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Immobilization of Alkaline Phosphatase on Microporous Nanofibrous Fibrin Scaffolds for Bone Tissue Engineering

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Abstract

Alkaline phosphatase (ALP) promotes bone formation by degrading inorganic pyrophosphate (PP_i), an inhibitor of hydroxyapatite formation, and generating inorganic phosphate (P_i), an inducer of hydroxyapatite formation. P_i is a crucial molecule in differentiation and mineralization of osteoblasts. In this study, a method to immobilize ALP on fibrin scaffolds with tightly controllable pore size and pore interconnection was developed, and the biological properties of these scaffolds were characterized both *in vitro* and *in vivo*. Microporous, nanofibrous fibrin scaffolds (FS) were fabricated using a sphere-templating method. ALP was covalently immobilized on the fibrin scaffolds using 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (EDC). Scanning electron microscopic observation (SEM) showed that mineral was deposited on immobilized alkaline phosphatase fibrin scaffolds (immobilized ALP/FS) when incubated in medium supplemented with β-glycerophosphate, suggesting that the immobilized ALP was active. Primary calvarial cells attached, spread and formed multiple layers on the surface of the scaffolds. Mineral deposition was also observed when calvarial cells were seeded on immobilized ALP/FS. Furthermore, cells seeded on immobilized ALP/FS exhibited higher osteoblast marker gene expression compared to control FS. Upon implantation in mouse calvarial defects, both the immobilized ALP/FS and FS alone treated group had higher bone volume in the defect compared to the empty defect control. Furthermore, bone formation in the immobilized ALP/FS treated group was statistically significant compared to FS alone group. However, the response was not robust.

Keywords

Alkaline phosphatase; Fibrin; Bone tissue engineering; Phosphate; Pyrophosphate

1. Introduction

Inorganic phosphate (P_i) has been reported to upregulate osteopontin (OPN) expression in MC3T3 cells [1] as well as cementoblasts by regulating OPN gene transcription [2,3] and could directly stimulate human aortic smooth muscle cells to undergo an osteochondrogenic

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phenotypic change [4]. It has been proposed as a signaling molecule for osteoblast differentiation [1]. Indeed, addition of P_i or β -glycerophosphate, which is broken down to P_i by alkaline phosphatase (ALP), has been shown to promote mineralization by osteoblasts *in vitro* [5–7], suggesting the important role of P_i in osteoblast differentiation and mineralization.

ALP is a metalloenzyme that is expressed in several tissues. ALP functions as an ectoenzyme bound to the plasma membrane through a phosphatidyl inositol-glycophospholipid linkage [8]. The catalytic mechanism involves the formation of a serine phosphate at the active site, which reacts with water at alkaline pH to release P_i from the enzyme [8]. Four ALP genes, intestinal, placental, placental-like and tissue non-specific ALP (TNAP), have been found in humans [8]. TNAP is a well-known marker for osteoblast differentiation. TNAP degrades inorganic pyrophosphate (PP_i), an inhibitor of hydroxyapatite formation, and generates P_i [9]. TNAP knockout mice show a hypophosphatasia phenotype similar to that of humans, especially the infantile form [10]. Primary calvarial osteoblasts isolated from TNAP knockout mice show decreased mineralization [11] and mineralization is delayed in primary calvarial cells isolated from TNAP heterozygous mice [11]. These results suggest a critical role for ALP in bone formation.

Pore size, pore interconnections and surface roughness of scaffolds have been shown to regulate osteoblast behavior including attachment, proliferation and differentiation [12–14]. Previously, we reported a sphere-templating technique to fabricate fibrin scaffolds with tightly controlled pore sizes and pore interconnections [15]. In these scaffolds, polymerized fibrin fibers make up the walls surrounding pores in the size range from 40 to 80 nm [15]. We observed excellent osteoblast cell adhesion, proliferation and differentiation on these scaffolds [16]. Incorporation of calcium phosphate crystals into the sphere-templating fibrin scaffolds promoted bone formation in a mouse critical calvarial defect model, indicating their osteoconductive property [16]. Bone formation was also enhanced by addition of rhBMP-2 [16]. Together, these data suggest that microporous nanofibrous fibrin scaffolds with tightly controllable architecture could be useful as bone regenerative materials.

The aims of this study were to establish a method to immobilize ALP on microporous nanofibrous fibrin scaffolds, and to determine the ability of these scaffolds to mineralization and osteoblast differentiation *in vitro* and *in vivo*.

2. Materials and Methods

2.1 Fibrin scaffold preparation and ALP immobilization

Microporous, nanofibrous fibrin scaffolds (FS) were fabricated using a sphere-templating method as described previously [15,16]. Briefly, poly(methyl methacrylate) (PMMA) (Polysciences, Inc.) beads (size 200–250 μ m) were close-packed and sintered at 145°C for 22 h. Bovine fibrinogen (Sigma) solution (200 mg of bovine fibrinogen in 1 mL of 0.9% NaCl solution) was cast onto PMMA bead templates. Bovine thrombin (Sigma) solution (267 U of thrombin in 2.67 mL, 133 μ L of 2N $CaCl_2$, 17.2 mL of Dulbecco's Modified Eagle's Medium (DMEM; Gibco)) was added to initiate fibrin formation at room temperature for 24 h. The PMMA templates were dissolved in several rinses of acetone over 72 hours and the scaffolds rehydrated and sterilized in 70% ethanol overnight, followed by several rinsings in sterile phosphate buffered saline (PBS).

The immobilization method was modified from Karageorgiou et al [17]. COOH groups of the fibrin scaffolds were activated by reaction with 0.4 mg/mL 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC)/0.6 mg/mL N-hydroxysuccinimide (NHS) (Pierce) solution in coupling buffer for 15 min at room temperature. The scaffolds were rinsed and incubated with intestinal ALP (Sigma) for 2 h at room temperature on a shaker. Scaffolds were

then rinsed four times with PBS for 15 min each on a shaker. In some experiments, control scaffolds with adsorbed ALP were prepared using the protocol described above but omitting the EDC/NHS activation step. Sodium dodecylsulfate (0.1% (w/v)) was used to remove nonspecifically bound protein for 30 min at room temperature in the protein immobilization stability experiment. All procedures were performed under sterile conditions.

2.2 Scanning electron microscope (SEM) analysis

Samples were fixed with 2% glutaraldehyde (Sigma) at room temperature for 1 h before dehydration. All samples were sputter coated with Au/Pd and then examined with SEM (FEI Sirion SEM) at accelerating voltage of 10 kV. The chemical composition of scaffolds was determined using energy dispersive x-ray analysis. The EDX spectra were randomly recorded in 5 areas for each sample (n=3).

2.3 ALP activity assay

The method used to determine the activity of immobilized ALP on the scaffolds was modified from Taylor et al [18]. Briefly, scaffolds were incubated in 0.5 mg/mL *p*-nitrophenol phosphate in glycine buffer solution. Every five minutes, a 90 μ L aliquot of the solution was removed and placed in 96 well plates containing 10 μ L of a stop solution (0.1 M NaOH and 0.1 M EDTA). The absorbance was measured at 405 nm. The enzyme activity was calculated using formula:

$$\text{Activity} = A_{405} (V_{\text{reaction}} / V_{\text{aliquot}}) / \beta$$

where A_{405} is the absorbance at 405 nm, V_{aliquot} is the volume of the aliquot read, V_{reaction} is the volume of the reaction and β is the extinction coefficient for *p*-nitrophenol (18.5 at 405 nm).

2.4 Calcium assay

The scaffolds were washed with sterile water, snapped frozen in liquid nitrogen, and lyophilized. The dry weight of the scaffolds was recorded. Calcium within the scaffolds was solubilized in 0.6 N HCl at 37°C for 24 h. Calcium content of the supernatant was determined using cresolphthalein complexone (Teco Diagnostics). The absorbance of samples was measured at 575 nm. The calcium concentration was calculated using a standard curve and normalized to dry weight.

2.5 Phosphorus assay

Aliquot of culture medium (10 μ L) was collected at 30 min, 24 and 48 h for each cycle of medium changing. Phosphorus content was determined using ammonium molybdate kit (Teco Diagnostics). The absorbance of samples was measured at 650 nm. The phosphorus concentration was calculated using a standard curve. For phosphorus content in scaffolds, the scaffold was solubilized in HCl and the supernatant was used for analysis. The phosphorus concentration was then normalized to dry weight.

2.6 Cell isolation and culture

Primary calvarial cells were isolated from the parietal bone of newborn C57/BL6 mice using methods described previously [16]. The calvarial cells from fraction 3–6 were pooled and cultured in growth medium (DMEM (Gibco) supplemented with 1% Penicillin-streptomycin (PS; Gibco), and 10% fetal bovine serum (FBS; Gibco)) and maintained in humidified 95%, 5% (v/v) CO₂ at 37°C. The medium was changed every 48 h. The cells from passage 2–4 were used in the experiments. Aliquots of calvarial cell suspension (2×10^6 cells/mL) (50 μ L) were seeded on the top surface of scaffolds and cells were allowed to attach for 30 min. The scaffolds

were transferred to fresh plates and osteogenic medium (DMEM supplemented with 1% PS, 10% FBS, 10 mM β -glycerophosphate (Sigma), 50 μ L/mL L-ascorbic acid (Sigma) and 100 nM dexamethasone (Sigma)) (500 μ L) was added.

For the mineralization experiments, MC3T3 cells were maintained in growth medium in humidified 95%, 5% (v/v) CO₂ at 37°C. The medium was changed every 48 h. Cells were trypsinized and seeded in 24 wells-plated at density 100,000 cells/wells and maintained in osteogenic medium. Intestinal ALP (Sigma) was added to yield final concentration 100 μ g/mL. In some experiments, levamisole (Sigma), an inhibitor of ALP, and foscarnet (Sigma), an inhibitor of sodium-dependent phosphate cotransporters, were added at final concentration 1 mM and 0.1 mM, respectively.

2.7 Mineralization assay

Mineralization was evaluated using a calcium assay, as described above, and Alizarin Red S staining. For Alizarin Red S staining, cells were rinsed twice with PBS and stained with 0.5% Alizarin Red S (Sigma) for 30 min at room temperature on shaker. Cells were then washed briefly with borate buffer and PBS.

2.8 Cytotoxicity assessment

An indirect cytotoxicity test was performed using an elution method as described previously [16]. NIH 3T3 cells were plated at 25,000 cells/wells in 96-well plates and maintained in humidified 95%, 5% (v/v) CO₂ at 37°C. At 24 h, the medium was removed and cells were rinsed twice with PBS. The scaffold's extraction medium (500 μ L) was added to the cells. At 24 and 48 h, cell morphology was examined using phase contrast microscopy. Cell viability was evaluated using an MTT assay.

2.9 Proliferation study

Cell proliferation was determined using an MTT assay. At designated time points (1, 3, and 7 d), scaffolds seeded with calvarial cells were transferred into new 48 well plates and rinsed twice with PBS. Fresh culture medium with MTT (Sigma; 0.5 mg/mL) was added and MTT assay was performed. Total cell number was calculated using a standard curve of absorbance versus cell number.

2.10 RNA isolation and RT-PCR

Scaffolds seeded with calvarial cells were collected at days 7, 14 and 21 and were then pulverized. Total RNA was extracted using Trizol reagent (Invitrogen), following the instructions provided. RNA (1 μ g) was used to synthesize first strand cDNA by reverse transcriptase (Invitrogen). For the polymerase chain reaction, aliquots of synthesized cDNA were added to PCR mixtures containing Taq polymerase (Sigma) and cycled on a DNA thermal cycler. Primers for PCR were as follows: 1) Alkaline phosphatase (ALP) fwd 5' GCCCTCTCCAAGACATATA 3', ALP rev 5' CCATGATCACGTCGATATCC 3', 2) Bone sialoprotein (BSP) fwd 5' GAGCCAGGACTGCCGAAAGGAA 3', BSP rev 5' CCGTTGTCTCCTCCGCTGCTGC 3', 3) Collagen type I (COL I) fwd 5' GAGGCATAAAGGGTCATCGTGG 3', COL I rev 5' CATTAGGCGCAGGAAGGTCAG 3', 4) Osteocalcin (OCN) fwd 5' CAGCTTGGTGACACCTAGC 3', OCN rev 5' AGGGTTAAGCTCACACTGCTCC 3' 5) Core binding factor I (CBFA1) fwd 5' CGCATTCCTCATCCCAGTAT 3', CBFA1 rev 5' GGTGGCAGTGTCATCATCTG 3', 6) GAPDH fwd 5' ACCACAGTCCATGCCATCAC 3', GAPDH rev 5' TCCACCACCCTGTTGCTGTA 3'. PCR products were then electrophoresed on a 1.2% agarose gel and visualized by ethidium bromide fluorostaining. The density of bands was determined using ImageJ software.

2.11 Scaffold preparation for in vivo experiments

Disc-shaped scaffolds 5 mm in diameter and 0.5 mm thick were employed in mouse calvarial defect experiments. Endotoxin test was performed prior to implantation. In some experiments, 3.75 µg rhALP (R&D systems) in 20 µl sterile PBS was immobilized on the scaffolds using the protocol described above.

2.12 Mouse calvarial defect studies

The protocol was described previously [16] and approved by IACUC, University of Washington. Briefly, C57/BL6 mice were anesthetized by an intraperitoneal injection of xylazine and ketamine cocktail. Twelve 5-week-old mice were used. Defect diameter of 5 mm in parietal bones was generated using a hand drill trephine burr with constant saline irrigation. Scaffolds were placed in the defects. At 45 days after surgery, animals were euthanized and the calvaria were removed and prepared for microcomputerized-tomography (µCT) and histological analyses. The mice were divided into groups as follows: a) Empty defect controls, b) FS alone, c) immobilized ALP/FS.

2.13 Microcomputerized-tomography analyses (microCT)

Tissues were fixed with 10% buffered formalin for 24 h at 4°C. Mineral formation within the defect was determined using microcomputerized-tomography. Samples were scanned at a resolution of 35 µm utilizing 50kV with 0.5 mm aluminium filter (Skyscan 1076). After reconstruction, analysis was done using CTAn software (Skyscan).

2.14 Histological analyses

After microCT imaging, the tissues were cut in half, and were decalcified in acid formalin solution (4% formalin, 10% acetic acid solution) for 2 weeks at 4°C. The samples were dehydrated in graded alcohol and embedded in paraffin. The sections (5 µm) were stained with H&E and Trichrome. For OPN immunohistochemistry, tissue sections were rehydrated in ethanol, then endogenous peroxidase was blocked with 0.2% H₂O₂ (Sigma). Four percent of serum (Vector) was applied to block nonspecific binding. Sections were incubated in primary antibody; OPN (R&D, 1:200). Normal IgG was used as control. Detection was performed using DAB substrate (Sigma).

2.15 Statistical analyses

The data are expressed as mean±standard deviation (SD). Statistical significance was assessed by analysis of variance (ANOVA) followed by Turkey's post hoc test. Dunnett post hoc test or Least Difference post hoc test were used to compare test groups in some experiments. Differences at $p<0.05$ were considered to be statistically significant.

3. Results

3.1 Effect of ALP on osteoblast mineralization

MC3T3 cells were cultured in DMEM supplemented with β-glycerophosphate and L-ascorbic acid. Addition of ALP (100 µg/mL) resulted in a significant increase in mineralization compared to control at both 7 and 14 days (Fig. 1). The mineralization was markedly inhibited by addition of foscarnet and partially inhibited by addition of levamisole. Although, foscarnet and levamisole treated groups showed higher mineralization compared to control at day 7, no statistical difference was observed. When incubating ALP in culture medium without cells, no positive staining of alizarin red was observed (data not shown).

3.2 Material characterization

Consistent with previous findings [15,16], the sphere-templating fabrication method used here led to the formation of nanofibrillar FS with tightly controlled pore sizes and interconnections. As shown by SEM in Fig 2a, FS exhibited a homogeneous, well-interconnected, highly porous structure. At higher magnification, the FS exhibited a fibrillar wall ultrastructure (Fig. 2b).

After immobilizing ALP on the fibrin scaffolds using EDC, ALP enzymatic activity was directly measured using p-nitrophenol phosphate as substrate. The ALP activity of immobilized ALP/FS increased with increasing concentrations of ALP immobilization solution (Fig. 3A). Significant increases in ALP activity were observed at ALP immobilization solutions of 1mg/mL and 2.5 mg/mL compared to those of control fibrin scaffolds.

The ALP activity of absorbed ALP/FS was lower than that of immobilized ALP/FS (Fig. 3B) when similar ALP concentrations were compared. ALP enzyme stability in the fibrin scaffolds was examined for 35 days. For absorbed ALP/FS, the enzyme activity decreased about 80% after 7 days of incubation and by 90% within 35 days (Fig. 3B). In contrast, 50% of ALP was retained on the immobilized ALP/FS at 7 days, and 30% at 35 days after incubation. At all time points, the ALP activity of immobilized ALP/FS was significantly higher compared to both control FS and absorbed ALP/FS.

Next, the efficiency of the immobilized ALP/FS to liberate P_i from an inorganic phosphate donor, β -glycerophosphate, was examined. The scaffolds were incubated in osteogenic medium without cells for 21 days, with fresh medium changes every 48 h. The P_i concentration in the culture medium was measured at 30 min, 24 h and 48 h following each media change as shown in Fig. 4A. P_i concentrations in the culture medium were similar between samples containing FS and no scaffold (media) over 21 days. In contrast, P_i levels in the culture medium containing immobilized ALP/FS increased significantly compared to control following the first media changes (Fig. 4A). At the second media change, P_i levels also increased, but not as great an extent as that seen after the first media change. By the third media change, P_i levels did not rise above controls (Fig. 4A). To determine why P_i levels decreased in the media with subsequent media changes, we examined the scaffolds for potential phosphate crystal deposition. As shown by SEM, mineral crystals were associated with the immobilized ALP/FS scaffold surface (Fig. 4Bb), whereas no mineralization was observed with FS (Fig. 4Ba). EDX spectra were obtained to determine the chemical compositions of the scaffolds. Carbon (C), nitrogen (N), and oxygen (O) were detected in both FS and immobilized ALP/FS (Fig. 4B). However, calcium (Ca) and phosphate (P) peaks were observed only in the immobilized ALP/FS. The calcium and phosphate content in the scaffolds were further investigated using the calcium and phosphorus assay. Both calcium and phosphate content in the immobilized ALP/FS were significantly increased compared to FS when incubated the scaffolds in osteogenic medium without cells for 21 days (data not shown).

3.4 Calvarial cell morphology, proliferation and differentiation on immobilized ALP fibrin scaffolds

To examine the ability of the scaffolds to support cellular growth and function relevant to bone formation, they were seeded with murine calvarial cells. Calvarial cell numbers increased to an equivalent extent on both FS and immobilized ALP/FS scaffolds from 1 day to 7 days (Fig. 5). Consistent with this, SEM analysis indicated a high cell density on the surface of the scaffolds at 7 days (Fig. 6). In addition, cells were able to adhere, spread, and form cell-cell contacts as indicated by the flattened and polygonal morphology of the cells covering the scaffold wall (Fig. 6a, 6b). At 21 days, multiple cell layers and the accumulation of fibrillar, extracellular substances were observed (Fig. 6c, 6d), suggesting that the cells could form

differentiated nodules. Mineral crystal precipitation was observed on the scaffolds and on cell membranes in the immobilized ALP/FS as early as 14 days in culture (Fig. 6e, 6f).

To further analyze calvarial cell differentiation on the scaffolds, RT-PCR was used to assess mRNA expression of osteoblastic markers at 7, 14 and 21 days (Fig. 7). Transcripts for ALP, BSP, COL I, and CBFA1 increased in cells exposed to either 0.1 mg/ml or 1.0 mg/ml immobilized ALP/FS at 7, 14 and 21 days. On the other hand, mRNA for the late differentiation marker, OCN, was observed only in the 1.0 mg/ml immobilized ALP/FS samples at 21 days.

3.5 Bone regeneration in mouse calvarial defect model

A critical size defect (5 mm) model in mouse calvarias was utilized to determine the ability of scaffolds to support bone regeneration. The defects were either treated with FS, immobilized ALP/FS or left empty and then examined using micro-CT as well as histological analyses at 45 days following injury.

No obvious fibrosis or ectopic calcification of the scalp in the calvarial defect was observed following gross inspection of the specimens. Micro-CT scans were performed to quantitate bone formation in the defects. In control empty defects, radiopacity was observed along the margin of defect with very small radiopaque areas noticed inside the defect (Fig. 8). FS alone showed scattered radiopaque areas in the defect. The immobilized ALP/FS exhibited both large and small radiopaque areas within the defect. The percentage of bone volume per total tissue volume (BV/TV) was calculated using CTAn software (Fig. 8). Defects treated with either FS or immobilized ALP/FS showed significantly higher BV/TV compared to empty defects. Moreover, more bone formation was noted in the immobilized ALP/FS vs FS alone treated group. However, while this effect was significant the differences between these two groups were not impressive.

Finally, paraffin-embedded tissues were sectioned and stained with H&E to evaluate tissue morphology. Consistent with previous findings [16], only a loose, thin connective tissue was observed within the empty defect, as shown in Fig. 9a-c. The majority of the defect filled with immobilized ALP/FS scaffolds contained dense connective and nodule-like tissue (Fig. 9d). The matrix in these nodules was composed of collagen and OPN as observed by Trichrome and OPN immunohistochemistry staining (Fig. 9e and f). However, some samples showed small areas of woven bone in the defect (Fig. 9g-i). FS scaffolds showed similar histomorphology (data not shown).

4. Discussion

In this study we describe the development and characterization of immobilized alkaline phosphatase containing fibrin scaffolds with tightly controlled pore sizes and pore interconnections. An ALP immobilization scheme that retained high levels of enzymatic activity was established, and shown to support the generation of P_i from an organic phosphate donor. Moreover, immobilized ALP/FS supported the proliferation and differentiation of primary calvarial cells *in vitro*. Using a murine calvarial defect model, immobilized ALP/FS promoted bone formation to a great extent when compared to the empty defect. A trend for an increase in bone formation compared to FS alone was noted. These observations support the idea that addition of factors to fibrin scaffolds facilitates bone healing and regeneration, and further, that factors enhancing local elevation of P_i may assist in enhancing bone formation. However, the results here suggest additional factors and/or procedures are required to develop a more robust response, *in vivo*.

Biomaterials modulating the local concentration of P_i such as bioactive phosphate glass have been investigated for the potential to support proliferation and promote differentiation of

osteoblasts [19,20]. Unfortunately, bioactive glass materials have a slow resorption rate and the material remaining at the site of the defects may result in a mechanical mismatch between materials and natural regenerated bone, leading to fracture. The fibrin scaffolds used in this study have been shown to degrade completely *in vivo* [16]. Therefore, they are useful as a scaffold base for modulating local P_i concentration or other factors required for bone regeneration.

We showed that addition of ALP into MC3T3 cell culture resulted in increased mineralization as early as 7 days. Addition of foscarnet or levamisole resulted in inhibition of mineralization. These results are consistent with previous studies showing that levamisole inhibits β -glycerophosphate-induced mineralization [5,7,21,22]. However, in our study, levamisole only partially inhibited ALP-induced mineralization, most likely because it was less effective in inhibiting intestinal ALP [23–25], which we used in this study. Foscarnet, a competitive inhibitor of sodium dependent phosphate cotransporters, also inhibited ALP-induced mineralization, consistent with previous reports that foscarnet can inhibit both β -glycerophosphate-induced and NaH_2PO_4 -induced mineralization [5,26]. Moreover, subcutaneous injection of foscarnet around calvaria of newborn rats resulted in local mineralization defects [26]. These results indicated that ALP-induced mineralization in osteoblasts required both the formation of P_i by ALP, and the influx of P_i through sodium dependent cotransporters. The requirement of sodium dependent phosphate cotransporters in mineralization of osteoblasts [26] and smooth muscle cells [27,28] has previously been demonstrated.

Addition of NaH_2PO_4 or β -glycerophosphate into a human osteosarcoma cell line (SaOS-2) resulted in the induction ALP mRNA expression and enzymatic activity [5,29]. In addition, glycerol, one of the products of ALP induced hydrolysis of β -glycerophosphate, had no effect on ALP activity [5]. Together, the expression of ALP in osteoblast was induced by signals obtaining from P_i . Previous studies reported that P_i induced expression of osteopontin [1,30] and sodium-dependent vitamin C transporter 2 in MC3T3-E1 cells [31] as well as matrix Gla protein in mouse chondrocytes [32]. In this study, we also show that primary calvarial cells cultured on immobilized ALP/FS exhibited higher ALP mRNA expression compared to control FS at 7 and 14 days, confirming previous finding. These results suggest that the immobilized ALP on the fibrin scaffolds degraded β -glycerophosphate and resulted in higher P_i concentration in culture medium. The increased concentration of P_i then induced the expression not only ALP but also other osteoblast marker mRNAs i.e. CBFA1, Col I, OCN.

Loss of enzyme activity over time is a major concern for potential use of the scaffolds to increase local concentration of P_i . In this study, we showed that the ALP activity was better retained in immobilized ALP/FS compared to absorbed ALP/FS at 35 days after incubation, indicating that enzyme stability was improved upon immobilization to the fibrin matrix. Loss of enzymatic activity during the incubation period might have occurred for several reasons. First, prolonged incubation at 37°C might have led to inactivation of the enzyme, potentially by denaturation or degradation. Second, the deposition of mineral on the scaffolds during the incubation period may have blocked substrate access to the active site of ALP. Last, covalent binding of the enzyme to the fibrin scaffold might have initiated a conformational change in ALP that negatively affected its enzymatic activity. However, we showed in this study that immobilized ALP/FS promoted mineral deposition when incubated in medium supplemented with β -glycerophosphate, suggesting that the biological activity of ALP on immobilized ALP/FS was not compromised by this coupling method.

To promote mineralization *in vivo*, the P_i/PP_i ratio should be increased. This environment can be obtained in two ways; the enzymatic breakdown of PP_i into P_i or simply delivery of P_i , to increase its concentration at local sites. Attempts to use P_i as an inductive molecule for bone

formation have been reported. Inorganic polyphosphates (Poly(P)), the other source of P_i , induced OPN, OCN, OPG and Col I α mRNA expression and also increases ALP activity as well as bone nodule formation [33]. In addition, using a rat periodontal defect model, Hacchou et al showed that Poly(P) facilitated bone regeneration to a great extent when compared with vehicle alone samples [33]. Further, a preliminary clinical trial using Poly(P) for bone regeneration has been reported. Yamaoka et al provided radiographic evidence for bone regeneration in some patients with periodontal disease treated by subgingival irrigation with Poly(P) [34]. However, no significant difference of clinical parameters between treatment and control group was observed [34]. Lack of improvement in clinical outcome observed in this study may be explained by several reasons including the delivery methods as well as the baseline of disease and health of an individual. These data, while limited, provide support for a role of P_i as a clinical therapy for bone regeneration.

In the study reported here, immobilized ALP/FS was expected to increase P_i /PP $_i$ ratio in calvarial defect by breaking down PP $_i$, resulting in an increase in the local concentration of P_i . We found that immobilized ALP/FS demonstrated a significant increase in the percentage of bone volume per total volume (BV/TV) compared to the empty defect. Although, a significant increase of BV/TV was noted when compared to FS, the response was not robust. There are several explanations for these results including the possible limit in the availability and concentration of local substrates with potential to yield P_i , local pH, and the possibility of inhibitors of ALP at the local site, naturally present locally or in response to the high concentration of ALP. Further investigations using immobilized ALP/FS in the calvarial model along with an active substrate will assist in determining why the outcome was not as significant as we anticipated.

5. Conclusion

ALP has been proposed as a crucial molecule in bone formation. The immobilized ALP/FS described in the present study are non-toxic, biodegradable, and support osteoblast-like cell proliferation and differentiation *in vitro*. Furthermore, the immobilized ALP/FS supported bone formation to a limited degree in a mouse calvarial defect model. Further studies are required to improve the outcome of ALP/FS prior to consideration for clinical use.

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Figure 1.

Effects of alkaline phosphatase (ALP) on mineralization. The upper panel showed alizarin red staining of cell culture for each treatment at 7 and 14 days after addition of alkaline phosphatase, foscarnet or levamisole or the combinations. The lower panel represented the calcium content per total protein of different treatments. The bars indicated statistical significance at $p < 0.05$. The asterisk indicated statistical significance at $p < 0.05$ compared to control

Figure 2.
(a) SEM micrograph illustrated the structure of microporous nanofibrous fibrin scaffolds. (b) Higher magnification SEM micrograph demonstrated the surface texture of the microporous nanofibrous fibrin scaffold wall structure.

Figure 3.

(A) Alkaline phosphatase activity of microporous nanofibrous fibrin scaffolds immobilized with various doses of alkaline phosphatase. (B) Alkaline phosphatase activity of immobilized alkaline phosphatase fibrin scaffolds incubated in PBS at 37°C. The bars indicated statistical significance at $p < 0.05$. The asterisk indicated statistical significance at $p < 0.05$ compared to control.

Figure 4.

(A) Inorganic phosphorus in osteogenic medium incubated with control and immobilized alkaline phosphatase fibrin scaffolds for various time points. (B) SEM showed the surface of (a) fibrin scaffolds and (b) immobilized alkaline phosphatase fibrin scaffolds. The EDX spectra illustrated the chemical composition of the scaffold surface. The asterix indicated statistical significant at $p < 0.05$ compared to control.

Figure 5.
(A) Proliferation of primary calvarial cells on control and immobilized alkaline phosphatase fibrin scaffolds. The bars indicated statistical significance at $p < 0.05$.

Figure 6. SEM showed (a,c) the control and (b,d) immobilized alkaline phosphatase fibrin scaffolds after seeding with primary calvarial cells for 7 (a,b) and 21 (c,d) days. Mineral deposition associated with primary calvarial cells after cultured on immobilized alkaline phosphatase fibrin scaffolds for 14 days was shown in (e,f).

Figure 7.

Osteoblast marker gene expression of primary calvarial cells cultured on the scaffolds in osteogenic medium for 7, 14 and 21 d. lane 1: control fibrin scaffolds; lane 2: immobilized alkaline phosphatase fibrin scaffolds concentration 0.1 mg/mL; lane 3: immobilized alkaline phosphatase fibrin scaffolds concentration 1 mg/mL.

Figure 8. Representative microCT images of mouse calvarial defects at 45 days after surgery. The graph illustrated the percentage of bone volume per total volume of the calvarial defect. The bars indicated statistical significance at $p < 0.05$.

Figure 9. Representative histological images of mouse calvarial defects at 45 days after surgery. (a-c) the empty defects; (d-i) immobilized recombinant human alkaline phosphatase fibrin scaffolds.