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PRE-MINERALISATION EFFECT OF NANOBIOCOMPOSITE BONE SCAFFOLD TOWARDS BONE MARROW-DERIVED STEM CELLS GROWTH AND DIFFERENTIATION

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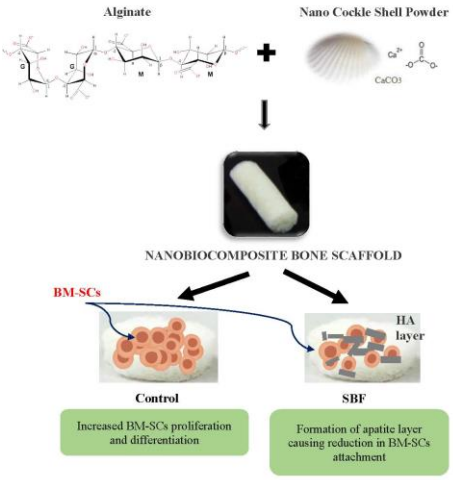
Graphical abstract



Abstract

Apatite layers formed by simulated body fluid (SBF) on the surface of calcium-based scaffolds have been proven to enhance the osteoblastic activity of pre-osteoblasts and osteogenic activity of bone marrow-derived stem cell (BM-SCs). Previously developed Alginate/Cockle shell powder nanobiocomposite bone scaffold (Alg/nCP) has been shown to possess excellent osteoconductive properties. The effect of pre-mineralization of the scaffold surface towards the growth and differentiation of BM-SCs' were evaluated using microscopic and biochemical methods in scaffolds divided into SBF pre-treated and control groups at two time points. MTT proliferation assay showed statistically significant decrease in cell proliferation in SBF group for both culture periods. SEM observation revealed growth of BM-SCs and scaffold surface mineralisation and calcium deposition in both groups with higher intensity observable in the control group. Supporting biochemical studies showed a significant decrease in alkaline phosphatase (ALP) level indicating a lesser osteogenic differentiation in the SBF group as compared to control. Pre-mineralisation of scaffolds in SBF produced a contradicting result in which it did not provide a better environment for growth and proliferation of BM-SCs. However, the Alg/nCP scaffold did show potentials in supporting the osteogenic differentiation of the stem cells.

Keywords: Nanobiocomposite bone scaffold, simulated body fluid, bone marrow-derived stem cell, mineralisation, osteogenic differentiation

Abstrak

Lapisan apatit yang dibentuk oleh larutan simulasi cecair badan (SBF) pada permukaan perancah berasaskan kalsium telah terbukti dapat meningkatkan aktiviti osteoblastik pre-osteoblas dan aktiviti osteogenik sel stem dari sumsum tulang (BM-SCs). Perancah tulang nanobiokomposit alginat/serbuk cangkerang kerang (Alg/nCP) dibuktikan melalui beberapa kajian terdahulu untuk memiliki sifat osteokonduktif yang baik. Kesan pra-mineralisasi permukaan perancah terhadap pertumbuhan dan pembezaan BM-SCs telah dinilai dalam perancah yang dibahagikan kepada kumpulan pra-rawat SBF dan kumpulan kawalan pada dua tempoh masa dengan menggunakan kaedah mikroskopik dan biokimia. Ujian MTT menunjukkan penurunan proliferasi sel secara signifikan dalam kumpulan SBF bagi kedua-dua tempoh. Pemerhatian SEM menunjukkan pertumbuhan BM-SCs serta mineralisasi permukaan perancah dan pemendapan kalsium dalam kedua-dua kumpulan dengan intensiti yang lebih tinggi dalam kumpulan kawalan. Kajian biokimia yang lain menunjukkan penurunan yang signifikan terhadap aras enzim alkali fosfatase (ALP) yang menunjukkan

pembezaan osteogenik yang lebih rendah dalam kumpulan SBF berbanding dengan kawalan. Kajian ini menunjukkan pra-mineralisasi perancah dalam SBF tidak menyediakan persekitaran yang lebih baik untuk pertumbuhan dan percambahan BM-SC yang didapati bercanggah dengan kajian terdahulu. Walau bagaimanapun, perancah Alg/nCP telah menunjukkan potensi dalam menyokong pembezaan osteogenik sel-sel stem.

Kata kunci: Perancah tulang nanobiokomposit, larutan simulasi cecair badan, sel stem mesenkim dari sumsum tulang, mineralisasi, pembezaan osteogenik

1.0 INTRODUCTION

Biological substitute material in the form of a three dimensional (3D) scaffold provides an attractive alternative in the quest for repairing and restoring damaged tissues. In the field of bone tissue engineering, the materials are able to provide support and help in the repair of damaged bones. An ideal bone scaffold should possess a few essential characteristics that include biocompatibility, biodegradability, osteoconductivity and a highly porous network that could mechanically enable proper cell growth while remaining stable over the period for in vivo tissue repair [1,2]. Seeding with cells and growth factors as well as surface modifications prior to implantation are alternate methods to improve the performance of the scaffold as a template for cell growth and differentiation. Studies have also shown that incorporation of bioactive component synergistically help in improving bone formation [3, 21, 22]. Surface modification of the scaffold is another method that could efficiently be undertaken using the simulated body fluid (SBF) solution to produce a simple mineralisation effect on the surface of the scaffold. SBF is a solution known to contain ion concentrations almost similar to those of human blood plasma in a condition of pH 7.4 and 37°C. SBF had been applied in bone tissue engineering researches to produce a mineralising effect on calcium-based scaffolds in which soaking scaffolds in the solution help the formation of apatite layer known as hydroxyapatite (HA). The presences of hydroxyapatite on the surface of the scaffold have an enhancing effect towards *in vitro* infiltration and growth of pre-osteoblast [4]. Moreover, the activity of osteoblastic cell also increases due to the presence of HA which can be found naturally in bone [5].

In our previous study, we have successfully fabricated a nanobiocomposite bone scaffold consisting of alginate, a natural polymer and nano cockle shell powder, a ceramic which had been proven to exhibit desired morphological and osteoconductive properties [6]. The fact that this nanobiocomposite scaffold is calcium based, we believe that the surface modification of the scaffold using SBF would produce a mineralising effect that could produce a favouring in situ environment for cell attachment. We attempted to test this effects of pre-mineralisation of the scaffold surface towards the growth and differentiation of bone marrow-derived stem cells (BM-SCs) and the possibility of enhancement in the osteogenic differentiation of the cells. BM-SCs had been widely used in tissue engineering sector for regeneration of damaged bone [7, 8] and is a type of multipotent stem cell with high osteogenic differentiation potential that makes them an excellent choice to be used in bone repair [8]. In the past two decades, the use of stem cells in bone tissue engineering has become a popular choice for bone repair [9]. Numerous studies have been reported in regards to incorporation of stem cells in various types of scaffold materials [10, 23], however it is note worthy that the easiest method of stem cell delivery would be through the use of a 3D structure such as a scaffold [11]. The presence of a scaffold would facilitate the attachement of cells and provide the necessary matrices for growth. In this study, we postulate the probability of a shorter cell growth duration and differentiation when cultured in a mineralized surface of the scaffold compared to an unmineralized scaffold surface.

2.0 METHODOLOGY

2.1 Alginate/Cockle Shell Powder Nanobiocomposite Bone Scaffold (Alg/nCP) Development

The scaffold was developed according to Bharatham *et al*. [6] using nano cockle shell powder and alginate in a ratio of 60:40 w/v. It is fabricated by mixing 0.6 g of nano cockle shell powder into 40 % alginate hydrocolloid solution, followed by stirring at 600 rpm until it is homogenised. The mixture was poured into a customised cylindrical mold and frozen at -20°C for 24 hours before being freeze-dried at -50°C for 24 hours. The lyophilised scaffold was removed from the mold and soaked in 1 % calcium chloride ($CaCl_2$) solution for 20 minutes, washed with deionized water thrice before it was soaked overnight in deionized water to remove unbound $CaCl_2$. The scaffold was re-lyophilised, sterilised and stored at room temperature prior to being used.

2.2 Simulated Body Fluid (SBF) Preparation

10x SBF was prepared according to the methods of Mavis *et al*. as well as Tas and Bhaduri [12, 13]. 60 mL 10x SBF solution was prepared by sequentially dissolving 3.5064 g NaCl (1000 mM), 0.0224 g KCl (5 mM), 0.2205 g CaCl2.2H2O (25 mM), 0.061 g MgCl2.6H2O (5 mM) and 0.0304 g NaH2PO4.H20 (3.62 mM) into 54 mL deionized water at room temperature. 6mL deionized water was added to make the total volume to 60 mL. The SBF stock solution was kept at room temperature in a covered glass bottle. 0.0504 g of NaHCO3 was dissolved in the stock solution prior to pre-mineralisation of scaffolds in it.

2.3 Nanobiocomposite Scaffold Pre-Mineralisation in SBF

18 scaffolds divided into 2 groups representing day 7 and day 14 of the study duration were soaked in 10x SBF for 24 hours in an incubator. SBF solution was removed after 24 hours and each scaffold was rinsed with deionized water to wash off salt residue prior to be used.

2.4 Bone Marrow-derived Stem Cell (BM-SCs) Isolation

Stem cells were isolated from the long bones of three to ten weeks old mice as approved by UKM Animal Ethics Committee (FSK/2017/HEMABARATHY/22-NOV./888-NOV.-2017-JUNE-2018-AR-CAT2). The bones were excised at both ends to expose marrow cavity from which the stem cells from the bone matrix were flushed out using 1-2 mL of Dubelcco's Modified Eagle Medium (DMEM) added with 2 % Penicillin/Streptomycin solution and 10 % fetal bovine serum (FBS). Isolated stem cells were filled into 15 mL centrifuge tube and were centrifuged at 2500 rpm for 7 minutes. The supernatant was removed, and the remaining cell pellet was resuspended with 3-5 mL of DMEM. The resuspended cells and media were then filtered using 40 µm cell strainer to remove unwanted substances. Cell count was carried out before the culturing of the cells in a T-25 flask under standard culture conditions.

2.5 BM-SCs Seeding on Nanobiocomposite Scaffold

36 cylindrical scaffolds (0.5 cm x 0.2 cm) were divided into four groups (9 scaffolds/group) consisting of pre-mineralised scaffolds and unmineralised scaffolds (control). The scaffolds were each seeded with $1x10^5$ cells/scaffold BM-SCs and cultured for 7 days and 14 days prior to microscopy and biochemical studies.

2.6 3-(4, 5-Dimethylthiazol-2-Yl)-2, 5-diphenyltetrazolium Bromide (MTT) Proliferation Assay

Proliferation of BM-SC was evaluated through standard MTT proliferation assay at days 7 and 14 of the culture period [6]. Culture media was removed and replaced by PBS to rinse off any unattached cells on the scaffolds prior the MTT assay.

2.7 Scanning Electron Microscopy with Energy Dispersive X-ray (SEM-EDX)

At days 7 and 14 the culture media was removed, replaced with 2.5 % glutaraldehyde and processed under standard methods for scanning electron microscopy (SEM) viewing. Prepared scaffold was first placed on stub, coated with a layer of gold/palladium (Au/Pd) prior to the imaging process. Energy dispersive X-ray (EDX) analysis was done to determine the presence of the mineral phases.

2.8 Alkaline Phosphatase (ALP) Biochemical Analysis

ALP concentration was measured in the cell lysate by adding 100 µL of p-nitrophenyl phosphate disodium, 40 µL of cell lysate and 60 µL of deionized water into a 96 well plate. The plate was then incubated at 37°C for 1 hour. The reaction was stopped with 100 µL of 0.3 M sodium hydroxide (NaOH) and the absorbance of the mixture was measured with a microplate reader at a wavelength of 405 nm. ALP concentration was obtained from standard curve prepared using p-nitrophenol.

2.9 Statistical Analysis

All quantitative results were analysed using One-way Analysis of Variance (ANOVA). Results were expressed as mean ± standard error of mean. Post hoc test were done for significant values ($p<0.05$) using Tukey's multiple comparison test.

3.0 RESULTS AND DISCUSSION

3.1 3-(4, 5-Dimethylthiazol-2-Yl)-2, 5-Diphenyltetrazolium Bromide (MTT) Proliferation Assay

Figure 1 shows the proliferation of cells of control and SBF group after being cultured for 7 and 14 days respectively. Proliferation of cells was found to have decreased in SBF groups for both the culture periods compared to control. While the proliferation of the stem cells increased significantly in the control scaffolds from day 7 to day 14, the reverse was quantitated for the SBF group. This negative effect of pre-mineralisation of the scaffolds in SBF could be postulated to the fact that high calcium concentration and prolonged exposure to excess calcium ion can exert an adverse effect towards cell proliferation [14]. Pre-mineralisation of the scaffolds in SBF would contribute to the formation of calcium and

phosphate apatites on the surface of the scaffolds which adds to the calcium content of the scaffolds. The additional source of calcium in this group contributes further to the increase in calcium concentration compared to control scaffolds which showed a favourable cellular response. Formation of apatite's on the scaffold surface as an effect of pre-mineralisation could have also contributed to the reduction in cell attachment sites. According to Salzig *et al*. [15], the attachment of certain cells to a surface depends on complementary ionic strength. High calcium concentration in the culture environment can affect the surface chemistry of the scaffold. This, in turn, affects the ionic strength of the scaffold's surface and results in disruption in cell attachment and proliferation on the scaffold matrix.

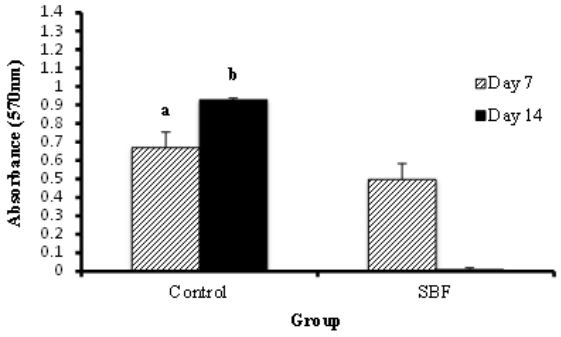


Figure 1 MTT assay on proliferation of BM-SCs on surface scaffold. Data expressed as mean ± standard error of mean, n=9. [a]Significant difference compared to SBF group on day 7 and 14; [b]Significant difference compared to control group day 7, SBF group day 7 and 14 at $p<0.05$

3.2 Premineralisation of Nanobiocomposite in SBF

Figure 2A shows the SEM micrographs of the scaffold surface undergone mineralisation by 10x SBF as compared to an un-mineralised scaffold surface (Figure 2B). Apparent changes in the texture of the scaffold matrix with presences of deposits indicate that the effects of mineralisation on the nanobiocomposite scaffold could be achieved within 24 hours of soaking in 10x SBF. Pre-mineralising of a scaffold increases the surfaces roughness which leads to a favorable cell attachment and proliferation [16].

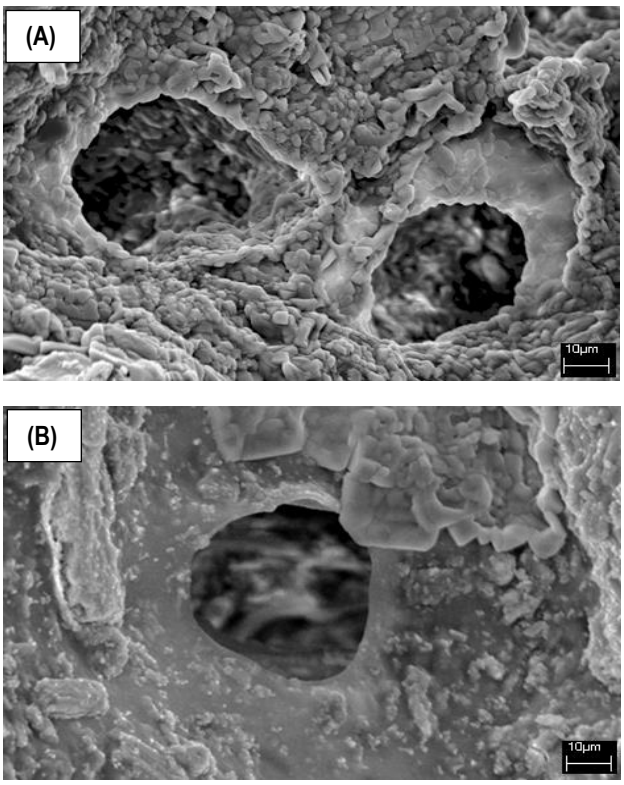


Figure 2 SEM micrograph of scaffold surface 24 hours post SBF mineralisation (A) and scaffold surface of un-mineralised nanobiocomposite scaffold (B) at X1000 magnification

3.3 Scanning Electron Microscopy with Energy Dispersive X-ray (SEM-EDX) of BM-SC's cultured on Nanobiocomposite Scaffolds

Figure 3 shows SEM micrographs of SBF and control scaffolds cultured with BM-SCs for 7 and 14 days respectively. Scaffold from both groups showed evidence of matrix mineralisation with mesh-like fibers observable on both scaffold surfaces by day 14 while control scaffolds showed surface morphological changes as early as day 7 (Figure 2A).

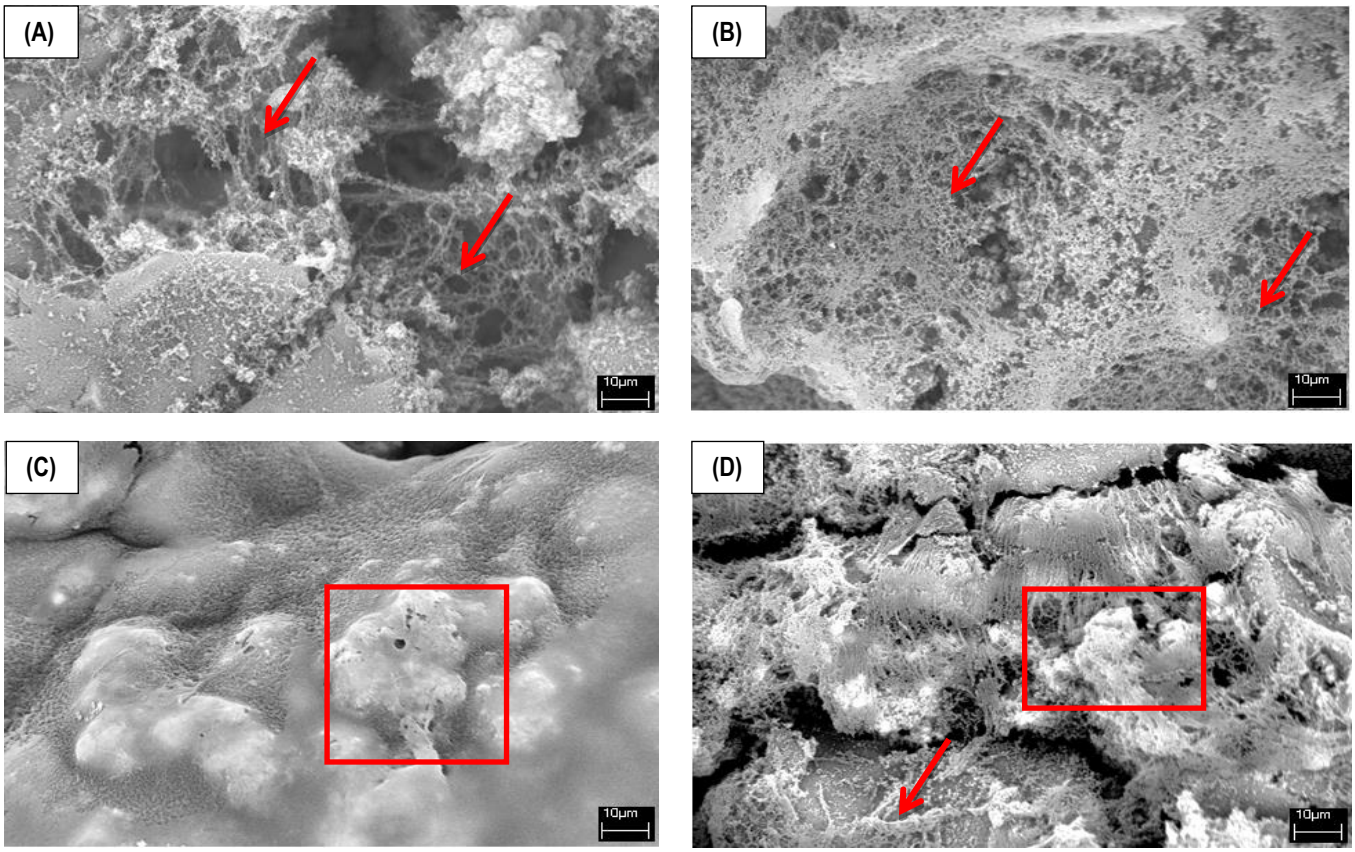


Figure 3 SEM micrograph of BM-SC's growth and proliferation on surface of scaffolds at X500 magnification. Presence of mesh-like mineralized fibers (arrow) and clusters of BM-SCs (box) are observed on surface of the scaffolds. Control scaffolds: Day 7 (A) and Day 14 (B); SBF scaffolds: Day 7 (C) and Day 14 (D)

Mineralisation and clustering of cells on the surface of the scaffolds showed that the nanobiocomposite bone scaffold supported the attachment, growth and osteogenic differentiation of BM-SCs. These mineralisation effects are justified with the detection of calcium (Ca) and phosphorus (P) elements through EDX analysis (Figure 4). The Ca/P ratio of the control scaffold at day 14 as shown in Figure 4B was 1.41, which was found to be within the physiological range of 1.4-1.7 in a healthy bone [17]. This data further supports the potential osteogenic properties of the nanobiocomposite scaffold in which both chemical and topographical factors could have contributed. Presence of calcium ions (Ca^{2+}) and carbonate ions ($(CO_3)^{2-}$) in the scaffold has possible osteogenic differentiation triggering effects towards BM-SCs. Ions such as calcium (Ca^{2+}), phosphate (PO_4^{3-}) and silicon (Si^{4+}) are known as osteogenic inductive stimulants [18, 19]. In addition, the topographical factor of the scaffold also contributes to support the attachment and growth of the cells. Presences of nanoparticle deposits on the matrix of the scaffold (Figure 2B) also contribute as a precursor for cell attachment. However, the surface morphology of the SBF group at day 7 produced scattered clustering of cells on a smooth matrix indicative of a reduction in cell attachment thus reducing the growth and proliferation of the cells on the scaffold by day 14. The pre-mineralised surface of the scaffolds was found to be unfavorable in supporting long term cell growth.

3.3 Alkaline Phosphatase (ALP) Biochemical Analysis

Osteogenic activity of BM-SCs cultured on the scaffolds was evaluated through ALP analysis as shown in Figure 5. ALP enzyme is a membrane-bound enzyme produced by a few types of tissues in the body and a prominent biomarker for bone tissues. Quantification of ALP activity is often taken as an early marker for osteogenic differentiation of stem cells [20]. The enzyme plays an essential role in mineralization of bone as it helps in the production of inorganic phosphate which is required for the formation of hydroxyapatite (HA) as well as to initiate the mineralization process. Amount of cells that attach, proliferate and differentiate on a scaffold surface will affect the concentration of ALP produced.

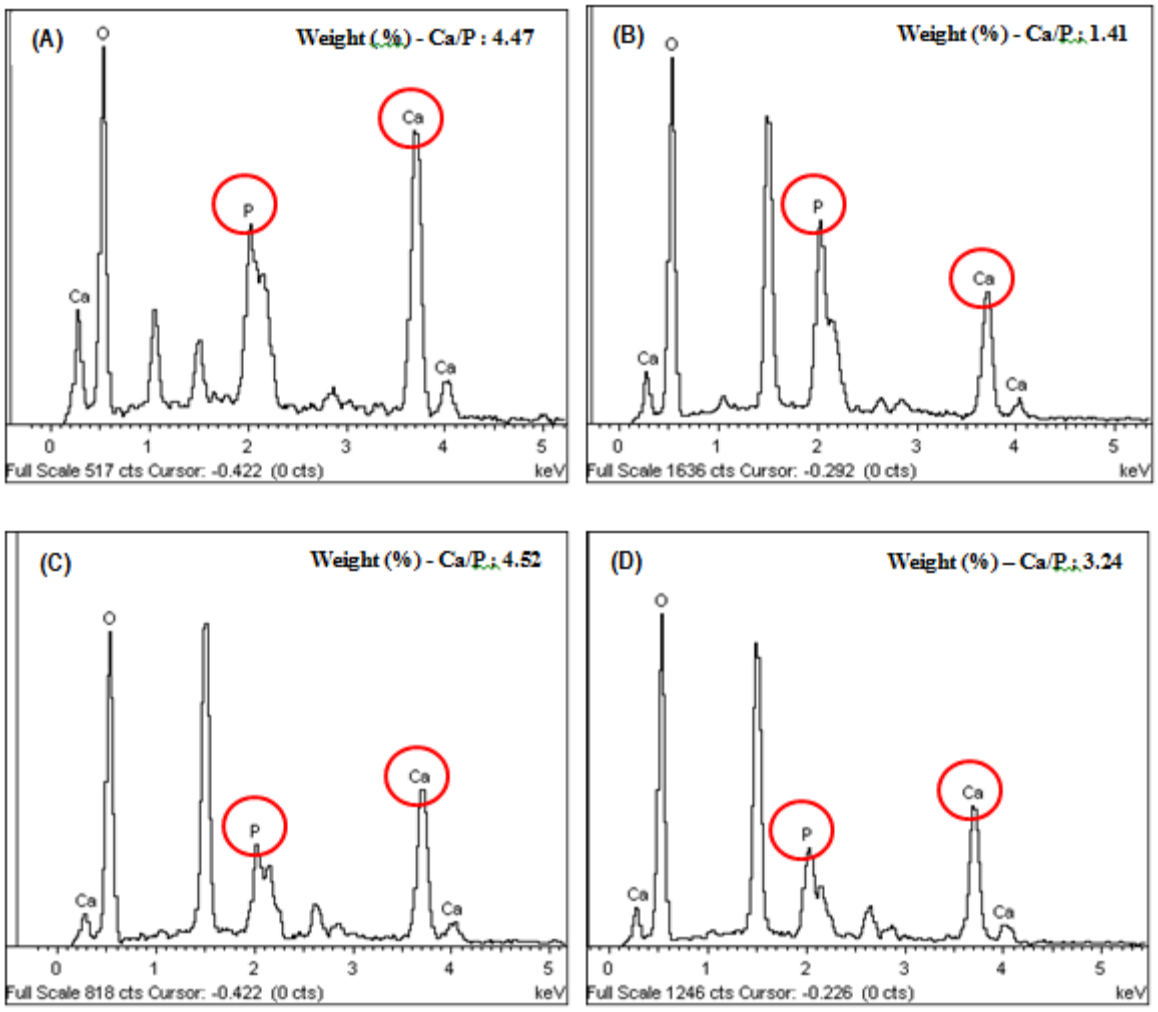


Figure 4 EDX spectrums indicating the presence of calcium (Ca) and phosphate (P) and Ca/P ratio on scaffold's surface. Control scaffolds: Day 7 (A) and Day 14 (B); SBF scaffolds: Day 7 (C) and Day 14 (D)

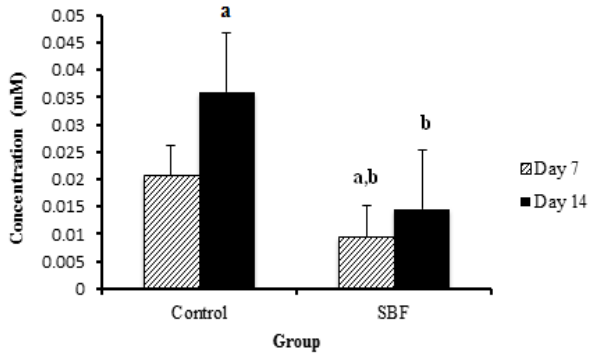


Figure 5 ALP enzyme activity of BM-SCs cultured on scaffolds. Data expressed as mean ± standard error of mean, n=9. [a]Significant difference compared to control group day 7; [b]Significant difference compared to control group day 14 at $p<0.05$

The concentration of ALP was found to be significantly lower in SBF scaffold group compared to control for both days 7 and 14, while a significant increase in the ALP activities was noted from day 7 to day 14 in the control scaffolds. These findings correlate with the results from MTT assay and SEM observation on the proliferation of the BM-SCs on the scaffold surface indicative of favourable cell growth and differentiation activity towards the osteogenic phenotype.

4.0 CONCLUSION

Pre-mineralisation of a scaffold surface is often postulated to produce better microenvironment for cell attachments and growth. However, findings from this study indicated pre-mineralisation of scaffolds in 10x SBF did not provide a better environment to support the attachment, growth and proliferation of BM-SCs on the nanobiocomposite scaffold. This contradicting results show that the possible reduction

in cell binding sites on scaffolds' surface due to prior deposition of minerals as a result from the pre-mineralisation process as well as the presence of high concentration of calcium could have negatively affected the cell binding capacity. However, the study did highlight the potentials of the nanobiocomposite scaffold used in the study to support the growth of BM-SC's and its possible osteogenic differentiation without the need of any enhancing factors.

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