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In Vitro Evaluation of the Cockle Shells Aragonite Nanocomposite Porous 3D Scaffolds For Bone Repair

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Abstract

The opinion of reinstating an injured body has been in existence right from the beginning of mankind with initial account of manifestation as traditions and magical. Three dimensional (3D) porous nanocomposite scaffolds with osteoconductivity and biodegradability properties was fabricated for bone repair using cockle shells aragonite nanoparticles and gelatin as their major components via freeze drying method. *In vitro* cell culture of hFOB (human Fetal OsteoBlast cells line) was seeded on the scaffolds to examine cytocompatibility of the organized scaffolds. Cell attachment, alkaline phosphatase (ALP) concentration, cell proliferation and capability to form mineralized bone nodules were assessed. The results showed a significant difference in ALP concentrations between and within the culture of different scaffold during the experiment period. Microscopic observations revealed that all four compounds sustain cell attachment and cell proliferation. Additionally, the cockle shells aragonite nanocomposite porous 3D scaffolds have shown ideal chemical and physical properties to sustain cell attachment and cell proliferation of the differentiated hFOB cells. Biological evaluation also showed that the scaffold 5211 did enhance the osteoblast proliferation rate and improved the osteoblast function significantly. The ALP concentration, calcium deposition and development of mineralized bone nodules were increased significantly. It can be concluded that the scaffold 5211 fulfilled all the requirements to be considered as an ideal bone replacement.

Keywords: 3D porous scaffolds, Nanocomposites, hFOB, cell behaviour, bone repair.

1- Introduction

Among the important properties and advantages of scaffold tissue engineering is its biological response. Scaffolds play a vital role in tissue engineering field in terms of providing the required three-dimensional (3D) architecture that facilitates the regeneration of new tissues. Tissue culture studies conducted on a 3D system (scaffolds) are known to provide more accurate findings in terms of physiological responses compared to the common 2 Dimensional (2D) culture systems [1]. *In vitro* studies are a useful way to test the suitability of the scaffold's architecture in terms of providing sufficient permeability that acts as a key factor that determines the efficiency of a scaffold [2]. The ability of the scaffold to sustain cells attachment, cell growth and cell differentiation into the

appropriate tissue depends on the pore interconnectivity, chemical properties, physical properties and permeability of the scaffold [3]. The architecture and permeability of the scaffold highly influence cell seeding effectiveness via determining the capacity of the scaffold to uptake nutrients and oxygen that are vital to support cell growth. A highly permeable scaffold is known to have a favorable biological outcome of improving *in vivo* bone formation [4, 5]. In order to determine these characteristics of the developed scaffolds, human osteoblast cell line provides an excellent choice as a cell culture system. These cell lines are proving to be superior candidates for bone scaffolds cell material studies due to their enhanced tissue reaction and high phenotypic similarities to the target tissues [6, 7, 8]. Alkaline phosphatase (ALP) is an initial indicator of differentiation which is accompanying calcification. This enzyme expression increases during the first few days of the defect and immediately before the mineralization of the matrix. It also favors the restricted enhancement of inorganic phosphate, a component of the mineral phase of bone [9]. While the calcium is measured as an indicator of osteoblast maturation and is thought to be capable of calcium ions in forming bones, the mineral that is significant in formation- resorption sequence of the bone [10]. This study is aimed to determine the biological properties of the cockle shell-derived $CaCO_3$ aragonite nanocomposite porous 3D scaffolds (CCANP3DSs) that were developed by assessing the architecture scaffold and evaluating the effects of materials used (cockle shell-derived $CaCO_3$ aragonite nanoparticles (CCAN), gelatin, dextran and dextrin) on cell behavior. In addition, to evaluate the osteoblast cells proliferation and differentiation within these scaffolds.

2- Materials and Method

2.1 The scaffolds

All scaffolds were prepared via freeze drying method [11]. The scaffolds dimensions were 1x1x1 cm and were sterilized using Ultraviolet (UV) irradiation for 5 hours prior to use.

2.2 *In vitro* Culture

hFOB cells 1.19 acquired from American Type Culture Collection (hFOB 1.19(ATCC®CRL-11372TM) which were fully-grown in the cell culture medium in controlled atmosphere according to manufacturer's guide. Cells from each vial were seeded into a T25 culture flask that contains Ham's F12 Dulbecco's Modified Eagle Medium with 2.5 mMl-glutamine (DMEM Ham's F‐ 12 with L & # 8208; Gin, sodium Pyruvate and HEPES, without phenol Red) complemented with 10% Fetal Bovine Serum (FBS: JRS12-43640, USA) and 1% Geneticin Solution (G418, nacalai tesque, Japan) at 34°C temperature and 5% CO_2. The medium was replaced every 2-3 days and the cells were consistently divided into 1:2 every 3-4 days at 80% convergence. The cells were separated using trypsin/EDTA (0.25%-Trypsin/1mM- EDTA Solution, nacalai tesque, Japan), concentrated through centrifugation process for 5 minutes at 1200 rpm and resuspended in fresh medium. Cells between the fourth and fifth passage were used for seeding purposes in the experiments (Figure 1).

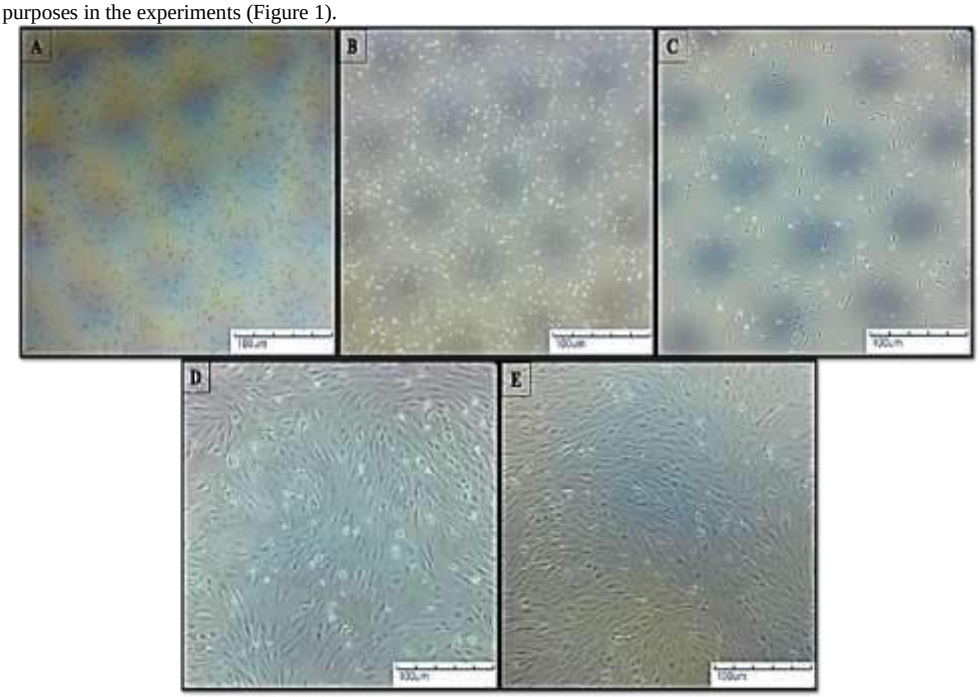

Figure 1: Inverted micrographs of human Fetal OsteoBlast (hFOB) cells, (A) reactivation, (B) 24 hours, (C) 48 hours, (D) 7 days and (E) 10 days of full confluence of cell line culture, (X400).

2.3 Preparation of Scaffolds Extracts

Extracts from all scaffolds were done in accordance to [12] for MTT assay, live/lifeless staining of cell as well as ALP analysis. Sterilized scaffolds prepared earlier were nurtured in 20 ml complete culture medium in different bottles for 24 hours at 34°C to achieve the scaffold leachable. The leachable from every scaffold were later collected into separately labeled falcon tubes kept for further use at 4°C.

2.4 MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphynyltetrazolium bromide) Colorimetric Assay

The assay of MTT was done in accordance to [12] methods. Six well plate were used to seed the hFOB cells at a density of 1 x 10^4 cells/well. Cells were nurtured for 24 hours in normal culturing settings this is to permit primary attachments. Medium were removed from every single well after 24 hours and exchanged with medium that contains the leachable extracts of scaffolds and which was again incubated for 24, 48, and 72 hours for the all seventh scaffolds, and continue the incubation for 5, 7, and 14 days for scaffold 5211. After the period of incubation, the medium with scaffold extracts were taken in every well and substituted with new medium which contains 10% of MTT solution ((3-(4,5-dimethyl-2-thiazolyl)-2,5-diphynyltetrazolium bromide, nacalai tesque, Japan). The cells were later incubated for 4 additional hours at 34°C this is done to achieve the standard MTT assay. Medium were removed after 4 hours and 0.8 ml dimethyl sulfoxide (DMSO, Mediatech, Inc.; A corning Subsidiary, Manassas, VA 20109, USA) was added to conclude the reaction as a solubilization buffer to all the wells for formazan crystals disolution. The lysate absorbance was read at a 495 nm wavelength using ELISA reader (TECAN-infinite M200 PRO) and the result was given as absorbance obtained from each well. This assay was done in triplicats with three replicates per scaffold and three replicates as a control which was cultured with standard culture medium.

2.5 Alkaline Phosphatase (ALP) Activity

ALP activity was resoluted in culture medium according to the methods of [13]. The activity of enzyme was determined using a pNPP (p-nitrophenyl phosphate) assay. The assay gives a sensitive and reproduceable method that is preferably suitable for determining ALP activity by incubating 0.8 ml of collected medium samples with 0.8 ml of 0.5 ml of Alkaline Buffer Solution (Sigma 221) and 0.5 ml of Stock Substrate Solution (40 mg *p*-nitrophenyl phosphate disodium (Sigma 104) diluted in 10 ml of sterilized purified water. The solution was incubated at 34°C for 1 hour in a 6 well plate to determine the ALP activity in regards to the formation of *p*-nitrophenol. In this technique, the ALP changes the hydrolysis of ρ-Nitrophenyl phosphate (pNPP) to ρ-Nitrophenol. pNPP which is neutral in color (colorless) but ρ-Nitrophenol has a high absorbance at 405 nm. The increased level of absorbance at 405 nm is relative to the activity of the enzyme (Ferrer, 2007). ELISA reader (TECAN-infinite M200 PRO) was used to read the absorbance of the solution and conveyed as absorbance reading from every well. This assay was carried out in triplicats with three replicates per scaffold and three replicates for control which was cultured with standard culture medium.

2.6 Live/Dead Cell Staining using Acridine Orange and Propidium Iodide AO/PI

The assay applies a simple binding of the AO stain with the cell's DNA to form a green fluorescence through electrostatic intercalation. A denatured DNA would cause the binding of the stain to its exposed sites thus producing red fluorescence as an indicator of dead cells. In addition, the PI stains enhances the dead cells identity by interacting with nucleic acids of cells with lysed membranes to from a red fluorescence. The hFOB cells having 1 x 10^5 density cells/well were cultured in a plate with six wells. Cells were cultured for 24 hours in a standard culturing conditions to allow primary attachments. Medium from all the well were discarded after 24 hours and substituted with another medium that contains scaffolds extracts. Subsequently cells were permitted to grow in the scaffolds extracts for 48 hours for all scaffolds, and for 24, 48 and 72 hours, and 5, 7, and 14 days for scaffold 5211 with medium change every 3 days. After incubation peroids, the cells were trypsinized and separated in standard medium which contains serum. All separated cells were sterily collected in 15 ml tube and spun at 1200 rpm for 5 minutes. The resultant supernatant was discarded leaving only the cell pellets. Staining solution was prepared through addition of 100 µl of 1 mg/ml propidium iodide (PI, nacalai tesque, Japan) and 100 µl of 1 mg/ml acridine orange (AO, nacalai tesque, Japan) to 10 ml phosphate buffer solution. Cells suspension were later mixed in an eppendorf tube at a ratio of 1:1 with the staining solution, 10 µl from the mixed suspension was poured into a glass slide and covered with slipped which was observed using fluorescence microscope (Nikon-ECLIPSE-Ti, Japan). Live/viable cells were observed to be stained green while dead/apoptotic cells were observed to stained red.

2.7 Cells Seeding onto Scaffolds

2.7.1 Pre-Wetting of Scaffolds

Each of the sterilized scaffolds was submerged into 10-20 ml of complete medium in a 6 well culture plate. The scaffolds were positioned into an incubator in 5% CO_2 at 34°C, for one hour to wet the scaffolds. Each of the scaffolds were then dried and gradually blotted with tissue paper to eliminate the excess medium on the surface and then transferred to a new 6 well culture plates before seeding. This was done to allow cell attachment to the scaffolds surface.

2.7.2 Cell Seeding for Attachment Studies

Cell seeding for attachment studies were done according to [12] methods. Prior to seeding the pre-sterilized scaffold blocks are placed in a 6 well culture plate, wetted with complete culture medium and incubated at 34°C for 1 hour. The hFOB cells with 80% confluence were separated using of trypsin/EDTA (0.25%-Trypsin/1mM- EDTA Solution, nacalai tesque, Japan). Cells were then concentrated using centrifugation process at 1200 rpm for 5 minutes and re-suspended in complete media. Cells were then seeded unto the scaffolds at a density of 100,000 cells/ scaffold that was allowed to absorb into the scaffold material. Following that, the cell seeded scaffolds were incubated at 34°C for an additional 1-3 hour prior to be fed with complete culture medium. The cell seeded scaffolds were visually assessed using an inverted microscope (Nikon-ECLIPSE-TS100) at appropriate intervals. The medium from each well is collected every other day for ALP analysis and replaced with fresh medium. Cells were grown on non-coated scaffolds for 24, 48 and 72 hours, 5, 7 and 14 days respectively. The experiments were carried out in 6 replicates, one of the replicates is used as a control.

2.7.3 Biochemical Analysis (ALP and Calcium)

The alkaline phosphatase activity (ALP) of seeded scaffold 5211 and non-seeded scaffold (control) was analyzed as a degree of osteoblast expression at 24, 48 and 72 hours, and continue at 5, 7 and 14 days post-seeding to evaluate the capability of the biosynthetic scaffold to generate bone *in vitro*. The alkaline phosphatase activity was measured using a 902 Hitachi automated clinical chemistry analyzer. The calcium (Ca^{+2}) content of scaffold 5211 was assessed to measure the quantity of mineralized matrix. The calcium (Ca^{+2}) was measured using the aforementioned analyzer.

2.7.4 Western Blotting and ELISA Colorimetric Detection for Total Proteins

The proteins activity of seeded scaffolds was examined as a measure of the osteoblast appearance after 3 days post-seeding to evaluate the capability of the biosynthetic scaffold to generate bone *in vitro* and compare levels between all non-coated scaffolds and for scaffold 5211 at 24, 48 and 72 hours and continue for 4, 7, 8, 9, 12 and 14 days and compare between these intervals. The proteins activity was measured using dot blot analysis, immunocytochemistry in which antibodies are used to identify proteins in cells and tissues by immunostaining and enzyme-linked immunosorbent assay (ELISA). The procotol was achieved using working solution through addition of 10 ml of solution A (Bicinchoninic acid solution) (nacalai tesque, product no.06385) and 200 µl of solution B (Copper sulfate solution) (nacalai tesque, product no.06385) into a clean beaker and were mixed completely, (Ratio; Solution A:Solution B = 50:1). Applied 25 µl of cultured medium into each well, 200 µl of working solution were added into each well, mixed for 30 seconds, then the plate were incubated at 37˚C for 30 minutes. Meaured absorbance was read at a wavelength of 562 nm using ELISA reader (TECAN-infinite M200 PRO) and the outcomes were shown as absorbance reading from all the wells. This assay was done in triplicats with three replicates for each scaffold and three replicates for control which cultured with normal working solution (as a negative control) for all scaffolds, and non-seeded scaffold for scaffold 5211(as a postive control).
The technique was for both the reduction of Cu^{+2} to Cu^{+1} by protein in alkaline solution with the choosy colorimetric detection of the cuprous action by the used of reagent that contains BicinChoninic Acid (BCA). The end result of the reaction was purple-colored. The purple-colored was formed due to the two molecules chelation of BCA with one cuprous ion. The working reagent contains 50 portions of Reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide) with 1 portion of Reagent B (4% cupric sulphate) [14].

2.7.5 Scaffold Preparation for Scanning Electron Microscopy Analysis

Cell spreading and attachment patterns were assessed on the freeze dried 5211 scaffold through Scanning Electron Microscopy (SEM) using VPSEM (Variable Pressure Scanning Electron Microscopy)-JEOL 1455 (Germany) coupled with Element Detection System (EDS). Samples of 5211 scaffold for microscopy studies were organized by eliminating the culture medium from the plates and at the determined period and replacing it with 2.5% glutaraldehyde solution. Samples were fixed in the fixation solution for 24 hour at 4°C. Three replicates of the cell seeded 5211 scaffold were processed further according to standard SEM sample preparation protocol and for comparative purposes. None seeded scaffold 5211 was used as a control. The cell growth pattern for scaffold 5211was assessed at 48 hours, 5, 7 and 14 days of culture period. Briefly, post fixed samples were first cut into smaller

blocks of 0.5 cm diameter x 0.5 cm length and placed into a glass vial. The samples were washed thrice using 0.1 M sodium cacodylate buffer at pH 7.3 for 10 minutes each followed by post fixation for 1 hour in 1% osmium tetroxide. The samples were later re-washed with 0.1 M sodium coccodylate buffer in three changes for 10 minutes prior to dehydration. Samples were then dehydrated in an ascending grade of acetone (35, 50, 75, 95 and 100%) and dried with a critical point dryer (CPD 030, Bal-TEC, Switzerland) for approximately 30 minutes. The dried samples were then mounted onto an aluminium stub and sputter coated with gold using a SEM coating unit (E 5100 Polaron, England), coated specimens were observed and analyzed using VPSEM-JEOL 1455 (Germany).

2.7.6 Scaffold Preparation for Histological Examination

For light microscopy studies, three replicates were prepared by removing the culture medium from the plates at the determined period and fixing the scaffold samples in 2.5% glutaraldehyde solution for 24 hour at 4°C. Following fixation, the samples were dehydrated with an ascending alcohol grade, cleared in xylene and embedded in paraffin wax using an analytical tissue processor (Leica, Microsystem Nussloch Heidelbergerstr D-69226, Germany). The blocked samples were then sectioned at 5 µm thick using a rotary microtome (Leica 2045, Germany), mounted onto glass slides and stained with routine Hematoxylin and Eosin for general histology and in the situation of porous scaffolds, it could show cell infiltration into the scaffold and cell supply at almost any distance, Massons Trichrome for demonstration of the collagen fiber and new bone tissue and Von Kossa for demonstration of osteoblast and new bone tissue (calicum salts) [15]. The stained slides were dipped in xylene and mounted with cover slips using DPX mounting medium prior to viewed under a light microscope (Motic, BA410, China) equipped with a camera (Moticam Pro, 285A, China).

2.8 Statistical Analysis

The quantifiable outcomes were analyzed using Explore for Normality of data then One-Way Analysis of Variance (ANOVA) and Kruskal-wallis H test. The results were shown as Mean ± Standard Error (SE). Post Hoc test was calculated for significant values $p<0.05$ using Tukey's multiple comparison test. All descriptive and inferential statistical analyses were conducted using Excel version 2013 and SPSS version 21.0, 22.0 and 23.0.

3- Results and discussion

3.1 MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphynyltetrazolium bromide) Colorimetric Assay

Figures 2 and 3 show the results of the MTT assay performed on hFOB cells grown in different scaffolds leachables for a period of 24, 48 and 72 hours for all seventh scaffolds, and continue the incubation for 5, 7 and 14 days for scaffold 5211. The absorbance reading from the assay correspondence to the cell viability when cultured in the medium containing the leachables of the scaffolds. Cell viability was observed to be higher in the presences of culture medium containing scaffolds leachables compared to normal culture medium (control) that was used as a blank for the experiment. A significantly ($p<0.05$) higher absorbance reading was obtained after 24 and 72 hours for cells grown in the medium containing leachable of scaffold 8100, after 48 hours for cells grown in the medium containing leachable of scaffold 6300 and after 14 days for cells grown in the medium containing leachable of scaffold 5211 compared to control . An increasing trend on cell viability was notable to be relative to the cumulative content of CCAN residue within the scaffolds composition after 24 and 72 hours except after 48 hours, scaffold 6300 which showed high cell viability even though having the lowest content of CCAN powder (60%) in its total composition.

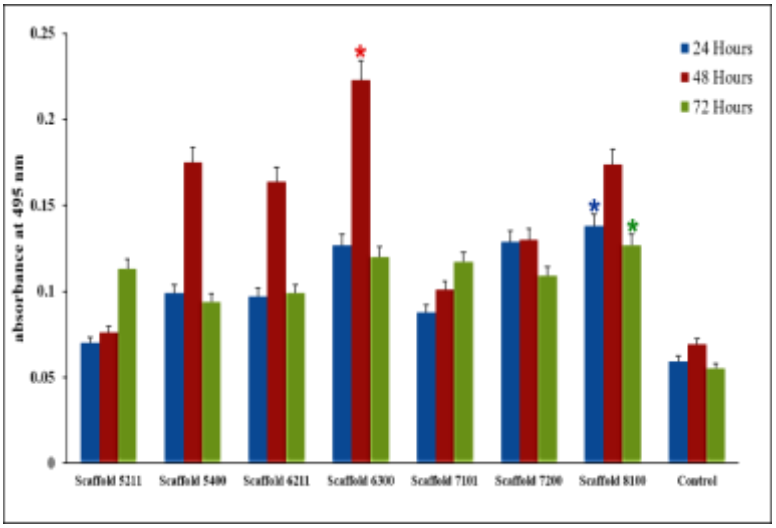

Figure 2: Results of MTT assay on hFOB cells grown in different scaffolds extracts after 24, 48 and 72 hours of the culture period. * Significant difference was observed as compared to control at $p<0.05$.

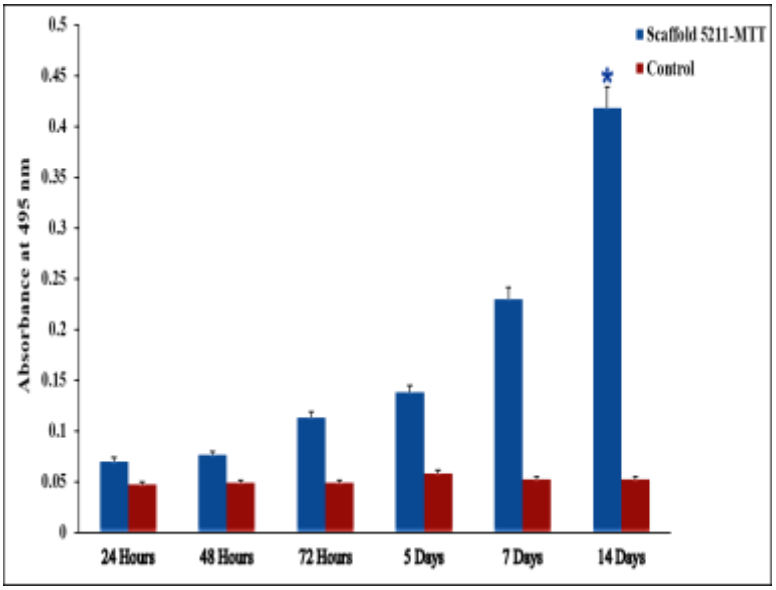

Figure 3: Results of MTT assay on hFOB cells grown in scaffold 5211extract after 24, 48 and 72 hours, 5, 7 and 14 days of the culture period. * Significant difference was observed as compared to control at $p<0.05$.

3.2 Alkaline Phosphatase (ALP) Analysis

Alkaline phosphatase (ALP) is a common biomarker of osteoblastic expression that gives an indication on the intensity of the bone forming osteoblast cells actions. These simple calorimetric assays using p-nitrophenyl phosphate as a substrate and protein assay bicichoninate kit give an indication on the osteoblast cells functional activity. There were significant differences in the ALP levels and proteins levels in the cells cultured in different scaffold extracts and structures, it is worth mentioning that a common trend was observable between the ALP levels and protein levels with those of the MTT and AO/PI staining. Increasing levels of ALP and proteins directly indicate the presences of a system that is favourable in supporting the cells proliferation rate as well as the subsequent mineralization of the matrices. Figures 4 and 5 show the ALP activity of hFBO cells cultured in medium containing the leachable of scaffolds measured on 24, 48 and 72 hours, 5 and 7 days for all the seventh scaffolds, and continue the incubation for scaffold 5211 for 14 days of the culture period. A significant ($p<0.05$) difference in ALP levels was observed between the culture of different scaffold leachable medium within the time frame of the study. However a significant ($p<0.05$) increasing trend of ALP levels were observed in cells cultured in medium containing leachable of scaffold 5400, 7101 and 8100 after 24 and 48 hours, scaffold 5211 and 7101 after 72 hours, scaffold 6211, 6300 and 8100 after 5 days, and scaffold 5400, 6211 and 7101 after 7 days. While cells cultured in medium containing leachable of scaffolds showed an increase in the ALP activities from 2nd to 5 days which was then followed by a decrease in the ALP activities at 7 days. A similar trend was also seen for the control group cultured in normal culture medium for the increasing of the ALP activity from day 2nd to 7 days. Generally, the cells that were cultured on the scaffolds extracts reached higher total ALP content on 5 days, which decreased significantly ($p<0.05$) on 7 days for all scaffolds and on 14 days for scaffold 5211.

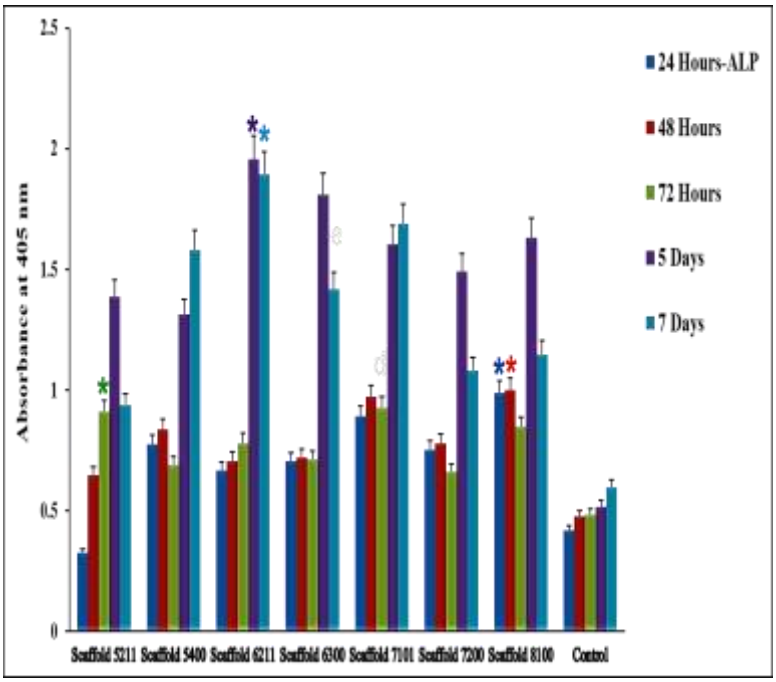

Figure 4: ALP activities of hFOB cells cultured in medium containing the extracts of scaffolds after 24, 48 and 72 and 5 and 7 days of the culture period. * Significant difference was observed as compared to control at $p<0.05$.

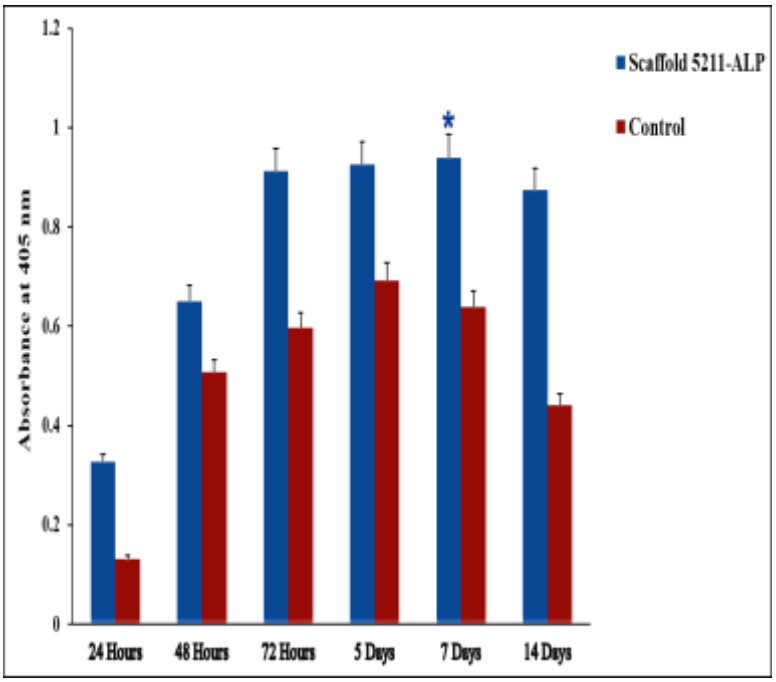

Figure 5: ALP activities of hFOB cells cultured in medium containing the leachable of scaffold 5211 after 24, 48 and 72, and 5, 7 and 14 days of the culture period. * Significant difference was observed as compared to control at $p<0.05$.

3.3 Live/Dead Cell Staining using AO/PI

The AO/PI staining technique was done to supplement the outcomes of the MTT assay by which the viable cells identification were seen to produces a green fluorescence after the stains were incorporated with the cell's DNA. Figures 6 and 7 show the images of cells cultured in medium containing the different scaffolds leachables obtained through an image analyzer. The staining procedure revealed that cells cultured with the extracts of the scaffolds as well as the control culture consisting of normal medium showed 100% viability during the 48 hours for all scaffolds, and during 14 days culture period for scaffold 5211. However, the number of cells noted correlated well with the results from the MTT assay that showed a higher number of fluorescence cells being present in the culture with leachable extracts of scaffolds 6300 and 8100. The cells cultured in the leachables of scaffolds revealed 100% viability in 48 hours culture period for all the scaffolds, and during 24 and 72 hours, and 5, 7 and 14 days for scaffold 5211. An increase in number of cells was observed to be comparative to the content of CCAN powder with the number of fluorescence cells observed found to be increasing from scaffolds 5211,5400,6211, 6300, 7101, 7200 to 8100 compared to the cells cultured in normal culture medium (control). The most number of cells was observed in the culture of medium containing leachable of scaffold 6300 during 48 hours, while for scaffold 5211, the numbers of cells was increased gradually during culture peroids.

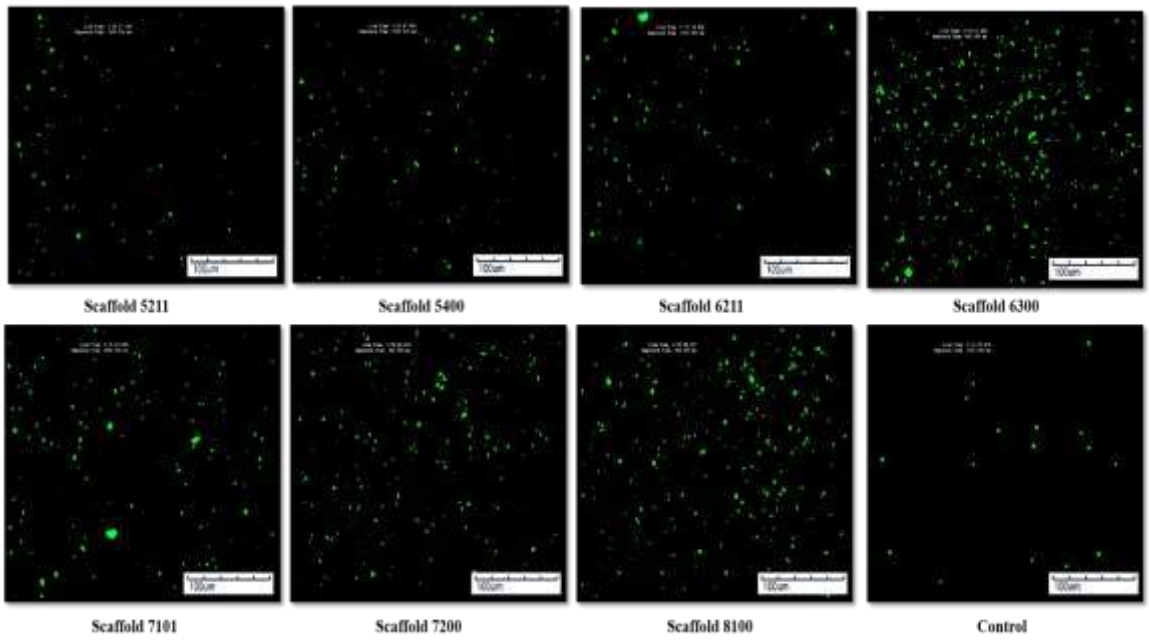

Figure 6: Fluorescence stained cells grown in the medium containing extracts of all scaffolds and normal culture medium as a control after 48 hours, (X100).

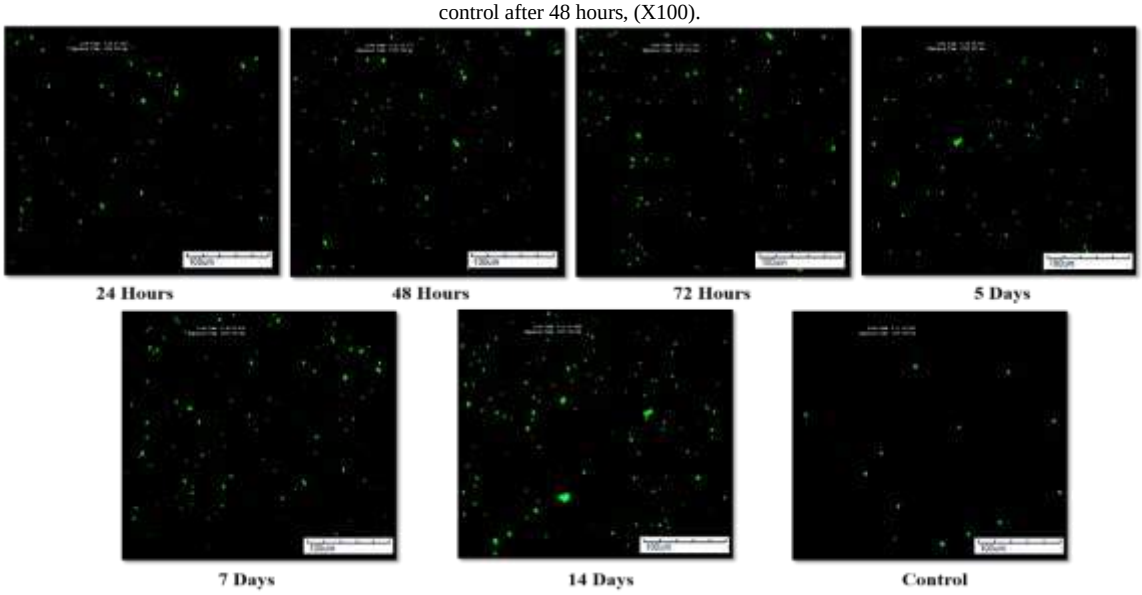

Figure 7: Fluorescence stained cells grown in the medium containing extract of scaffold 5211 and normal culture medium as a control during 14 days, (X100).

3.4 Microscopy Studies on Cell Attachment

The adherence and growth of the hFOB cells towards (CCANP3DSs) were investigated using the inverted microscope for all scaffolds during culture peroid (Figures 8 & 9) and using Scanning Electron Microscopy (Figures 13-17) as well as histological staining of scaffold 5211 (Figures 18-20). Based on previous findings, the osteoblast cells were seeded onto the scaffold consisting of 50% CCAN powder and 25 % gelatin, 10 % dextran and 15 % dextrin composition (scaffold 5211).

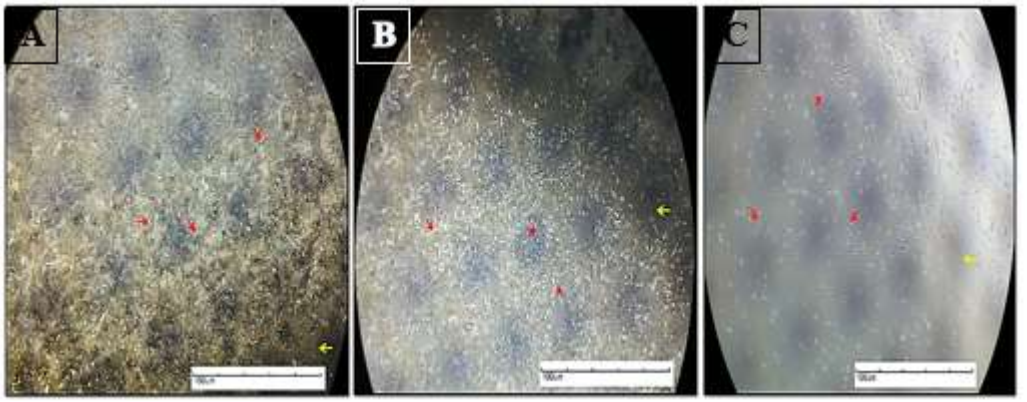

Figure 8: Growth of human Fetal Osteoblast cells (hFOB) (red arrows) in (A) the nanocomposite 6300 scaffold extract (yellow arrow) after 24 hours, in (B) the nanocomposite 5211 scaffold extract (yellow arrow head) after 14 days, in (C) normal culture media (yellow arrow) as a control after 24 hours, (X400).

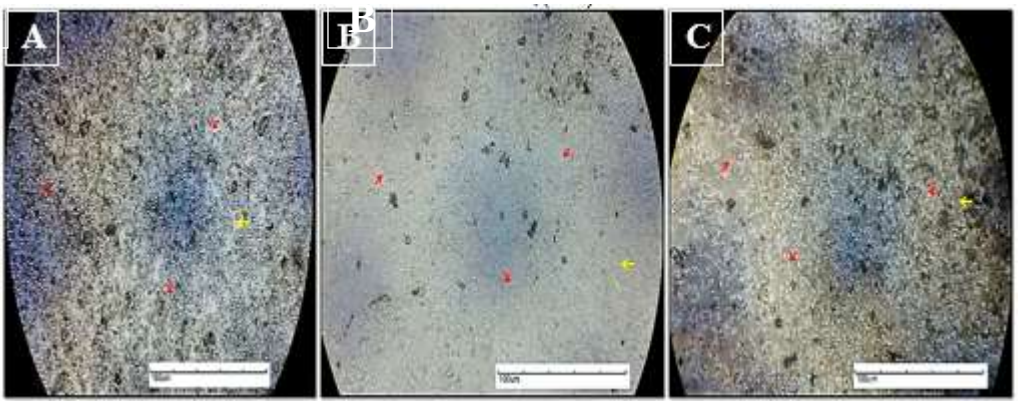

Figure 9: Culture of hFOB cells (red arrows) onto (A) the nanocomposite scaffolds structure (yellow arrow), in (B) the nanocomposite 5211 scaffold structure (yellow arrow), in (C) the nanocomposite 6300 scaffold structure (yellow arrow) showing a favorable adherence towards the scaffold material after 3 hours incubation and before adding the complete media, (X400).

3.4.1 Alkaline Phosphatase (ALP) and Calcium Analysis

Figure 10 shows the ALP and calcium activity levels in the culture medium of the 5211 scaffold seeded with hFOB cells and control scaffold without seeding. Alkaline phosphatase (ALP) is the most commonly familiar biochemical marker for activities of osteoblast. The absorbance value represents the intensity of the ALP activity within the culture medium. A significant ($p<0.05$) increase in ALP levels were observed from 1st day and onwards in the nanocomposite scaffold and peaked on 5 days, followed by a gradual decline in the ALP activity from day 7 onward. The control scaffold showed a different trend of ALP activity with a gradual increase in the activity levels observed only on day 3 and onward. The highest levels were obtained on 3, 5 and 7 days followed by a gradual decrease after 14 days. Significant ($p<0.05$) difference in the ALP activity levels in the culture medium was observed compared to the control during cultural period. However, the ALP levels of the nanocomposite scaffold medium were found to be

much higher at the initial stage of cell culture compared to control scaffold that showed an increase in the ALP levels at a much later stage of the culture period.The calcium deposition was a marker of full maturing osteoblast cells in the culture. Figure 10 shows the calcium measurements in the culture medium. During 1st and 2nd day of cultured scaffold, there were no significant ($p>0.05$) changes in the level of the calcium deposition when compared with the control group. In contrast, after 72 hours, 5 and 7 days, the calcium deposition increase was observed. The calcium deposited on the scaffold cultured in 14 days was significantly higher ($P<0.05$) than the calcium deposited on the control group.

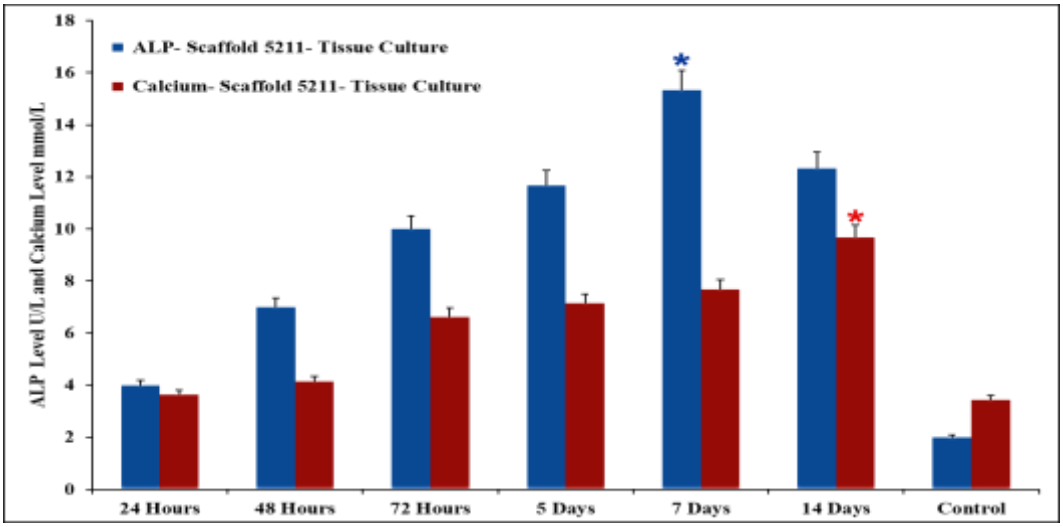

Figure 10: ALP and Calcium levels in the culture medium of nanocomposite scaffold 5211 and control scaffold after 24, 48 and 72 hours and 5, 7 and 14 days of the culture period. * Significant difference was observed as compared to control at $p<0.05$.

3.4.2 Western Blotting and ELISA Colorimetric Detection for Total Proteins

Figures 11 and 12 show the total protein activity levels in the culture medium of all the non-coated (CCANP3DSs) seeded with hFOB cells for the first 3 days and scaffold 5211 for 24, 48 and 72 hours and continued for 4, 7, 8, 9, 12 and 14 days and control scaffold without seeding (as a positive control). Proteins activity is the other commonly recognized biochemical marker used to identify osteoblast activity. The absorbance value represented the intensity of the proteins activity that expression of three proteins (collagen, osteopontin and alkaline phosphatase) complex in bone building within the culture medium. An increase in proteins levels were observed for scaffold 5211 from 1st day onwards with the intense peak absorbance noted at 2nd and 12th day. Significant ($p<0.05$) difference in the protein activity levels in the culture medium was observed from postive and negative control. The protein levels of the nanocomposite scaffolds medium were found to be higher for the scaffold 5211 after 3 days of cell culture compared to other scaffolds and the negative control. Significant ($p<0.05$) difference in the protein activity levels in the culture medium was observed from the negative control.

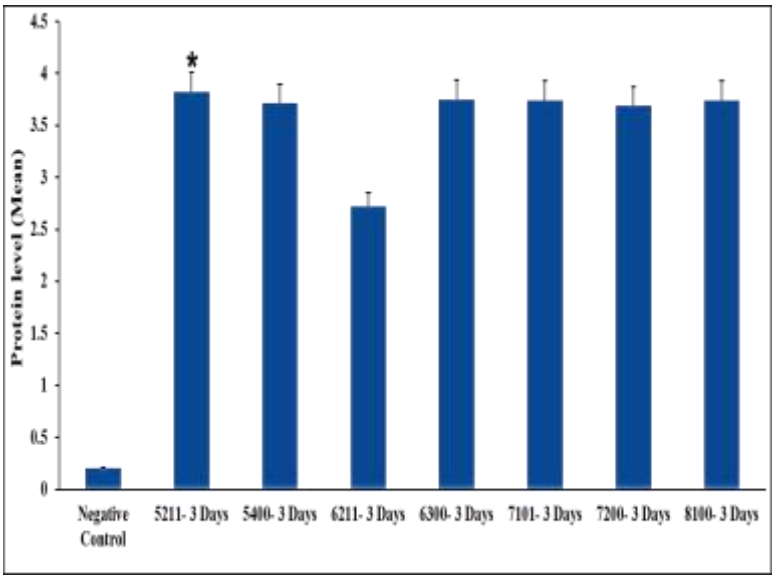

Figure 11: The mean of protein activity levels in the culture medium of seeded nanocomposite scaffolds and control after 3 days of the culture period. * Significant difference was observed as compared to control at $p<0.05$.

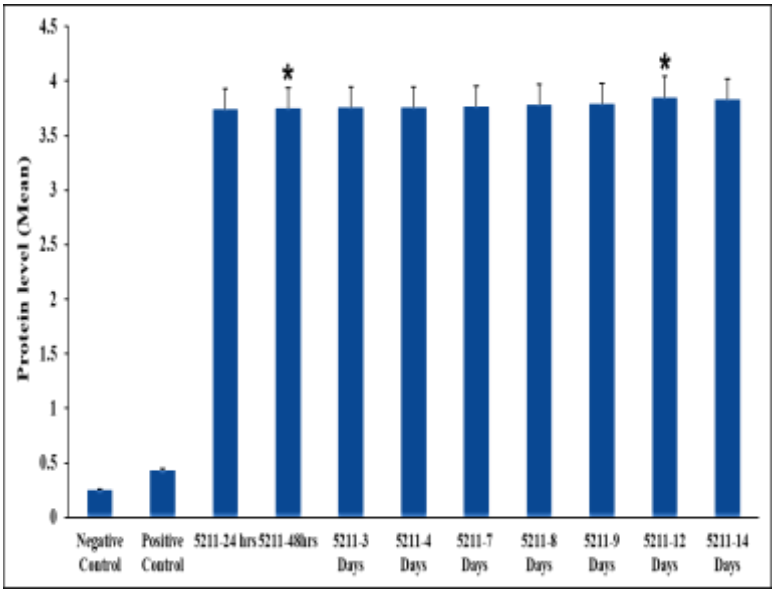

Figure 12: The mean of protein activity levels in the culture medium of seeded nanocomposite scaffold 5211 and non-seeded scaffold (control) after 24, 48 and 72 hours and 4, 7, 8, 9, 12 and 14 days of the culture period. * Significant difference was observed as compared to control at $p<0.05$.

3.4.3 Scanning Electron Microscopy (SEM) Analysis

The VPSEM examinations were done at the top of the scaffold surface (the place of seeded osteoblast cells) and the bottom of the scaffold at 48 hours, 5, 7 and 14 days post culture in the 6 wells plate. Notwithstanding for the point that any preparation of sample treatment (dehydration and critical point drying) or the high vacuum within the VPSEM could brake or tear some structures of cell, the primary structure of the cells is obviously observable.

A quantity of microvilli appeared on the dorsal exteriors of the cells (Figure 14 (C)). Majority of the cells on both scaffold sides gather in one place forming a clusters, whereas the others casually attached on the surface of the material as shown in Figure 13 (B) and comparison to control scaffold without seeding (Figure 13 (A)). Mineralized collagen fibers abundance can be observed on scaffold surface within the pores, bounded by a huge quantity of bone-forming cells. These appeared after culturing for 5 days and more obvious at 7 days (Figures 14 (A and B) and 15 (A and B)). The cultured scaffold was enclosed by a thick matrix coating the top and bottom surfaces. As previously noted, the cells formed a dense layer on the phase-separated scaffold surfaces, which appear to be more sparingly growing on the scaffold surfaces, are the primary porosity was not observable at 5 and 7 days. The cells were capable of wandering through the scaffolds and occupied the whole construct.

Numerous cells were found inside of the phase-separated scaffold through the VPSEM investigation as shown in Figure 14 (A, B and C), while these scaffold harboured great number of clusters of osteoblast cells bounded by their extracellular matrix as shown in Figure 15 (A and B). After 5 and 7 days of culture, the structures mostly became organized. A condensed extracellular matrix layer was existing on and in between material scaffolds, of a collageneous nature. After 14 days of culture, the structures mostly became completely mineralized matrix and a close resembles of bone like structure (Figure 16). The EDX (Energy-Dispersive X-ray) analysis in this study confirmed that these structures consisted of calcium carbonate, which will be described in the next paragraph.

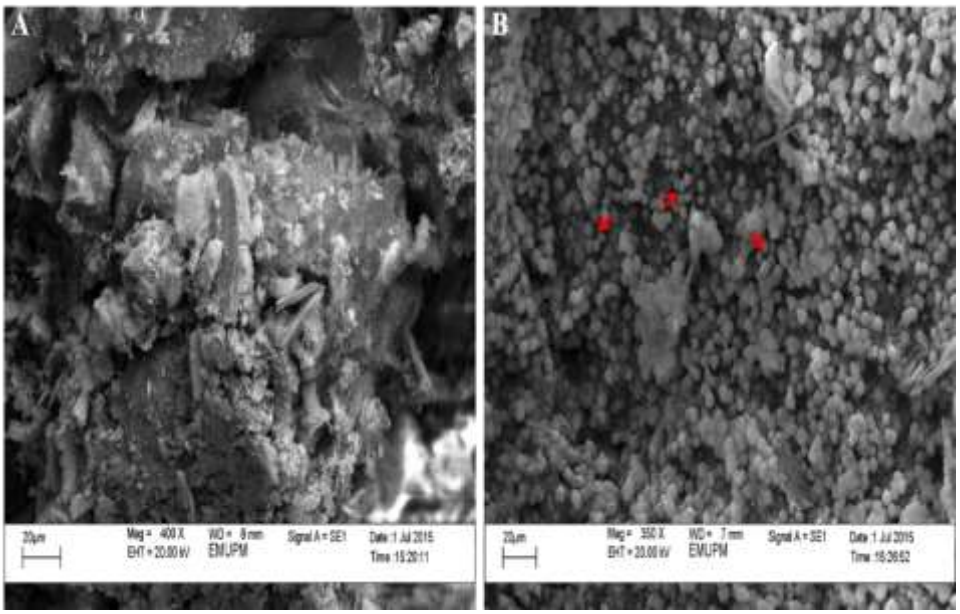

Figure 13: VPSEM micrographs of scaffold 5211 section after 48 hours post-seeding show attachment of clusters of active cells (red arrows) onto the surface of the 5211 scaffold (B) and comparison to control scaffold without seeding (A).

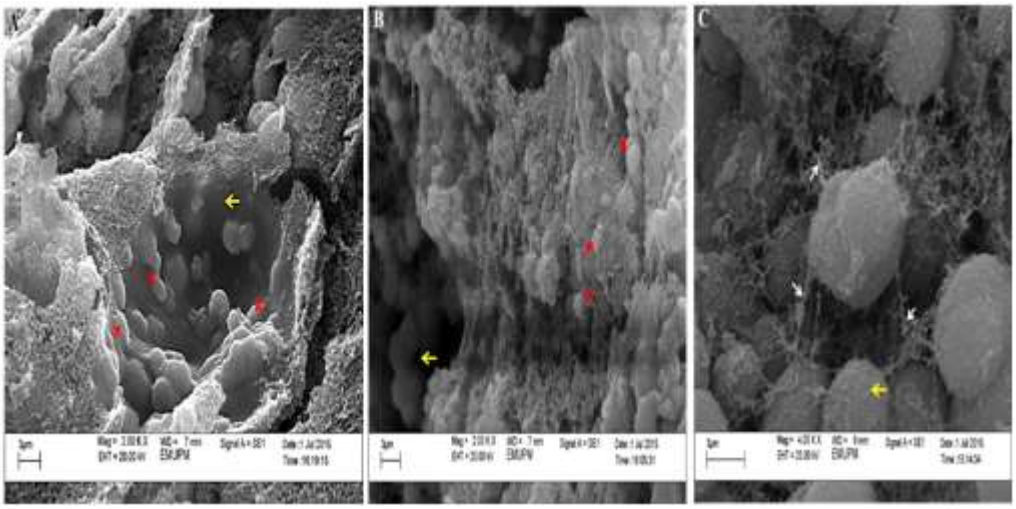

Figure 14: VPSEM micrograph of scaffold 5211 section after 5 days post-seeding shows (A) pore (yellow arrow) containing clusters of osteoblast cells and changed their shapes to flat cells (red arrows), (B) clusters of osteoblast cells (yellow arrows) and some of them change to flat shape (red arrows) and (C) the cytoplasmic processes are very obvious (white arrows).

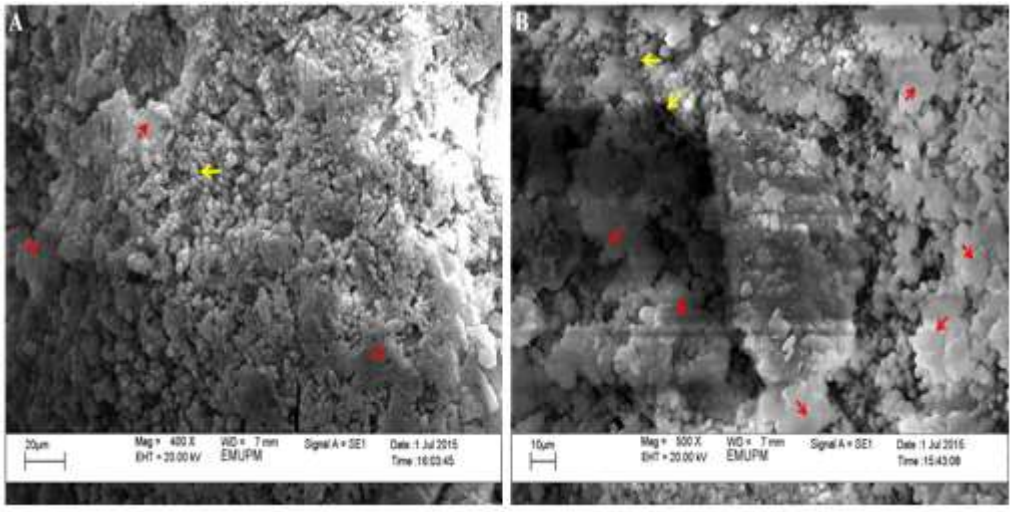

Figure 15: VPSEM micrograph of scaffold 5211 section after 7 days post-seeding (A) shows attachment of clusters of active cells (yellow arrow) onto the surface of the 5211 scaffold and (B) creation large amount of extracellular matrix (red arrows).

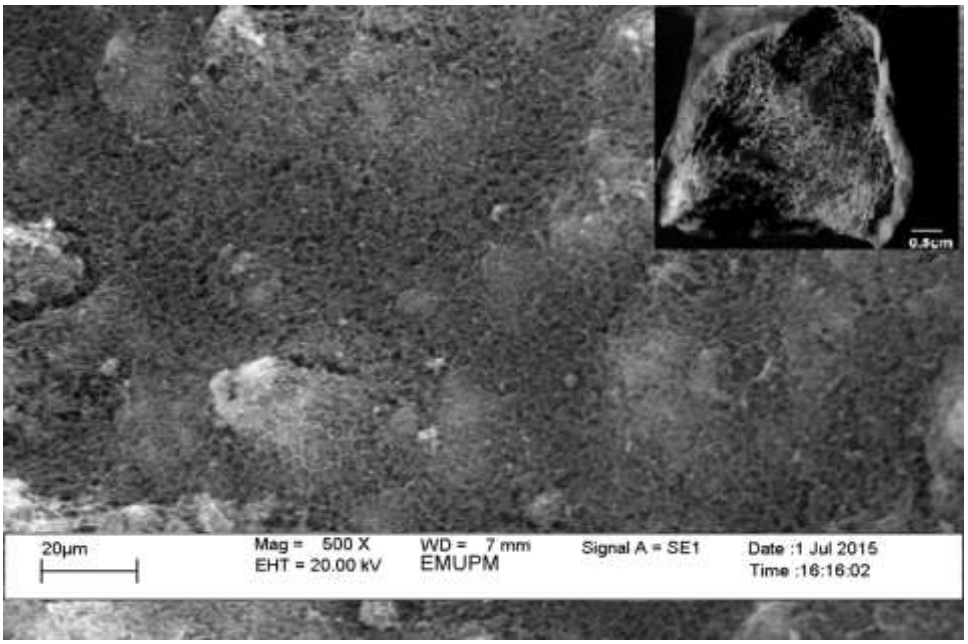

Figure 16: VPSEM micrograph of scaffold 5211 section after 14 days post-seeding shows a completely mineralized matrix and a close resembles of bone like structure. Insert in the left side up is an image of a bone structure adapted from "Bone structure and formation: A new perspective" (Olszta *et al*., 2007).

3.4.4 Energy-Dispersive X-ray Spectroscopy (EDS) Analysis

Figure 17 shows the results of the element analysis of the 5211scaffold and the control scaffold before and after 14 days of cell culture studies obtained from the surface of the cell as well as the scaffold matrices within the VPSEM analysis. The spectrum shows the elements present in the scaffold structure prior to cell seeding indicating the presences of calcium with carbon and oxygen. After 3 days of culture period, the spectrum of the nanocomposite scaffold showed the presences of peaks corresponding to the phosphate and calcium elements. After 5 days of culture period, the spectrum showed the presences of both phosphate and calcium as the dominant peaks in the scaffold structure, but the peak of the calcium is slightly higher than the phosphate. After 7 days of culture period, the spectrum showed the presences of both phosphate and calcium as the dominant peaks in the scaffold structure, but the peak of the calcium is higher than the phosphate. After 14 days of culture period, the spectrum showed the presences of both phosphate and calcium , but the dominant peaks in the scaffold structure was the calcium. The findings from the element analysis further support the results of the SEM analysis proving the presences of the bone like tissues on the surface of the nanocomposite scaffold.

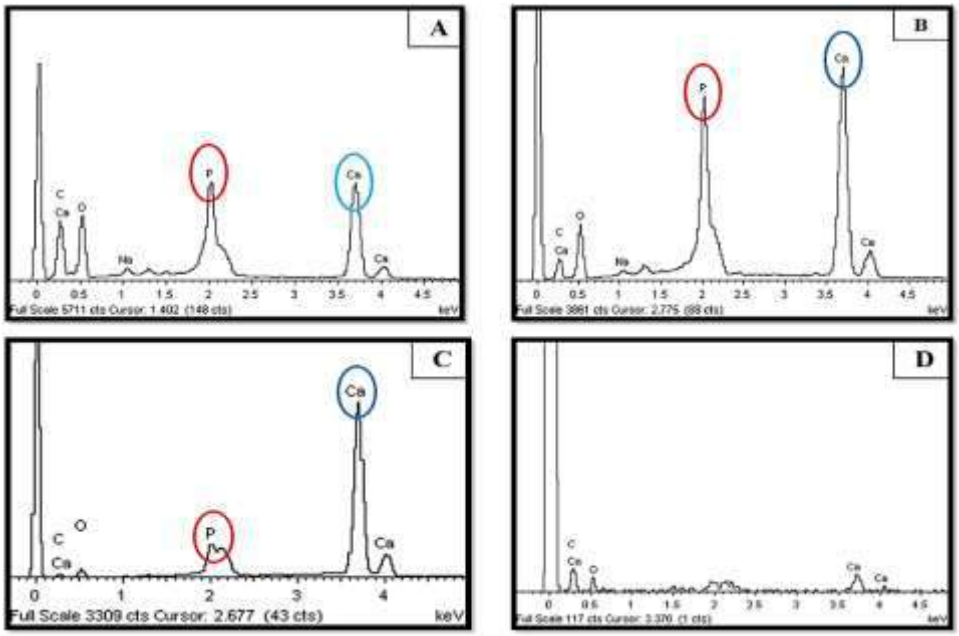

Figure 17: EDS analysis of the 5211 scaffold, (A) after 3 days, (B) after 7 days and (C) after 14 days post seeding and comparing with (D) the control.

3.4.6 Light Microscopy

The histological examination is considered as one of the most important examinations. The histological study in this trial was assessed through evaluating qualitatively the bone matrix-like tissue formation induced by the interaction between the hFOB cells and the porous 3D nanocomposite scaffold material. The differences observed in tissue formation for 14 days. The new products of scaffold constructs, which had an initial cell seeding were sectioned, stained with H&E and counter stained with Van Kossa and Massons Trichrome for osteoblast, extracellular collagenous and calcium deposition. The clusters of osteoblast cells were visible through the histological stained section of the scaffold at 48 hours as shown in Figure 18 post seeding specially around the pores, the way that the cells penetrate inside the scaffold (highlighted circle). Therefore, monolayers of cells were found lining the pore walls and a thicker tissue was observed throughout the scaffold volume.
The histological section of the scaffold structure further supports the finding of the VPSEM evaluation. The histological section of the nanocomposite scaffold indicating the presence of osteoid like tissues stained brownish black at 5^{th} and 7^{th} day post-seeding (Figure 18 B-D). While Figure 19 (D-F) shows heavy calcium deposition on the surface of the scaffold matrices observed with Von Kossa stains at 10^{th}, 12^{th} and 14^{th} day post-seeding. The red color seen in Figure 18 (D-F), and the blue color seen in Figure 20 (A-F) was collagen and was more abundant around the pores of the scaffold. The osteoblast cells formed a continuous thick layer which became like multilayered sheets with the presence of the fibrous matrix.

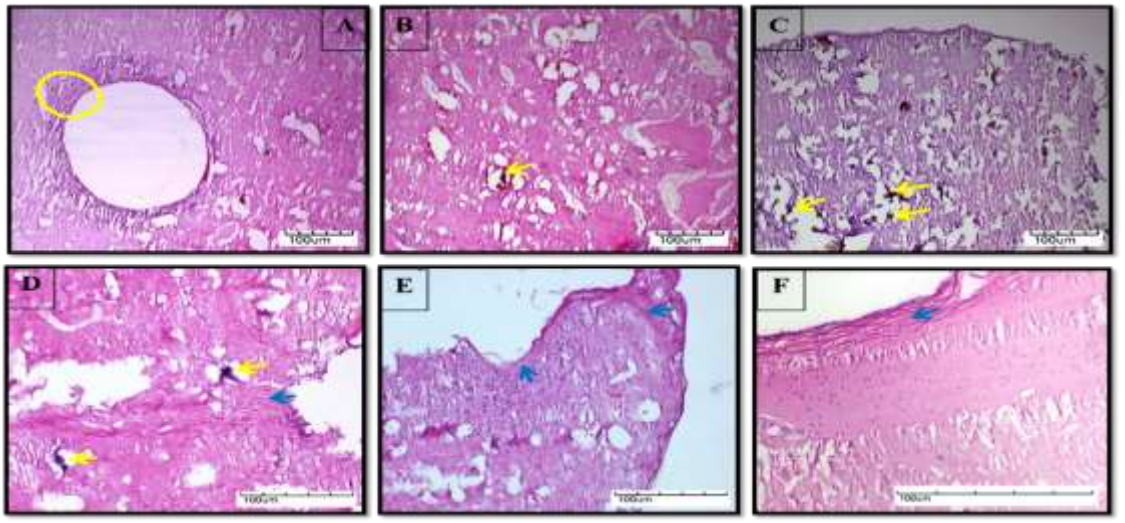

Figure 18: H&E stained histological section of undecalcified nanocomposite scaffold 5211 after 48 hours post seeding showing the clustering of osteoblast cells within the scaffold structure, especially around the pore (highlight circle, (A)), after 5 and 7 days showing the presences of osteoid like (yellow arrows), (A, B and C, X100) and after 10 days showing the presence of collagen fiber (blue arrows) (D and E, X200 and F, X400).

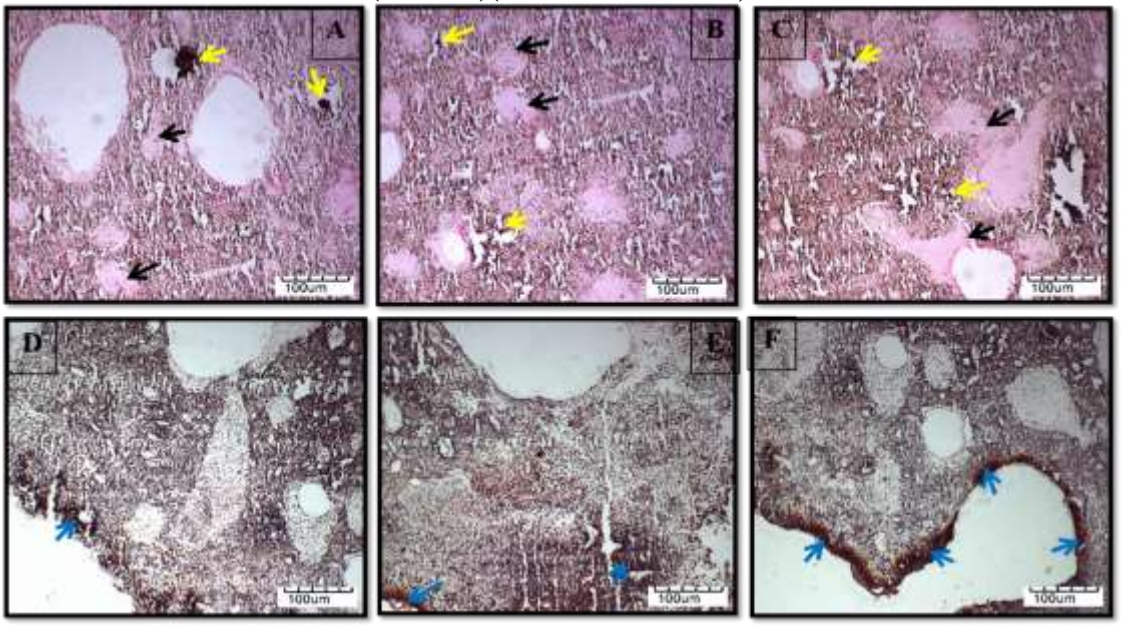

Figure 19: Von Kossa stained histological section of undecalcified nanocomposite scaffold 5211 after 3, 5 and 7 days showing the presences of osteoblast cells embedded in ExtraCellular Matrix (ECM) (black arrows) and osteoid like (yellow arrows) (A, B and C, X100) and after 10, 12 and 14 days showing the presence of calcium deposition (black) (blue arrows) on and in scaffold matrices (D, E and F, X100).

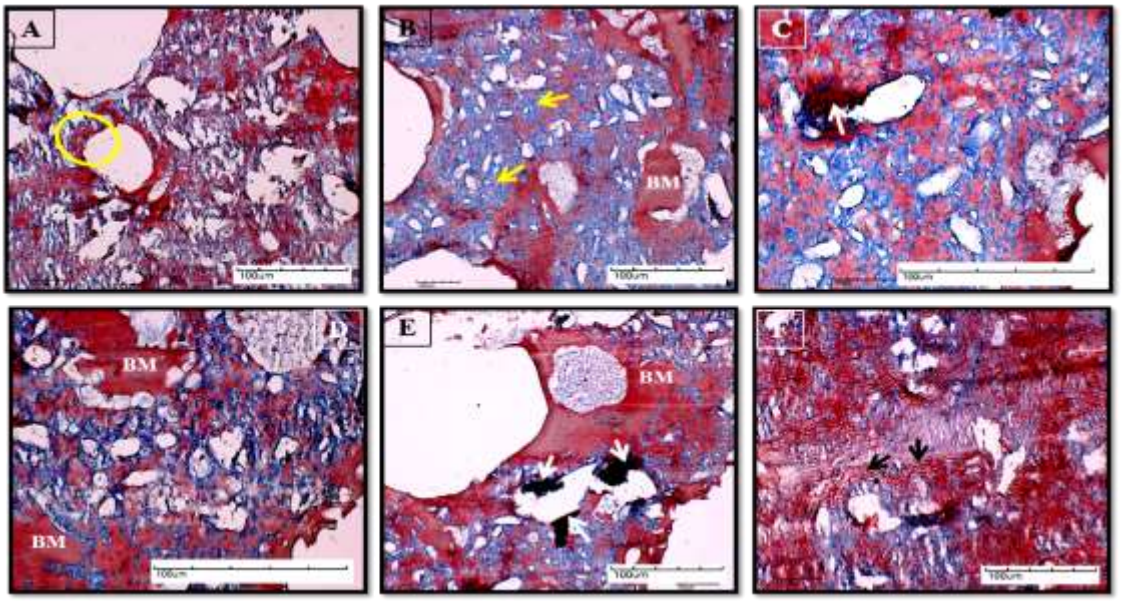

Figure 20: Masson's Trichrome stained histological section of undecalcified nanocomposite scaffold after 48 hours post-seeding showing the clustering of osteoblast cells within the scaffold structure, especially around the pore (highlight circle) (A, X200), after 5 and 7 days showing the collagen deposition (yellow arrows) in scaffold matrices (B, X200 and C, X400) and after 10, 12 and 14 days showing the presence of calcium deposition (white arrows), bone matrix (BM) and collagen fibers (black arrows) (D, X400, E and F, X200).

The evaluation of tissues *in vitro* gives ample required platform as a basal study on the biological performance of a developed material that test the potentials of interaction of cell with the scaffold materials in addition to study of the biocompatibility of the materials which are essential in safeguarding successfull development of biomaterial based scaffolds [13, 16].

The established records on the use of human osteoblast cells in testing of cell-biomaterials interactions has been previously agreed by some studies [3, 17, 18]. It is also to be justified that the long term aim of the developed scaffolds is to be used in clinical applications thus, influencing the choice of using this cell lines as a representative of the target tissues.

The culture medium containing the scaffold leachable was used as a representative of the scaffolds biodegradation model to test the viability and proliferative properties of the cells in the presents of substances leached out from the scaffolds. The use of the scaffold extracts tests the cellular response in terms of cell growth and proliferation as being directly affected form the ionic releases of substances from the nanocomposite scaffolds that could potentially change the properties of the culture medium. The results obtained from this study revealed that there is a increasing rate of the growing cell as the CCA N powder in the scaffolds composition was increasing which correlate positively with the calcium concentration increase leaching out from the scaffolds. Calcium ions leaching out from the scaffolds directly improves the cells propagation rate as a result of calcium ions are well known biomolecules that are vital in defining early cell behavior [19, 20, 21, 22, 23, 24]. This readily available reserve of calcium ions from the cockle powder in the scaffolds provides an added advantage to the developed scaffolds in regards to encouraging early cell differentiation for a faster healing property. This phenomenon could be as a result of the changes in the culture medium pH values with the leachable extracts of scaffold 6300, 8100 having higher calcium content that could have changed the pH value of the medium to be highly basic in nature as supported by the findings from the degradation studies [11].

The capability of cell proliferation and growth in the extract from the scaffolds acts as a direct measure of cytotoxic effect absences from the products leaking out from the scaffold materials. A similar study by [12] concluded that the capability of growing cells in the extract of a scaffold as being cytocompatible in nature thus supporting the findings of this study in proving the cytocompatible effects of the scaffolds.

In addition to MTT assay as an indicator for proliferation properties of the cells, a simple AO/PI staining was also done to visually inspect the number of live and dead cells when the cells are cultured in the presences of the scaffolds leachable extracts.

The researchers [25, 26] also showed the release of substances from a nanobiocomposite material could possibly stimulate cells proliferation as indicated by the expression of the ALP biochemical marker for osteoblast cells proliferative activity. The researcher [27] further support that the increase in ALP levels which is one of the appearance of three proteins (alkaline phosphatase, collagen and osteopontin) involved in bone remodeling as an indicator of osteoblast cells activity that indirectly increases the concentration of phosphoric acid in addition to supporting matrix mineralization. Report shows that ALP is related with calcification with an

improved manifestation of this enzyme is outwardly vital earlier than the commencement of matrix mineralization and providing restricted enhancement of inorganic phosphate which is one of the apatite constituents of the mineral phase of bone [28]. In this study, the ALP obtained is similar to the results reported by (9, 29, 30, 31].

In this study, it was shown that the highest activities took place within 5 days, this observation remained similar to [29 and 31] who also stated that a peak ALP expression was observed at 3rd day. Nonetheless, this result is not in agreement with [32] observations who demonstrated that ALP activity improved as the experiment progresses for all samples and particularly after 14 days of incubation. In current study, ALP in scaffolds 5400 and 7101 extracts increased after 7 days and this is similar to the works done by [31 and 32] who reported the unceasing action of the cells in spite of deposition of calcium in the culture.

Consequently, in this work an initial increase in Ca^{+2} activity was shown which was followed corresponding by reduction to increase cell differentiation when the production of alkaline phosphatase reduced. The decrease action of ALP may be as a result of the point that further cultured of cells on the scaffolds may lead to the next phase of differentiation. This is in addition to the intracellular calcium rise which might decide an inhibitory outcome. The decline of ALP may possibly symbolize a reoccurrence to osteoprogenitor cells or maturation to osteocytes which usually express small amounts of this enzyme. The obtained result in this study is similar to the result obtained from previous studies by [9, 28, and 31]. High reduction in ALP activity throughout matrix vesicle mineralization was observed. The ALP activity reduction is related with fast Ca^{+2} buildup by the matrix vesicles. This is greatly reproducible and constant result which transpires in all instance of vesicle mineralization which is referred to starting of mineralization of bone scaffold.

The increasing levels of ALP observed from 1st to 7th day prior to its decline corresponds well to the SEM findings of the nanocomposite scaffold. Changes in the nanocomposite scaffold indicate that early osteoblast cell differentiation and proliferation is taking place between 1-6 days prior to declining on day 7 due to down regulation of the ALP expression thus marking the start of the mineralization process in scaffold. This findings support the SEM observations of the scaffold in which formation of calcium nodules were highlighted as an indication of the mineralization process at 7 days. According to [33] the increase in ALP levels on porous scaffolds is noted as an indication of early cell differentiation followed by a drop that is observable with the initiation of the mineralization process. This further validate the findings from this study. The researchers [31 and 34], also reported similar trends in ALP levels that were found to correspond well to the increase and decrease in the levels in regards to the proliferation and growth pattern of the osteoblast cells on the respective scaffold materials.

The CCAN powder in the scaffolds provides sufficient cues in the form of calcium ions to promote and facilitate the proliferation and differentiation of the osteoblast cells. Literature findings show that the slight increase in calcium ion could enhance the osteoblast cellular responses due to its potential involvement in extracellular signaling [26]. Based on this fact, the nanocomposite scaffold could be regarded to possess this "built-in" signaling system that most likely contributed to its excellent early stage mineralization properties as observed in the SEM images.

The accumulation of mineral is as a result of progressive pre-osteoblastic cells increase and stages of matrix maturation and differentiation which is a vital phase to further up-regulate or express genes that are responsible for extracellular matrix mineralization. This result is accord to result reported by [35 and 36].

The cells attachments and proliferation patterns were observed through light microscopy as well as Scanning Electron Microscopy (SEM). Cells that attached onto the surface of the scaffold structure during seeding provide an interesting observation on the cells adhesion and morphological properties in a two dimensional view. Based on the findings from the microscopy studies a favourable adherence of cells towards the nanocomposite scaffold was evident on the scaffold structure. The researcher [37] found similar kind of observation, they showed higher number of proliferating cells aggregating to one another to form stratified cell layers towards the biomaterial scaffold in which the author concluded that the scaffold material as non-toxic and biocompatible in nature. However, the three dimensional growth pattern observed through SEM micrographs provides the much needed informations on the actual attachment and progressive (growing) osteoblast cells properties.

At 48 hours post seeding, a larger number of cells were found to have well attached to the surface of the scaffold material. Round clusters of cells with distinct microvilli were observable within the network of the nanocomposite scaffold as evident through the SEM images as well as through light microscopy studies on the H&E stained sections. This profound attaching of cells on the surface of the nanocomposite scaffold indicates that the presences of the CCAN in the scaffold composition as well as the other materials highly influences the initial osteoblast attachment properties by providing important cues in promoting cell adherence and attachment. This study hypothesized that the presences of the CCAN may have contributed to the increase in binding sites for the osteoblast cells. The presences of the CCAN in the scaffold composition also leads to surface structure modification in which an increase in surface structure roughness is observable due to the deposition of the CCAN as noted through the higher magnification of SEM images [11]. This finding is similar to those of [3 and 38] who also showed that the topographic surface of a substrate can affect the cells attachment to it. Images from the VPSEM also confirm cell adaptation to the 3D environment in the scaffold, connecting the pores and attaching to the wall of the pore. The VPSEM revealed an important deposition of calcium in the bone matrix. This observation was comparable to the observations reported by [39 and 40].

The boundary existing between scaffold and the matrix of bone is an interfacial sheet of collagenous unmineralized matrix with several micrometers thickness. The sheet is apparently fashioned from the discharge of extruded osteoblast progressions and well developed osteoblast cells. These results are similar to the one reported by [41]. Nevertheless, [3 and 38] stated that additional protein are seen when cultured osteoblast are not closely stick to the substrate, but assumed stand-off morphology with significant surface activity. Thus, these findings reconfirmed this study hypothesis that the cultured scaffold enhanced cell distribution *in vitro*.

Based on the increase surface area to volume theory, an appropriate increase in surface structure roughness is known to provide a beneficiary effect to the scaffold material as adequate surface roughness which has been shown to facilitate better cell attachment

and proliferation [42, 43, 44]. The researchers [45] also reported the improvement in cell attachment properties in the presences of a surface with nano level roughness, thus further supporting the findings in this study on the early stage cell attachment.

The SEM image of the nanocomposite scaffold at the same time frame also showed the presences of large clusters of cells. These clustering of osteoblast cells were also reported by [31 and 46] and in a study on chitosan-alginate scaffolds and on cockle shell microparticles scaffolds respectively at 3 and 7 days of culture, thus further supporting the findings from the current study on the behaviors of the osteoblast cells. Furthermore the clustering effect of these cells has been shown to have a desirable outcome in regards to enhance the differentiation phase, thus indirectly reducing the needs for larger number of cells for seeding purposes [47]. These criteria could be favourable for future application purposes in regards to producing a cell seeded scaffold system.

The (CCANP3DS) showed an interesting observation comparatively within these large clusters of cells as observed at 48 hours. Scattering of small nodules were noted among the cell clusters that indicates an important finding in regards to the early matrix mineralization process. These nodules indicate the presences of calcium rich mineral salt observed on the surface of the cells prominently occurring in the nanocomposite scaffold. The EDS analysis further supported these findings. According to [48], the presents of calcium nodule secreted by cells is regarded as a sign for successful bone regeneration capacity. Such observations were also reported by [31, 46, 49 and 50]. The presence of these nodules also supports the high biocompatibility nature of the scaffold that could induce a better cell response without the need of any form of stimulating agents or growth factors.

VPSEM observations on the scaffold structure at 14 days post-seeding gave a full understanding on the performance of the nanocomposite scaffold in regards to supporting its intended function. The images obtained at 14 days of the study clearly show the excellent osteoconductive nature of the scaffold observed through a well mineralized surface structure of the scaffold resembling closely to the trabecular bone structure. These findings may denote the late stage of matrix mineralization following ExtraCellular Matrix (ECM) deposition by the osteoblast cells that has likely reached the maturation stage much earlier [34]. The ability of the scaffold to show mineralization of its matrix is often used as an indicator for a potentially good bone repair process [45].

Histological staining sections detected in the (CCANP3DS) indicating presences of osteoid like tissues with high calcium deposition that underlies the maturation of the matrices. Verification by EDS further confirms the structure observed on the nanocomposite scaffold are completely mineralized ECM with the detection of high levels of calcium and phosphate. The ability of the osteoblast cells to promote the ECM deposition and mineralization has been shown in many studies at varying intervals, for instance, by 20^{th} day in the presences of Bone Morphogenetic Proteins (BMPs) glass ceramic scaffold [48], 3 to 4 weeks in porous poly ε-caprolactone scaffold [51], by 17^{th} day on porous titanium scaffold [34], by 21^{th} day on chitosan/poly(lactic acid-glycolic acid) scaffolds [43], from 7 to 28 days on a bi-modal porous scaffold [52], from 19 to 21 days on a porous 3D-scaffolds [31] as well as by 14 days on porous calcium phosphate-alginate cement scaffolds [53] and alginate/cockle powder nano biocomposite scaffold [18]. This finding on the unique architecture displayed on the surface of the nanocomposite scaffold gives it an additional credit in possessing remarkable potentials in inducing early osteoblast cell maturation and matrix mineralization without the requirement of additional promoting agents.

The analysis of EDX showed that numerous nodules containing of calcium carbonate exist. The nodules are rich in calcium minerals as seen by analysis using EDX. Calcium carbonate or mineral deposition formation is an initial osteoblast cells function. The examination of the energy dispersive of an osteoblast on this scaffold revealed calcium signs between 3-14 days of the culture and also the extracellular matrix, and their quantities improved from 7 to 14 days. These findings advocate that the cell clusters and also the individual cells on the new scaffold contribute to the calcium production. These findings are similar to the one obtained by [46, 54-57].

Examination histologically revealed that the scaffold was enclosed and also packed with new bone or osteoid tissue. Also in this work, histological examination established that formation of bone began after seeding of scaffold with the osteoblast cells. Consequently, osteoblast cells secret collagen and ground substance which constitutes the early osteoid or non-mineralized bone. Also osteoblast is accountable for calcification of matrix. The process of calcification appeared to start by the osteoblast through the matrix of small membrane-limited matrix vesicles secretion. These vesicles are alkaline phosphatase rich and are secreted keenly when the cell is producing the bone matrix only. The vesicles are classically situated at a definite length from the cells where mineralization is to take place. These results and observations are similar with the observation of [58].

Organization and maturation of the bone like extracellular matrix shows the biological implication of the beginning of mineralization as a subsequent conversion point in the osteoblast progressive sequence. Studies which demonstrates the association between mineralization and the chronological genes expression during the progressive osteoblast phenotypic growth was also carried out by [59-61]. Additionally, it is acknowledged that the entire cellular procedures rest on the communications between extracellular matrix (ECM) and cells, also the ECM protein may change the surface chemistry of tissue-engineered substrates to improve adhesion of cell and growth promoting [3, 62-66].

From the cellular perspective, cell attachment and dispersion are vital occurrences in effective tissue-graft material formation. Conclusively, these findings revealed that there is significant difference between scaffolds; this was due an indication that not all scaffolds configurations have the same prospective and even quality that can be used *in vivo* but at 7^{th} and 14^{th} day, a sharp increase of ALP, cell proliferation and calcium deposition respectively were seen on scaffold 5211 which was cultured under usual conditions; obviously suggest that this scaffold was accountable for the improved hFOB cells mineralization.

4- Conclusion

The findings from the current study and those from previous work (our published data, [11] showed that scaffold 5211 was favorable characteristic in terms of its physiochemical evaluation, morphology, mechanical strength as well as biological performance (cytocompatiblity and biodegradability). Furthermore, this study concluded that 5211 scaffold is an excellent

osteoconductive scaffold of the third generation biomaterial based scaffold category in regards to its *in vitro* performance. The latter provided sufficient evidence on pursuing further on assessing the performance of the scaffold in an actual biological system. This study also concluded that the MTT and ALP level that were observed in the nanocomposite scaffolds during 14 days of the culture period are significant and clear evidence of its biocompatible composition and porous structural design that is capable of promoting and facilitating cell attachment, migration and proliferation. Hereby, this study presents scaffold 5211 to be considered as a bone replacement.

Acknowledgements

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