at days 2 and 20 (4-fold, Fig. 3a). Furthermore, our results showed that both GCM treatments up-regulated endogenous protein expression of OCN (Fig. 3b, days 20–30 [45S5 GCM], days 24–30 [6P53-b GCM]). Extracellular OCN expression (Fig. 3c, 3-fold [6P53-b GCM], 5-fold [45S5 GCM]) and total overall protein yield (Fig. 3d) showed similar trends and reflected the results observed for endogenous OCN expression. In analyzing differences between the GCMs, gene and protein expressions of osteocalcin were found to be greater in 45S5 GCM treated cells relative to 6P53-b GCM treated cells at early time points and appeared to converge to similar levels towards day 30.

3.4. The effect of bioactive glass ion treatments on mineralized tissue formation

Alizarin Red staining confirmed evidence of enhanced mineralized tissue formation by GCM treatments. The degree of redness represents the quantity of ECM Ca^{2+} incorporation, thus illustrating the extent of mineralization. While control treated (Fig. 4a) and 6P53-b

Fig. 1. Transcription factor gene expression in MC3T3-E1 cells exposed to GCM (6P53-b + AA + β -GP and 45S5 + AA + β -GP) and control + AA + β -GP treatments. a) Runx2, b) ATF4, c) MSX1, d) SP7 (OSX) (ANOVA, $p < 0.05$, * denotes statistical significance, ** $p < 0.01$, and *** $p < 0.001$).

GCM treated cells (Fig. 4b) did not exhibit any degree of redness, 45S5 GCM treated cells showed a slight redness in their ECM on day 20 (Fig. 4c). Moreover, while only a slight increase in the degree of

redness occurred for control treated cells on day 30 (Fig. 4d) relative to day 20, a marked increase in redness occurred for 6P53-b GCM treated cells (Fig. 4e) and 45S5 GCM treated cells (Fig. 4f) at day 30.

Fig. 2. Growth factors (BMP2 [a] and BMP6 [b]) and cell signaling (SMAD3 [c]) gene expression after MC3T3-E1 subclone 4 exposure to GCM and control treatments (ANOVA, $p < 0.05$, * denotes statistical significance, ** $p < 0.01$, and *** $p < 0.001$).

Fig. 3. Effect of 45S5 and 6P53b GCMs on osteoblast matrix synthesis of osteocalcin. a) OCN gene expression, b) intracellular OCN protein expression, c) OCN extracellular protein expression, d) total OCN protein expression (extracellular + intracellular) (ANOVA, $p < 0.05$, * denotes statistical significance, ** $p < 0.01$, and *** $p < 0.001$).

3.5. The effect of Mg^{2+} on Osteocalcin

For these studies, only AA was used to induce differentiation. β -GP, which releases phosphate ions, was not included in these latter studies to isolate the individual ionic effects without additional phosphate ions in each treatment. Individual and combined ion effects involving Mg^{2+} had different roles on osteoblast gene expression. Mg^{2+} down-regulated OCN expression (Fig. 5: 0.25-fold) and inhibited the up-regulation of OCN when combined with Si^{4+} or Ca^{2+} . In the absence

of Mg^{2+} , OCN expression was up-regulated for Si^{4+} , Ca^{2+} , and Si^{4+} : Ca^{2+} treated cells (4.7-fold, 2.8-fold, and 4.5-fold, respectively).

3.6. The effect of bioactive glass dose on OCN and biomineral synthesis

After 21 days in culture, it was found that OCN expression increased with increased bioactive glass particle concentration (Fig. 6). Significant increases in OCN expression for each GCM were observed at a particle concentration of 1.6 g/50 mL (0.032 g/mL). Although

Fig. 4. Enhancement of specific osteoblastic biomineralization by MC-3T3 cells. All images were captured at 20× objective and 10× base magnification. a) control d20, b) 6P53-b GCM d20, c) 45S5 GCM d20, d) control d30, e) 6P53-b GCM d30, f) 45S5 GCM d30.

Fig. 5. The effects of individual (Mg^{2+} , Ca^{2+} , Si^{4+}) and combinatorial ($Si^{4+}:Mg^{2+}$, $Ca^{2+}:Mg^{2+}$, $Si^{4+}:Ca^{2+}$) ion exposure on OCN expression by differentiating MC3T3-E1 subclone 4 cells. (ANOVA, ** $p < 0.01$; $p < 0.05$ denotes statistical significance).

not significant for 6P53-b GCM at 2.4 g/50 mL (0.048 g/mL), an upward trend was still reported here.

At day 28, both 45S5 GCM and 6P53-b GCM treated cells showed EDS and Raman spectra indicative of mineral formation (Figs. 7–10). At the higher concentration levels (2.4 g/50 mL), distinct nodules were formed (Fig. 7b, d) while the lowest concentration (0.8 g/50 mL) studied in both treatments showed evidence of both indistinct nodule formation and weak, dispersed mineral formation (Fig. 7a, c). To compare the type of mineral formed, the ratio of the areas of the 1072 cm^{-1} carbonate band and the 960 cm^{-1} phosphate band was compared (Fig. 8). For the 0.8 g/50 mL treatments, only the nodule-like mineral was considered, rather than the less distinct mineral formations. Higher carbonate concentration, which is related to mineral maturity, was found to correlate with higher concentrations of GCM. Raman mapping of the 45S5 GCM treated samples also showed evidence of collagen formation (Fig. 9e), while the control sample showed evidence of neither collagen nor mineral formation (Fig. 8f). Similar results were obtained for the 6P53-b samples (Fig. 10).

4. Discussion

To address the aims of the study, we found that 45S5 GCM treatments out-performed 6P53-b GCM treatments in inducing early onset (≤ 20 days) of biomineralization, higher OCN gene expression,

and higher up-stream gene expression of markers that regulate OCN expression. This difference was attributed to the release of Mg^{2+} in 6P53-b GCM, which down-regulates OCN expression and dampened enhanced osteogenesis by Si^{4+} and Ca^{2+} . After 21–30 days, both GCM treatments out-performed control treatments in enhancing biomineralization and showed a dependence on bioactive glass particle concentration.

Early outperformance by 45S5 GCM as compared to 6P53-b GCM on biomineralization can be attributed to early enhanced OCN gene and protein expression. This early enhanced OCN gene expression was probably owed to early enhanced SMAD3 expression (day 2), which has been implicated in the promotion of mineralized tissue formation in MC3T3-E1 subclone 4 cells [23,24]. Since OCN binds extracellular matrix Ca^{2+} to the developing bone matrix, a lack of OCN up-regulation would inhibit enhanced biomineralization. This probably explains the delay in enhanced biomineralization tissue by 6P53-b GCM treated cells as compared to 45S5 GCM treated cells at day 20. Thus, the relative differences in initial biomineralization between each GCM treatment reflected the relative differences in enhanced early OCN expression.

Although Mg^{2+} is stored within bone and is important for bone remodeling [25], it may down-regulate osteogenic gene expression. In this work, it was found that Mg^{2+} induced a lack of OCN up-regulation, which has not been previously shown in other studies of Mg^{2+} on osteogenesis. Abed et al. [26] linked Mg^{2+} to reduced osteoblast differentiation owed to the reduced expression of Runx2, which is partly involved in OCN regulation. Thus, the presence of Mg^{2+} in 6P53-b GCM probably led to its under-performance in enhancing OCN expression at earlier time points. At later time points (> 21 days) and higher particle concentrations (> 0.08 g/50 mL), these relative differences between each GCM become smaller for OCN and biomineral content. Yet, the appearance of more developed and larger nodule in 45S5 GCM treated cells as compared to P53-b GCM treated cells indicates that this delay by Mg^{2+} also impacts biomineralization.

Interestingly, it was found here that the threshold value (0.08 g/50 mL) increased OCN expression while also increasing the rate of well-developed mineral. In previous work [21], it was found that the increase in OCN expression is coupled to an increase in Si^{4+} concentration; therefore, it is possible that a similar effect occurred in this work. Si^{4+} release and silanol network formation follow the reaction proposed by Hench and colleagues [18],

In this generalized model, it is assume that the amorphous silica (SiO_x) is nearly stoichiometric ($x = 2$). As n increases, the spontaneously polymerized silanol network precipitates as a silica-gel layer. But, the formation of a silica-gel layer is insufficient to enhance biomineralization. It is the coupling of this effect with increased osteoblast OCN expression that binds with Ca and P to form mineral that is essential. Thus, the increased release of Si^{4+} enhances biomineralization rate through the enhanced formation of a silica-gel layer and increased expression of the key Ca-binding protein, OCN.

Interestingly, the increased $1072\text{ cm}^{-1}/960\text{ cm}^{-1}$ indicates an increase in the amount of carbonate apatite formation relative to crystalline apatite formation with ECM. The 960 cm^{-1} peak represents the phosphate bond stretch indicative of crystalline apatite formation while the 1072 cm^{-1} peak represents the carbonate bond stretch indicative of B-type carbonate apatite formation [27]. This result suggests that as the mineralized matrix matures with increasing bioactive glass

Fig. 6. The effect of increasing bioactive glass particle concentration on MC3T3 osteocalcin expression at 21 days. (* $p < 0.05$ for statistical significance).

Fig. 7. SEM with EDS imaging of mineral at day 28: a) 0.8 g/50 mL 45S5 GCM, b) 2.4 g/50 mL 45S5 GCM, c) 0.8 g/50 mL 6P53-b GCM, d) 2.4 g/50 mL 6P53-b GCM. All images were taken at 500× (19 kV, 15 mm WD).

particle concentration, more carbonate apatite is being incorporated. The increased presence of carbonate apatite is likely due to the probable increase in carbonate ion release from the higher bioactive glass particle concentrations. The improved maturation of the matrix (evidenced by more distinctive carbonate peak) is probably owed to the enhanced osteoblast activity associated with the likely increase in Si concentration (evidenced by increasing OCN expression with increasing bioactive glass particle concentration observed here and in previous work with Si alone [21]).

To address the central hypothesis, the effect of Mg^{2+} in combination with Si^{4+} or Ca^{2+} fits into the combinatorial principle of control over osteogenic gene expression. The combinatorial principle states that gene expression is regulated by gene regulatory proteins (GRPs) in that each GRP is involved in the control of the expression of multiple genes while multiple GRPs are involved in the control of the expression of individual genes. In our previous work, we found that Si^{4+} and Ca^{2+}

combinatorially enhanced OCN expression at levels between each of the individual ion treatments [21]. In this study, we showed that this Mg^{2+} plays a down-regulatory role on OCN expression and dampens

Fig. 8. Area ratios of carbonate (1072 cm^{-1}) and phosphate (960 cm^{-1}) Raman bands in mineralized nodules at 28 days ($p < 0.05$ denotes statistical significance, ** $p < 0.01$, and *** $p < 0.001$).

Fig. 9. Representative Raman spectra obtained from 45S5 GCM, day 28 samples: a) indistinct mineral 0.8 g/mL, b) 0.8 g nodule, c) 1.6 g nodule, d) 2.4 g nodule, e) collagen nodule (found in all treatments), f) control.

Fig. 10. Representative Raman spectra obtained from 6P53-b GCM, day 28 samples: a) indistinct mineral 0.8 g/mL, b) 0.8 g nodule, c) 1.6 g nodule, d) 2.4 g nodule, e) collagen nodule (found in all treatments), f) control.

the up-regulatory role of Si^{4+} and Ca^{2+} . Thus, these ions affect gene expression during osteoblast differentiation in accord with the combinatorial principle.

Based on our results, the use of 6P53-b would be suitable as an implant coating despite its delay in enhancing gene expression relative to 45S5. Since these materials bond well to the titanium surface and enhance osteogenesis, these materials would serve well in applications as an implant coating. Furthermore, these materials could be used to enhance bone defect healing. A threshold concentration (0.08 g/50 mL) yields relatively well-developed mineral formation. Still, it is worthy to note that such mineral is not as well developed as that of 45S5 and we attribute this to the presence of MgO in the glass and its associated dissolution leading to delays in OCN expression. This in vitro experiment thus illustrates the osteogenic potential of 6P53-b in the absence of ECM signals that arise during in vivo experiments or clinical application.

Since Mg^{2+} may play a regulatory role on osteogenesis, it may be possible to use this feature in the development of new medical devices to control new bone formation. It is proposed here that multilayered bioactive glass designs could be used to take advantage of Mg^{2+} release and regulation of osteogenesis. Biomaterials with multilayered membranes have been shown to be beneficial for controlling bone formation and remodeling [28,29]. Alternating layers of this device would consist of layers of osteogenic releasing ions (i.e. Si^{4+} or Ca^{2+}) to stimulate newly formed bone and layers containing Mg^{2+} to propel the remodeling process. This sequential releasing biomedical device, by enhancing and strengthening new bone formation, could have potential clinical applications in tissue regeneration procedures or implant-bone osseointegration. Such biomedical device designs

will be further investigated to determine if such control over new bone formation can be achieved.

5. Conclusions

Both 45S5 and 6p53-b glass conditioned mediums contained increased levels of ions as previously reported. These differences in ion concentrations led to enhanced gene expression of key osteogenic markers at various time points throughout osteoblast differentiation and mineralization. These genes include transcription factors, growth factors, and signaling proteins involved in OCN expression. 45S5 GCM treated cells were found to significantly up-regulate OCN expression and enhance biomineralization while 6P53-b GCM treated cells had a similar, but delayed and less pronounced effect. It was also found that as the bioactive glass particle concentration increased, the content of biomineral that remained carbonate-rich also increased.

This regulation of OCN and delayed biomineralization enhancement by 6P53-b GCM were attributed to the presence of Mg^{2+} in 6P53-b GCM treatments. The presence of Mg^{2+} either individually down-regulated OCN expression or combinatorially limited Si^{4+} or Ca^{2+} up-regulation of OCN expression. Thus, we conclude that Si^{4+} , Ca^{2+} , and Mg^{2+} combinatorially attenuate the expression of upstream markers of OCN expression and that such combinatorial attenuation controls the level of osteoblast biomineralization.

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