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Cockle Shell-Based Biocomposite Scaffold for Bone Tissue Engineering

Zuki Abu Bakar, Bahaa F. Hussein and Noordin Mohamed Mustapha
Faculty of Veterinary Medicine, Universiti Putra Malaysia
Malaysia

1. Introduction

Bone transplantation is a rapidly growing and expanding field, and has a significant impact on improving the quality of life of patients suffering from bone tissue damage and disease. Bone is the second most frequently transplanted tissue in humans after the blood. Secondary bone tumour, trauma or deformity often presents a significant problem for bone surgery (Boyne *et al.*, 2002). Grafting is regularly used in medical procedures to replace damaged tissue. Bone grafting involves replacing damaged bone with harvested bone from donors or from another location within the body. Traditionally, bone graft treatments, such as autografting, allografting, and xenografting were used to replace or repair damaged bone tissue. These processes can be long, painful, and have the possibility of being rejected by the body. Autografts are osteoinductive, osteoconductive and have osteogenic properties (Cypher and Grossman, 1996). Although autografts are considered the gold standard of bone transplantation, they also have certain limitations due to possible donor site morbidity, low tissue availability and may introduce additional medical complications (Younger and Chapman, 1989; Moore *et al.*, 2001). Allografts are grafts made of tissue from a human donor, usually during post-mortem. This method rules out the limitations of auotgrafts technique, but have their own limitations including donor shortages and risks of infections. Xenografts are bone grafts from different species such as bovine, porcine or coralline bone that can be implanted into human graft site. Xenografts offer the advantage of availability in a variety of shapes and sizes, but they are also subjected to problems of immunogenicity and have the tendency to denature or decompose in room temperature (Vaccaro *et al.*, 2002). Distraction osteogenesis is another technique for bone treatment used to promote bone growth using the body's innate bone-healing mechanisms. The process is lengthy and painful, in which the two sides of a bone fracture are separated by a short distance every few weeks until the desired length of bone has been regrown (Chang *et al.*, 2004). Other treatment options, the man-made devices, such as bone cement fillers and prosthetics made of metals, ceramics and polymers are also used for bone defect repair or replacing damaged bone tissue. All the conventional methods for bone repair and replacement can be long, painful and have the possibility of being rejected by the body (Ducheyne *et al.*, 1992). Alternative approaches have been heavily researched and investigated based on a tissue engineering strategy, in an effort to overcome the inherent limitations of the currently available solutions to bone defects. In this approach, a tissue engineered bone is produced by seeding cells that can become osteoblasts on highly porous biomaterials (Hardin-Young *et al.*, 2000).

Tissue engineering is one of the most exciting and rapidly growing areas of biotechnology. According to Williams (1987), tissue engineering is defined as an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain or improve tissue function. These substitutes are generally known as "scaffolds". The concepts of tissue engineering is to engineer autografts, by expanding autologous cells *in vitro* guided by a scaffold (artifical matrix), or by implanting an acellular scaffold *in vivo* and allow the patient's cells to repair the tissue guided by the scaffold. In both ways *in vitro* or *in vivo*, tissue engineering utilize a scaffold (supporting structural device), osteogenic cells and bioactive factors either alone or in various combinations (Meyer *et al.*, 2004b). The scaffold should be made of a biocompatible and bioresorbable material that degrades and disappears over time with the regeneration of tissues, so that once the tissue has developed the scaffold no longer exists as such and the newly formed tissue can perform the function of the damaged tissue (Ross, 1998). Therefore, tissue engineering offers a potential approach to create tissues, organs and synthetic graft products under laboratory conditions to overcome the problems of implant rejection, transmission of diseases associated with xenografts and allografts, and shortage in organ donation.

The main target for bone tissue engineering is bone repair, and it can be used for healing or repairing wide range of bone defects. There are many different strategies which may be used to develop bone tissue engineering. One such strategy involves seeding autologous osteogenic cells *in vitro* along a biodegradable scaffold to create a scaffold–cell hybrid that may be called a tissue-engineered construct. Osteoblasts, chondrocytes and mesenchymal stem cells obtained from hard and soft tissues of the patient may be expanded in culture and seeded onto a scaffold that should in some way fade away to allow an entirely natural bone tissue replacement (Langer and Vacanti, 1993). Millions of patients with bone problems worldwide each year could benefit from this new therapeutic strategy (Buckwalter, 2004; Holtorf *et al.*, 2005).

Scaffolds are the key to the development of tissue engineered bone construct for implantation into the bone critical defect. Successful tissue generation *in vitro* and/or *in vivo* requires highly specialized artificial bone substitutes, since they serve as a model in three dimensions for the interposition of tissue mimicking the extracellular matrix for adhesion and cell proliferation, differentiation and must also provide support to protect the tissue in the early stages of healing (Freed *et al.*, 1994b). Many artificial bone substitutes that introduced to maintain the function of bone containing metals, ceramics and polymers. Each material has specific disadvantages, and none of them can fully substitute for autografts in current clinical practice. More importantly, the bone graft substitute must be biocompatible, nonimmunogenic and noncarcinogenic to minimize host response. The bone graft substitute must also possess mechanical properties similar to that of the bone to prevent stress shielding (Hench and Wilson, 1993). Many of artificial bone substitutes, with its high biocompatibility and good bioaffinity, stimulate osteoconduction and are slowly replaced by host bone after implantation. An osteoconductive material allows a series of events of protein adsorption resulting from the anchor of osteoblasts to the surface and subsequent deposition of extracellular matrix and bone (Dziedic *et al.*, 1996). This allows for intimate contact to be made between the implant and surrounding tissue, thus accelerating the natural healing response. Finally, a potential bone graft material should be consistent in use and readily available in unlimited quantity in order to be considered suitable for surgical implantation (Hatcher, 2004).

The success of a tissue engineering scaffold will come into play to determine whether it can support cell attachment, growth and eventually cell differentiation into the appropriate tissue. For these reasons, the bioresorbable scaffold must be biocompatible and having porous interconnected network to facilitate vascularization and rapid growth of newly formed tissue (Laurencin *et al.*, 1999; Mooney and Mikos, 1999). Hence, several needs are identified as crucial for the production of scaffolds in tissue engineering. The scaffold should possess a) interconnecting pores of a scale appropriate to promote integration and vascularization of tissues, b) controlled biodegradability or bioresorbability, so that the host tissue will eventually replace the scaffold, c) appropriate surface chemistry to promote cell attachment, differentiation and proliferation, d) mechanical properties sufficient to match the space provided for the implementation and handling, e) not induce a negative response, and f) easily manufactured in a variety of shapes and sizes (Li and Li, 2005). Given these requirements in mind, several materials have been adopted or synthesized and fabricated into scaffolds. The basic principle of our research work was to apply the tissue engineering strategy that combines osteoprogenitor cells with a new bioceramic scaffold for regeneration of critical size bone tissue defect. The cockle (*Anadara granosa*) is by far, the most abundant species cultured in Malaysia. A possible advantage of using cockle shell as a biomineral is that they may act as analogs of calcium carbonate present *in vivo*.

2. Morphological and biomechanical properties of cockle shell-based biocomposite scaffolds

The information from the experimental study showed that the physical and mechanical properties including stiffness, degradability, porosity and mechanical integrity of this scaffold were appropriate for use as extracellular matrix materials for bone tissue engineering. All the scaffolds prepared have pore size between 20-420 μm. The ESEM examinations of the prepared scaffolds as shown in figure 1 revealed that the fabricated scaffolds possessing different sizes of porosity. The physical characteristic of porosity formed in the scaffolds are mainly attributed to the voids containing trapped air that make way to provide spaces for the swelling effect when the scaffolds get wet, and these voids formed between the granules or particles in the bulk material. Our observation on the formation of porosity is in accord with Lam *et al*. (2002). The macro porosities in the scaffolds were created as the result of homogeneously dispersed air bubbles throughout the mixture by stirrer magnetic machines at 800-900 rpm. The mixture was observed to contain a fine dispersed of air bubbles, after the formation of a dough-like solution. Different wt% of scaffolds solution had a suitable interconnected pores size between 20-420 μm. After evaporation of water by heating the mixture becomes more concentrated and thick, which could be sufficient to block the air bubbles to release and this will lead to an increase in the pores after the mixture dried. Similar finding was reported by Kang *et al*. (2006). In this study, we used dextran, gelatin, and dextrin in addition to the cockle shell powder as scaffold materials for tissue engineering applications because we thought that we could take advantage of the powders in microsphere processing for scaffold design. The dextran was particularly chosen since it has been shown to be resistant to both protein adsorption and cell adhesion, and allowed designing a scaffold with specific sites for cell recognition (Le´vesque *et al*., 2005). The used of dextran for scaffold development not only due to high number of pores created but also their morphology which was more rounded, and the

degree of interconnectivity observed were highly influenced by the amount of dextran. In fact, the natural dissolution of dextrin, dextran and gelatin in water was responsible for the porosity formation. Furthermore, it could be attributed to the fact that an increase in gelatin concentration increased the capacity for entrapment of air bubbles and particle-induced pores in the matrix (Askarzadeh *et al.*, 2005; Le´vesque *et al.*, 2005). Therefore, an inversely proportional relationship between the compressive stiffness and porosity was established due to the increased in pores and the degree of interconnection between pores. Also, there is a proportional relationship between the amount of pores in the scaffold and the degradation rate due to an increase in the surface area. The scaffolds prepared by freeze-drying had dual pore structures caused by air bubbles and ice crystals, for freezing the dispersion or solution results in the formation of ice crystals that force and aggregate the collagen molecules into the interstitial spaces. The ice crystals were then removed by freeze-drying. Also, the conventional freeze dried scaffold formed only small pores as a result of a fast freezing rate, and the ice crystal agglomeration can controlled the pore size as showed in figure 2. These results are consistent with the results of Le´vesque *et al.* (2005) and Sachlos and Czernuszka (2003).
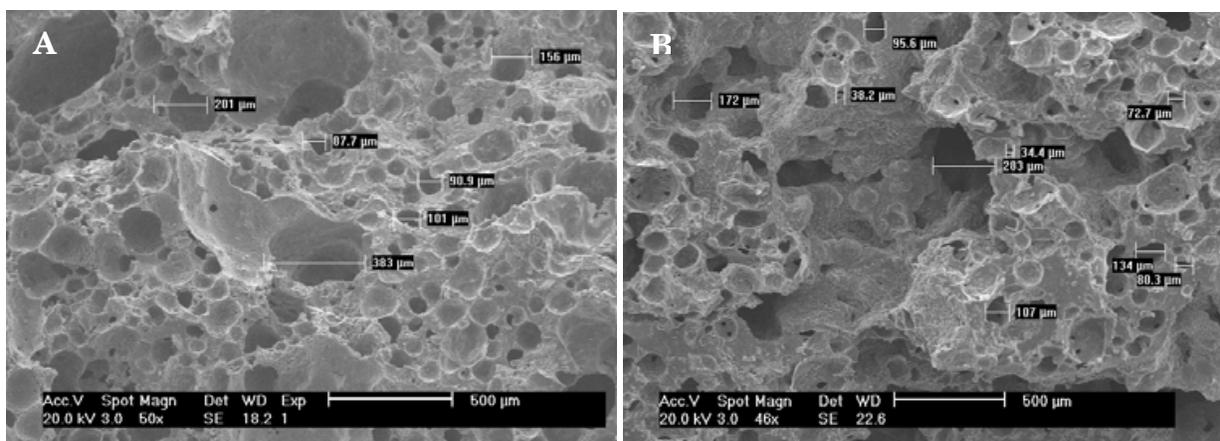

Fig. 1. ESEM microphotographs of scaffolds prepared by heated method showing porous structure. A) scaffold 244 and B) scaffold 334
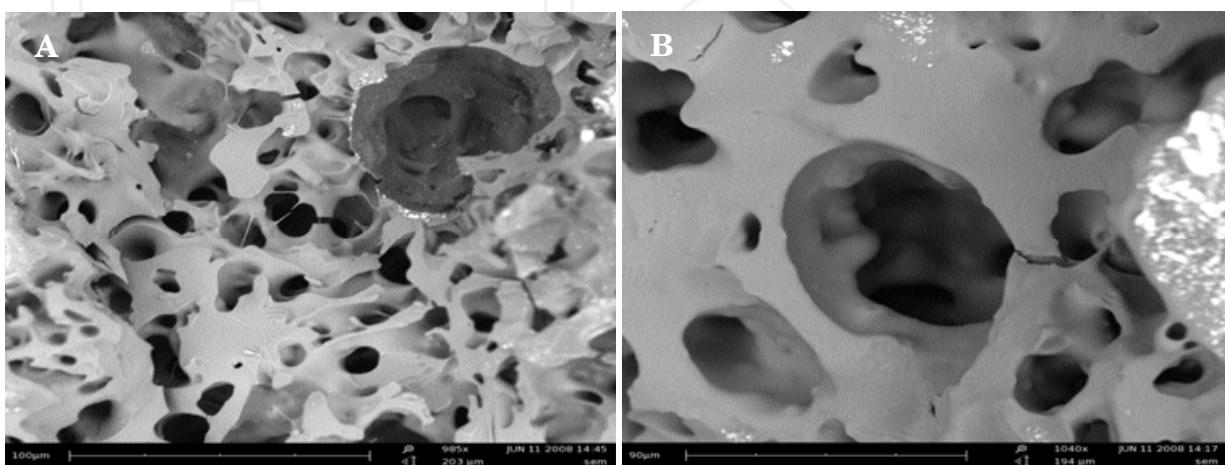

Fig. 2. Phenom SEM of the scaffolds prepared by freeze drying method show high porosity with different pore sizes and high interconnectivity. A) scaffold 244 and B) scaffold 352

Micro porosities may increase the degradation rate of the scaffolds due to the increased surface area available for attack. Scaffolds prepared with the freeze dryer method exhibit higher porosity. Accordingly, different scaffolds porosity and outer solid layer thickness was achieved by these two methods, which have direct influence on their degradation behaviour. In our study the scaffolds cross linked produced by heating method show a decrease in the acceleration and degradation rate. This result is in contrast with Oliverira and Reis (2004), who mention that most of the scaffolds lose half of their strength almost after the first days of immersion in the liquid, and the continuous soaking in the liquid will displayed only 5% of their initial strength and had lost almost half of their starting mass. In contrast, our study showed that the scaffolds dimensions did not change significantly during the degradation evaluation for up to 10 and 30 days for scaffolds prepared by freeze drying and heating methods, respectively. Thus, the decline in mechanical properties for these scaffolds is due to excessive mass loss, which was caused by the increased in porosity of the scaffolds. Scaffolds prepared by freeze drying method characterized by a rapid degradation than those fabricated by heating method, which was related to the short chains of amorphous product, consequently, this leads to increase in the number of sites susceptibility to chain scission (Porter *et al.*, 2001). The scaffolds prepared by freeze drying method exhibit significantly (P<0.05) higher water uptakes, compared to the samples prepared by heating method which have higher porosity. Nevertheless, the scaffolds produced in our study by both methods might be very useful in situations where a high mechanical property is necessary.

There is a conflict between a high porosity and the mechanical compression properties of the scaffolds. Dry infiltrated scaffolds 262 and 226 demonstrated lower mechanical properties (Table 2), which probably due to the higher porosity and interconnection between pores of the scaffolds prepared with this method. Although these porous structures are most appropriate for the final application of these scaffolds, still these scaffolds provide a good mechanical property when compared with other scaffolds that obtained from other biodegradable polymers which presented a compression module below 12 MPa, and proposed for use in tissue engineering of bone, for example, PLLA/hydroxyapatite composite foam (Zhang and Ma, 1999). Maria (2004) method for preparing more than one type of scaffolds was consistent with our technique. She used different processing methods to produced different scaffolds of different geometries, and the samples tested have also different geometries and dimensions.

The compression tests of scaffolds were carried out to obtain the stress-strain relations from which the yield strength and modulus were evaluated. The significant increase in modulus and yield strength for the scaffold can be attributed to the strong interactions between the four materials that form the scaffolds, and one of the major challenges in the fabrication of porous scaffolds is the tradeoff between adequate material porosity and mechanical strength (Thomson *et al.*, 1995b; Porter *et al.*, 2000; Zhensheng *et al.*, 2005). However, some of the scaffolds posses the similar yield strengths, which suggested that polymer infiltration, did not affect the yield strength of the dry sample. It was noted that for the porous designs, there was a significant increase in the yield strength in some because of different porosity scaffold after polymer infiltration. This reinforced the theory by infiltration with polymer to improve the mechanical properties of the scaffolds (Lam *et al.*, 2002). The role of gelatin in compression mechanical test was to provide support of the mechanical compression, and these were formed by the changes of chain of amino acids by cross linked. Thus the initial modulus of scaffolds gradually increased with increasing concentrations of gelatin in the

mixture solution for both freeze-dried and heating stirrer-processed (heat method). This varying in mechanical property may be due to the increase and decrease of the scaffolds porosity, as showed in table 1. The decrease in the modulus MPa of scaffolds was due to the increase in scaffold porosity. Our finding agreed with what was reported by Kang *et al*. (2006). The variation in the ratio of gelatin to other materials, the degree of porosity composite and the degree of crosslinking may influence on modulus and ultimate compression test of the specimens.

No.	Sample	Yield strength MPa	Modulus MPa
244	Dry uninfiltrated	11.43	144.5
244	Dry infiltrated	13.95	182.3
244	Wet infiltrated	1.946	0.696
262	Dry uninfiltrated	3.628	74.57
262	Dry infiltrated	3.429	20.74
262	Wet infiltrated	0.132	0.428
352	Dry uninfiltrated	13.19	187.5
352	Dry infiltrated	9.676	71.41
352	Wet infiltrated	0.394	0.751
334	Dry uninfiltrated	7.271	123.1
334	Dry infiltrated	5.801	33.24
334	Wet infiltrated	0.007	4.077
226	Dry uninfiltrated	4.894	48.05
226	Dry infiltrated	5.250	148.8
226	Wet infiltrated	2.779	0.639

Table 1. Compressive stiffness (dry uninfiltrated, dry infiltrated and wet infiltrated) measured at 25°C for scaffolds prepared by heat method

The DSC analysis can be used to determine the physical transformations or phase transitions of the bioceramic scaffolds. A melting or glass transition temperature of the material can be determined, which gives an overview of the physical nature of these scaffolds. The melting transition temperature (T_m) is influential when the materials used are crystalline or semi-crystalline. When a glassy region and the stage of glass transition is followed a significant decrease in the graph without other peaks present, an average glass transition temperature (T_g) can be determined. During its melting, the material blend shows two peaks next to each other; the peak found at 97.78-112.97°C is attributed to gelatin, dextran and dextrin melting (Figure 3). These temperatures are compatible with those used to extrude the blends. Also, there are peaks less intense, approximately at 204-245°C which probably related to glass transition temperature (T_g). As the temperature increases the sample eventually reaches its melting temperature (T_m). The melting process results in an endothermic peak in the DSC curve. The ability to determine transition temperatures and enthalpies (thermodynamics and chemistry) makes DSC an invaluable tool in producing phase diagrams for various chemical systems. These results were in accord to that of Marcos *et al*. (2005). Glass transitions (T_g) may occur as the temperature of an amorphous solid is increased. These transitions appear as a step in the baseline of the recorded DSC signal. This is due to the

sample undergoing a change in heat capacity; no formal phase change occurred as showed in figure 3. As the temperature increases, an amorphous solid will become less viscous. The main application of DSC is in studying phase transitions, such as melting, glass transitions, or exothermic decompositions. These transitions involve energy changes or heat capacity changes that can be detected by DSC with great sensitivity. The melting points and glass transition temperature for these materials are available from standard compilations and the method can show up possible materials degradation by the lowering of the expected melting point. Depends on the molecular weight of these materials, thus lower grades will have lower melting points than expected. This is possible because the temperature range over a mixture of compounds melts is dependent on their relative amount da Silva (2009).
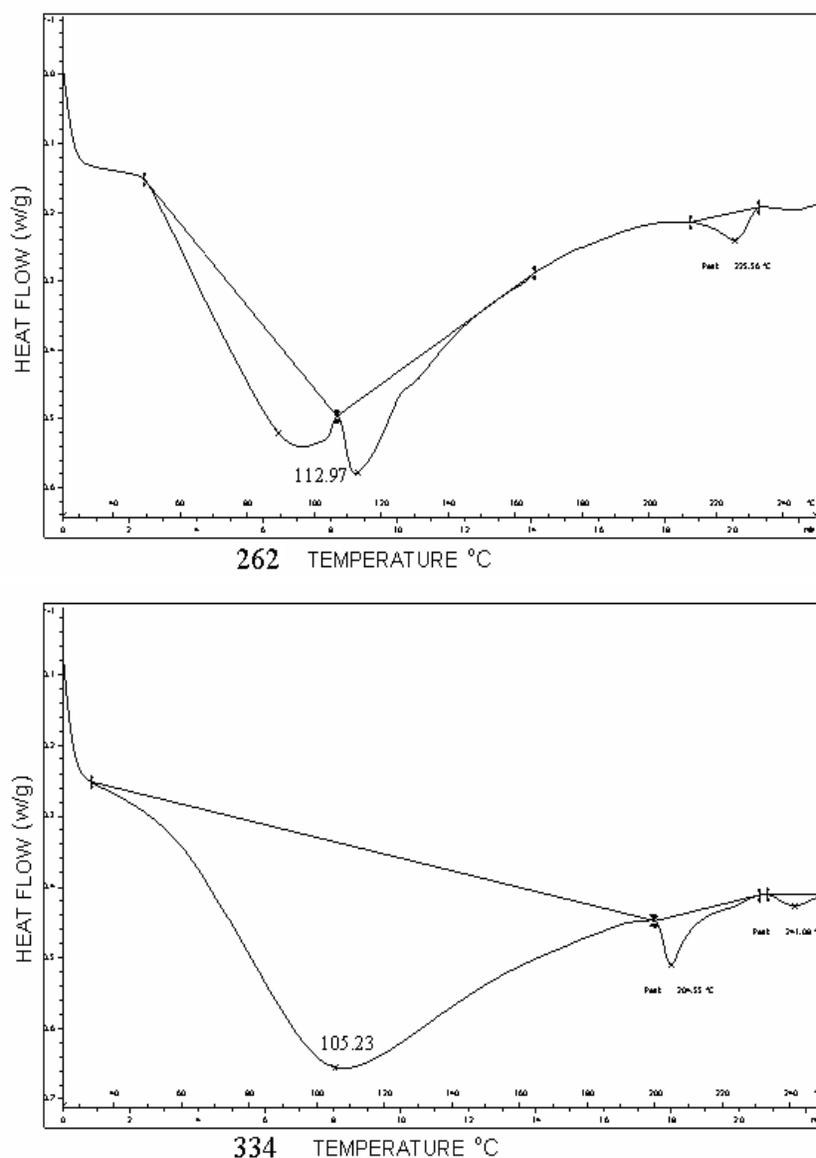
Fig. 3. The graphs show the endothermic DSC peak (denaturation) of the scaffold materials (gelatin, dextran, dextrin and CaCO$_3$) dispersion of the three samples with a maximum at 97.78-112.97°C. The first and second minimum melting peaks of the three samples observed to be semi symmetrical. The thermal signature was that of the scaffolds with the first and second peak of three samples (262, 334, 352)
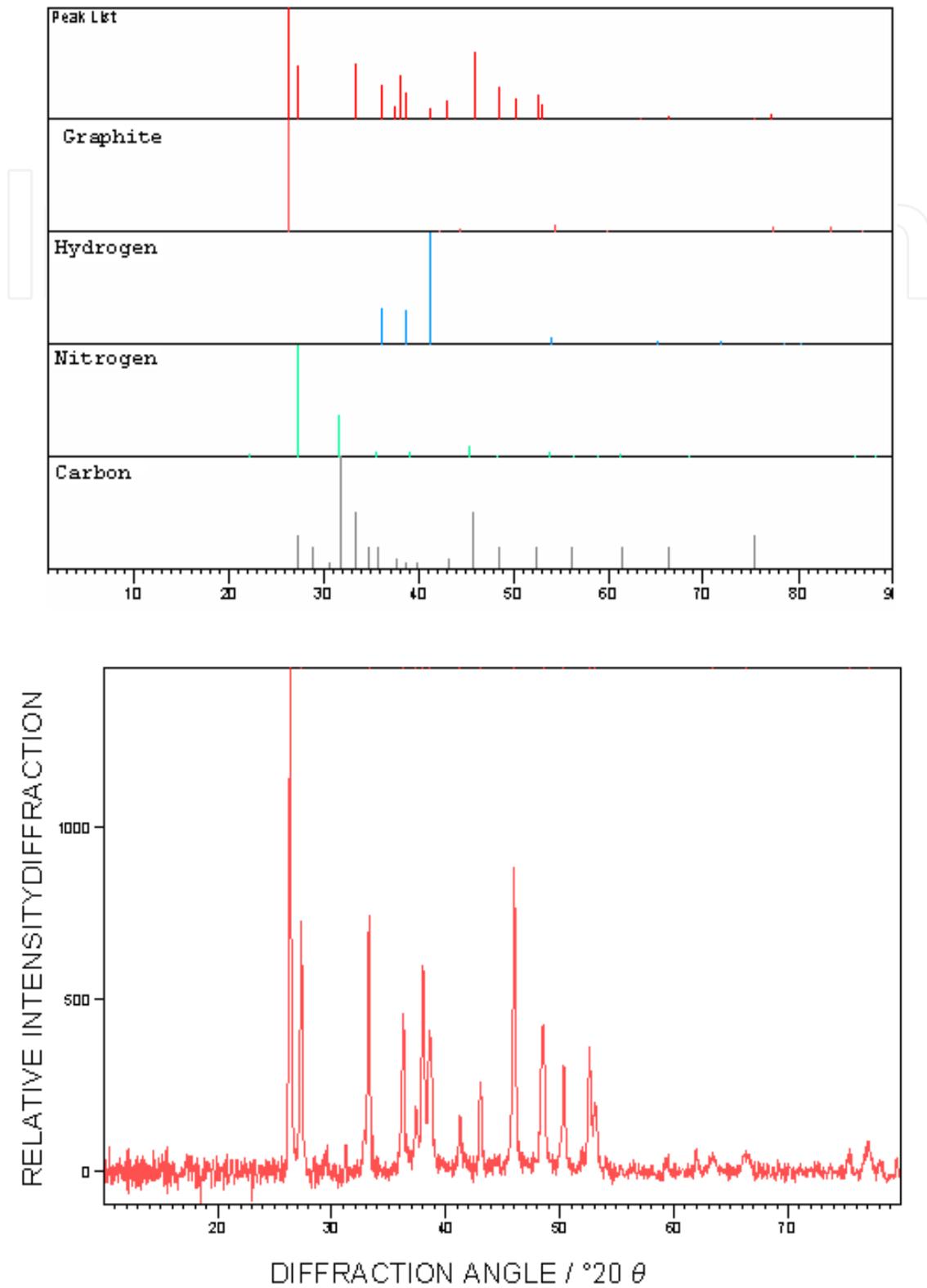
Fig. 4. The graphs show the XRD pattern of the scaffold products. The peak at 26°θ and 28°θ are characteristic of calcium like graphite, and other peaks are characterized of Calcium. This corresponds well to the specification (Graphite, Ca)

No.	Sample	Yield strength MPa	Modulus MPa
244	Dry uninfiltrated	10.10	2037
244	Dry infiltrated	12.62	2075
262	Dry uninfiltrated	23.99	1952
262	Dry infiltrated	23.70	1898
352	Dry uninfiltrated	7.141	268.5
352	Dry infiltrated	4.621	152.4
334	Dry uninfiltrated	10.80	825.3
334	Dry infiltrated	9.33	735.4
226	Dry uninfiltrated	4.871	550.2
226	Dry infiltrated	5.231	649.4

Table 2. Compressive stiffness (dry uninfiltrated and dry infiltrated) measured at 25˚C for scaffolds prepared by freeze drying method

The XRD data show an increase in the crystal size for all the composites that do not exhibit an increase in compressive strength. This increase in crystal size, however, is not apparent in the micrographs, as the size of the crystals reduced from the XRD data is near the limit of resolution of the ESEM. Thus, the X-ray diffraction pattern of the scaffolds at different samples, no changes in peak pattern of crystallinity could be detected as shown in figure 4. On the other hand, the XRD data seem to indicate a similar mechanism at work in all the composites, namely improved mechanical properties due to smaller crystallites (Mickiewicz, 2001).

3. *In vitro* evaluation of the osteoblast seeded cockle shell-based biocomposite scaffolds

The ability of bone cells to colonize artificial bone graft substitute material is of clinical importance. Alkaline phophatase is an early differentiation marker. The ALP activity is a transient early marker of osteodifferentiation in MSCs and is generally a good indicator of differentiation. It was reported that ALP is associated with calcification and an enhanced expression of this enzyme is apparently needed just before the onset of matrix mineralization, providing localized enrichment of inorganic phosphate, one of the components of apatite, the mineral phase of bone (Genge *et al.*, 1988). In the current research work, the ALP results as showed in figure 5 agree with the results reported by many investigators, namely, Toquet *et al.* (1999), Kose *et al.* (2003), Park *et al.* (2004), Datta *et al.* (2005), and Wang *et al.* (2007). During the early period (from 5 to 14 days) the extracellular matrix undergoes a series of modifications in composition and organization that renders it competent for mineralization. During this matrix maturation phase, every cell has become alkaline phosphatase positive. Similar results were observed by Lian and Stein (1992). In the current study we observed that the peak activity occurred within 5-7 days, this was in agreement with Park *et al.* (2004) who reported that a high expression of ALP was prominent at 3 days of the culture. However, this finding disagreed with Kose, *et al.* (2003) who reported that ALP activity increased over time for all their samples and especially, after 14 days of incubation. The level of the ALP in sample 226 increased again after 14 days and this agrees with Kose *et al.* (2003) who reported the continuous activity of the cells despite

the calcium deposition in the culture. Thus, in this study an initial rise in activity was expected, followed by a decrease corresponding to further differentiation of the cells, when alkaline phosphatase production slowed. The reduced activity of ALP could be attributed to the fact that more cells cultured on the scaffolds stepped into the next differentiation stage. This was in addition to the intracellular calcium increase which could determine an inhibitory effect. ALP decrease could represent a return to osteoprogenitor cells or maturation to osteocytes, which normally express small quantities of this enzyme. These results were in accord with those of Genge *et al.* (1988) and Wang *et al.* (2007). There was a major decline in ALP activity during matrix vesicle mineralization. The decline in ALP activity is very closely coupled with the rapid accumulation of Ca^{2+} by matrix vesicles. It is a highly reproducible and consistent finding and occurs in every instance of vesicle mineralization. ALP is known to be a transient marker of osteoblastic differentiation, being up-regulated initially and down-regulated as differentiation progresses. Figure 5 depicted the ALP activity during the period of osteoblast culture of different samples and this explains why it was possible to see a statistical difference in end-stage markers, such as calcium deposition, when none was seen for the earlier stage marker ALP.
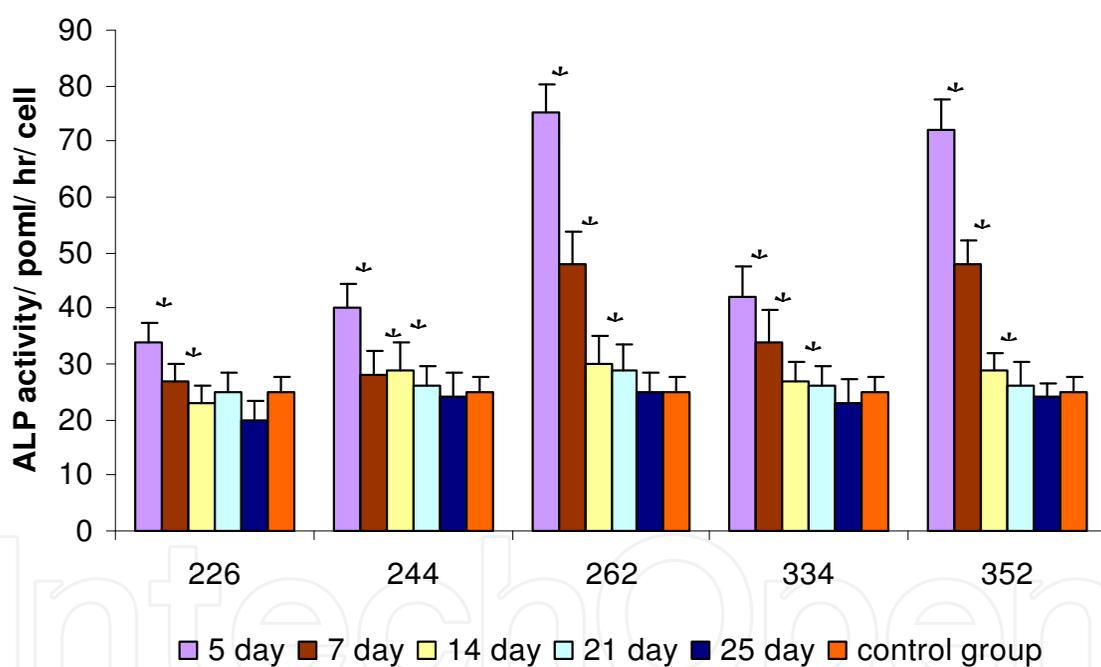

Fig. 5. The ALP activity of osteoblast cells at 5, 7, 14, 21 and 25 days post seeding on different scaffolds. Each bar denotes 1 standard deviation. * Significant difference (p<0.05) (one-sample t-test) within groups

All studies regarding calcium deposition in cultures of osteoblastic cells is a marker of full maturation. There is an important relation between the calcium deposition accumulation cultures and ALP activity. The studies reported here explore this phenomenon and revealed that a consistent marked loss of ALP activity does occur during the matrix vesicles mineralization. The time of onset and the extent of decline in ALP activity were found to mirror almost exactly the time of onset and the extent of Ca^{2+} accumulation by the matrix vesicles of all the scaffolds as shown in Figure 6. Such observations were also reported by

Genge, *et al*. (1988). The mineral accumulation is a consequence of the progression of pre-osteoblastic cells through the proliferation and matrix maturation stages of differentiation and it is an essential step for the further up-regulation or expression of genes responsible for the mineralization of the extracellular matrix. The findings here agreed with those reported by Lian and Stein (1992) and Maria (2004). After 15 days, a dramatic increase in calcium deposition was observed on all types of scaffolds that were cultured under normal flask conditions, clearly suggesting that scaffold 244 was responsible for the enhanced mineralization of marrow stromal cells more than other scaffolds. Possibly the dextrin and cockle shell ($CaCO_3$) concentrations were responsible for the observed, enhanced mineralization including the stimulation of the seeded cells on scaffolds. Additionally, the mitigation of potential nutrient transport limitations experienced by the cells cultured under these conditions enhanced the mineralization. The results clearly suggest that the new product composition is responsible for the enhanced mineralization of osteoblast cells. The higher calcium deposition reported for the scaffolds of some samples may be explained by the higher number of cells and cell density registered in these scaffolds. These observations were in agreement with the results of investigations carried out by Maria (2004). Therefore, calcium content of the scaffolds is indicative of the late stage differentiation of osteoblasts and a continual increase in calcium content over the culture period is expected. All constructs that were seeded with osteoblast cells showed a consistent increase in calcium content, with the highest value occurring at day-25 as seen in this study. Such results were in good agreement with the observations made by Datta *et al.* (2005) and Sikavitsas *et al.* (2005). It is well known to many researchers that the expression of calcium, a marker for mature osteoblasts, is strongly correlated with bone mineralization in osteoblast cultures. In the absence of mineralization, high levels of calcium cannot be expressed, and levels of ALP cannot decline. Such findings suggest that mineralization itself is closely associated with calcium expression, which may support our results in which the increased level of calcium (Ca^{2+}) occur during matrix vesicles mineralization formation. Such findings are in accord with that reported by Stein and Lian (1993) and Maeno *et al.* (2005).

From a cellular viewpoint, cell attachment and spreading are critical phenomena in formation of successful tissue-graft material interactions. In conclusion, this study has shown that there is no significant difference ($p > 0.05$) between groups, and this was an indication that all scaffold compositions have the same potential and even quality and can be used *in vivo*.

In the ESEM analysis, all the figures showed that all the osteoblasts on the cultured scaffold were covered by a dense matrix coating on the bottom and the top surfaces together. The cells were able to migrate throughout the scaffolds and filled the entire construct. The top surface of the scaffolds exhibited a thin layer of extracellular matrix, after 3 to 7 days in culture. ESEM examination of the surface of the seeded scaffolds revealed that cells had attached and begun to spread. However, the cells did not adhere closely to the irregular surface of the substrate but instead made numerous contacts via cytoplasmic extensions as seen in Figures 7. These results agreed with that of Sautier *et al.* (1990), who reported that the topographic surface of a substrate can influence the manner in which the cells attach to it. The ESEM images also confirm the adaptation of the cells to the 3D environment in the scaffolds; bridging the pores and attaching to the pore walls. Figures 8 showed a significant amount of calcium deposition in the bone matrix. Similar observations were also reported by Yoshikawa *et al.* (1997) and Yoshikawa *et al.* (2000). In addition, the attached cells exhibited numerous processes and surfaces studded with microvilli and ruffles. It is possible that fixation and dehydration caused cellular collapse and that the lack of close contact was an

artifact. At the interface between the bone matrix and scaffold is an interfacial layer of collagenous unmineralized matrix, several micrometers thick. This layer is reportedly formed from secretion of extruded osteoblast processes and was seen in figures 9. These results agreed with those obtained by Yoshikawa *et al.* (1998). However, Sautier *et al.* (1990) reported that more protein synthesis occurred when cultured osteoblasts did not adhere closely to the substrate, but adopted stand-off morphology with considerable surface activity. Thus, the results confirmed the conclusion that the cultured scaffolds enhanced cell distribution *in vitro*.
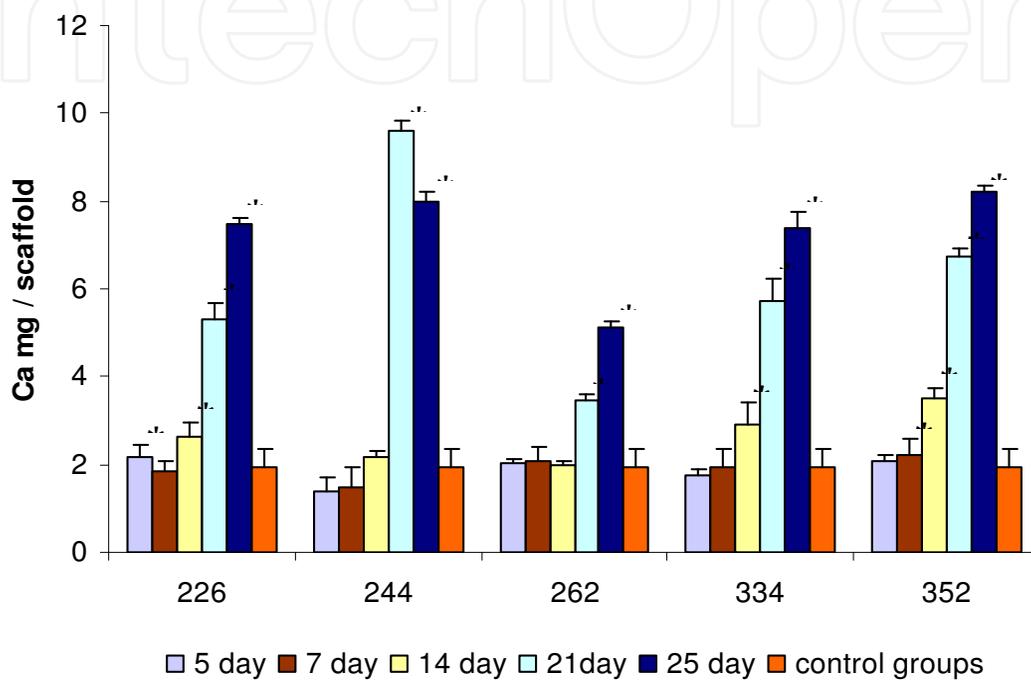
Fig. 6. Calcium depositions of osteoblast cells at 5, 7, 14, 21 and 25 days post seeding on different scaffolds. Each bar denotes 1 standard deviation. * Significant difference (p<0.05) (one-sample t-test) within groups
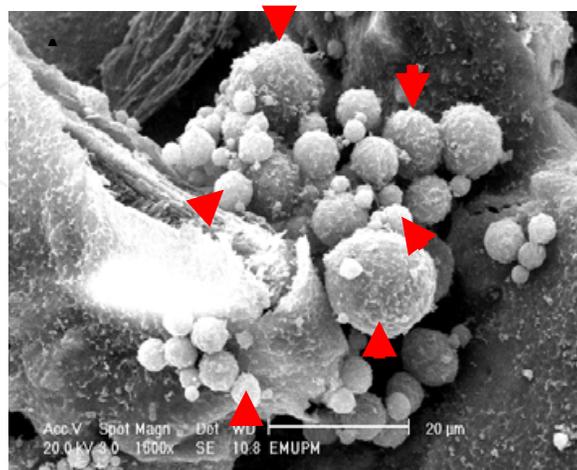
Fig. 7. ESEM microphotographs of rabbit osteoblasts cultured on different scaffolds for 3 days (arrowhead) and 7 days (arrow) show the structure of the osteoblast cells on scaffold 262 (bars = 20µm)
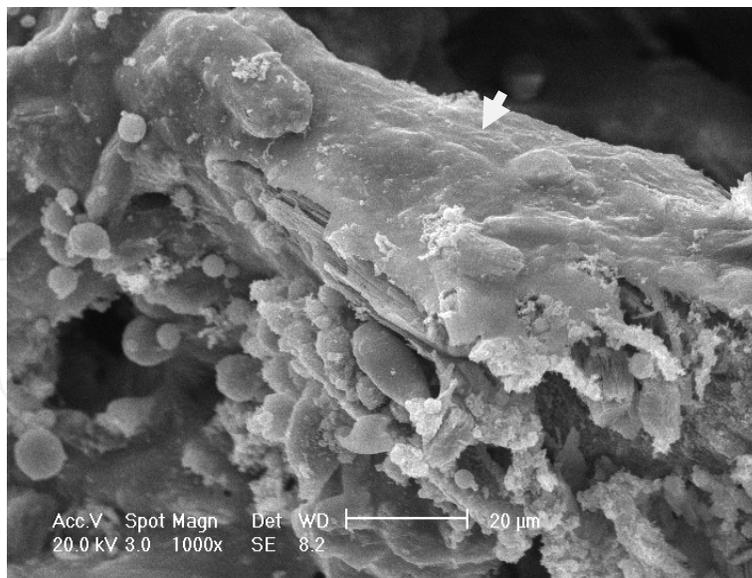
Fig. 8. ESEM microphotographs of scaffold section at 19 days post-seeding. Subculture composite *in vitro* shows the structure of the cells and their processes, and also the activity and creation of extracellular matrix (arrow) (bar = 20µm)
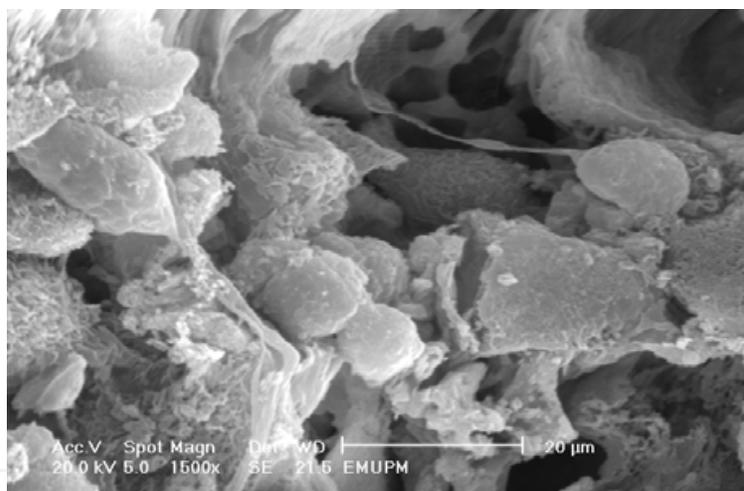

Fig. 9. ESEM microphotograph of rabbit osteoblasts cells on the cultured scaffold at 21 days post-seeding shows that some cells are well developed flat in shape and others are spindle or rounded during this period (bar = 20µm)

The EDX analysis showed that there are many nodules consisting of calcium carbonate. These nodules are calcium-rich minerals, as identified by energy-dispersive X-ray analysis. The formation of calcium carbonate, or mineral deposition, is a primary function of osteoblast cells. The energy dispersive analysis in Figure 10, were taken from the cell surfaces as well as the scaffold matrices (marked with a blue rectangle) and shown on the accompanying SEM images. Energy dispersive analysis of an osteoblast on these scaffolds showed signs of calcium after 19 days culture as well as the extracellular matrix, and their amounts increased from day 14 to day 25. These results suggest that both the individual cells and cell clusters on these new scaffolds contributed to the production of calcium. These results conform with Lu *et al.* (2003) and Li *et al.* (2005).
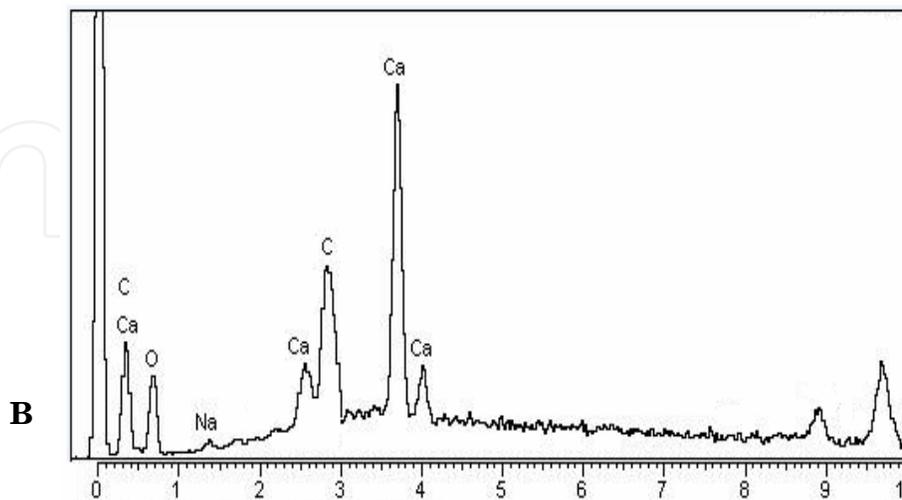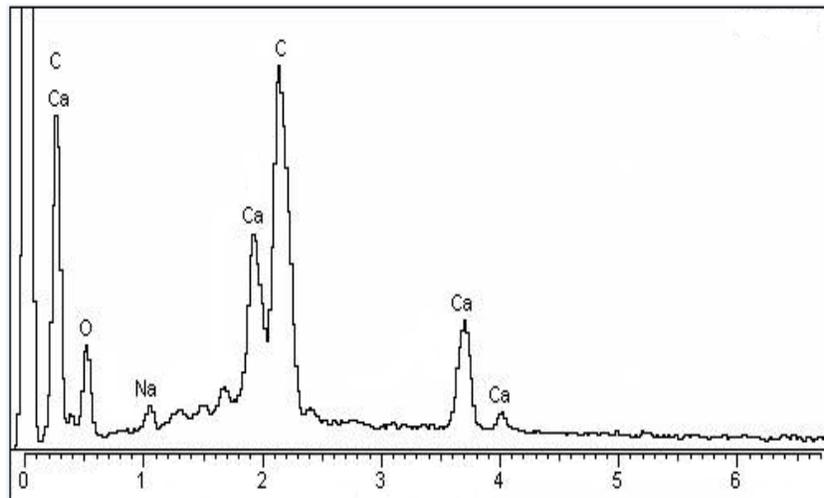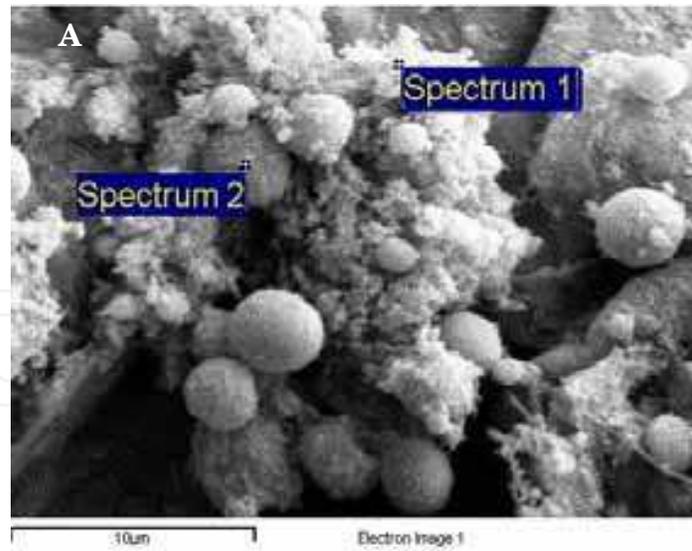
Fig. 10. SEM microphotograph (A) shows the osteoblasts grown on bioceramic scaffolds at 19 days of dynamic proliferation, (B) spectrum 1 depicts the extra cellular matrix and spectrum 2 the calcium carbonate as determined by EDX analysis

Histological examination showed that the scaffolds were covered and filled with new bone or osteoid tissue. Histological examination confirmed that the bone formation commenced after seeding of the scaffolds with the osteoblast cells. Thus, the osteoblast cells secreted both the collagen and the ground substance that constitutes the initial unmineralized bone or osteoid. The osteoblast is also responsible for the calcification of the matrix. The calcification process appeared to be initiated by the osteoblast through the secretion into the matrix of small, membrane-limited matrix vesicles. The vesicles are rich in alkaline phosphatase and are actively secreted only during the period in which the cell is producing the bone matrix. These vesicles are typically located at some distance from the cells where mineralization is to occur. These observations were in good accord with what was observed by Ross and Pawlina (2006). As the process continued, the newly organized tissue, at the presumptive bone site became more visualized, and the aggregated mesenchymal cells became larger and rounded as shown in Figures 11. Because of the collagen content, the bone matrix appeared denser than the surrounding mesenchyme in which the intercellular spaces revealed only delicate connective tissue fibers. The maturation and organization of the bone like extracellular matrix demonstrates the biological significance of the onset of mineralization as a second transition point in the osteoblast developmental sequence. This is further supported by studies that directly demonstrate a relationship between mineralization and the sequential expression of genes during the progressive development of the osteoblast phenotype (Chantal *et al.,* 2000). Furthermore, it is known that all cellular events depend on interactions between the cells and the extracellular matrix (ECMs) and that ECM protein can modify the surface chemistry of tissue-engineered substrates to enhance cell adhesion and promote growth (Jäger *et al*., 2005).
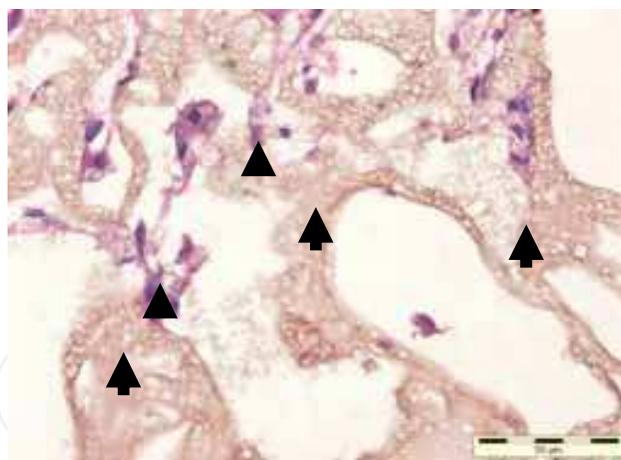


Fig. 11. Microphotograph of the scaffold stained with counterstained van Gison at 60 days post seeded show the osteoblasts (arrowheads) and the osteoid tissue presented in the most area (arrow) with (x 400)

4. *In vivo* evaluation of the osteoblast seeded cockle shell-based biocomposite scaffolds

Rabbit was the animal model used in this experiment because it correlates well with the clinical situations, and the model is well documented and reproducible. We used the segmental bone defect model of rabbit radius to evaluate the bone healing capacity for this

new product. The radius is easily accessible; no fixation is needed because of the ulna support, and the short duration of fracture healing in rabbit model. Only male rabbits were chosen to standardize the sexes and to avoid the presence of hormonal fluctuation in female rabbits, which could give some influence on bone healing. The animal model, it is recommended to use adult rabbits to prevent epiphyseal slipping. For this we used 4-6 month-old rabbits. Such observations also reported by Herold, (1971) who was the first to described this model. The rabbit also is a well-established animal model in the field of BMP research (Zegzula *et al.*, 1997; Texeira and Urist, 1998; Yuehuei and Friedman, 1999). The critical-size bone defects (CSD) normally do not heal when left untreated, thus we used this size of defect to proof that the new developed scaffolds can be used to treat large bone defect. It is well accepted that the length of the CSD in long bone is twice the bone diameter, and bone defects of 15 millimeters were used in this study.
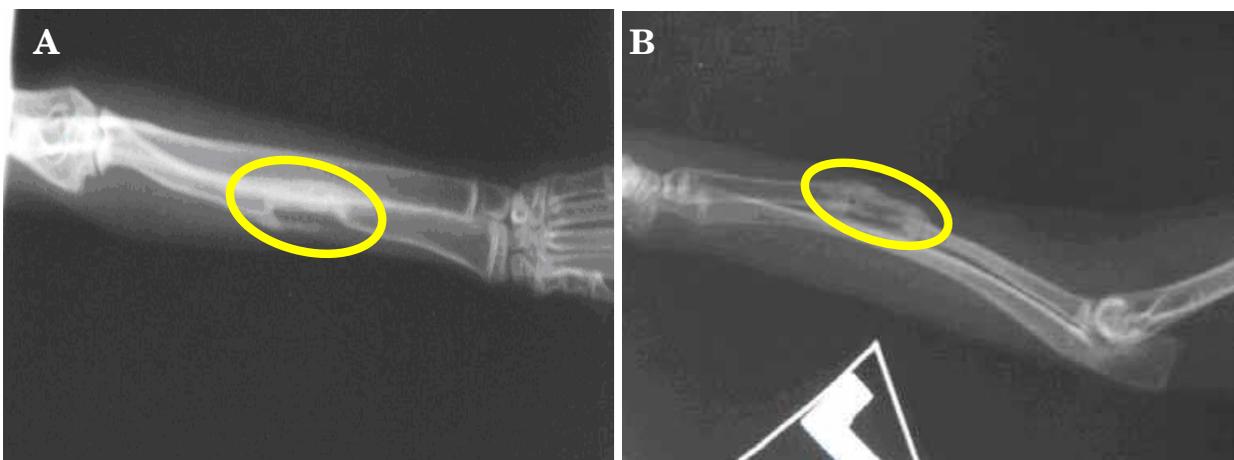

Fig. 12. Radiographs of the radial bone defect in group B at 4 weeks post-implantation show the extensive bone formation is seen over the defect implanted with a freeze dried scaffold which filled up the defect space (yellow circles). Radiograph of the radial bone defect in group B at 8 weeks post-implantation show the radio density of the implant is increased, and the borders appear smooth indicating bone remodeling
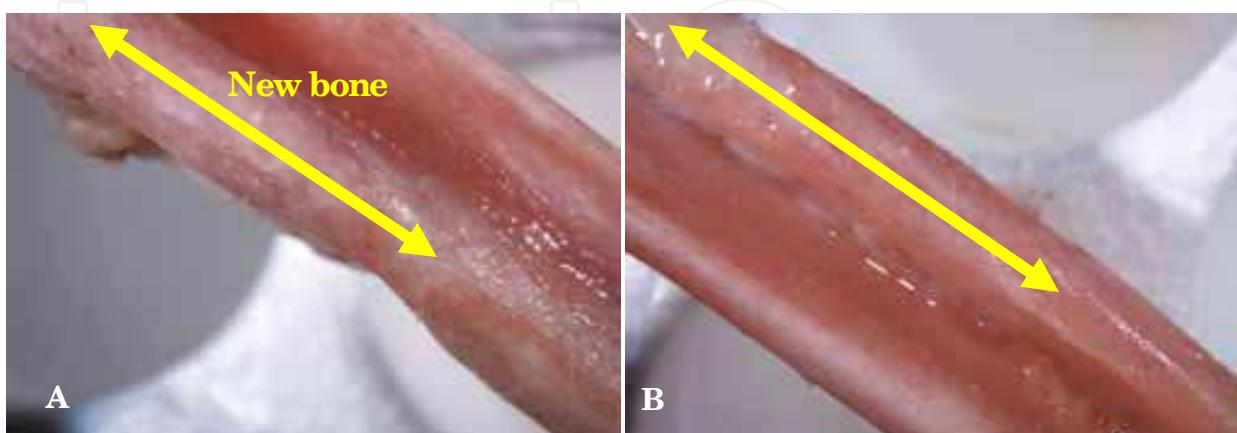

Fig. 13. Photographs of the radial bone implanted with seeded scaffold in group A at 8 weeks post-implantation show the new bone formation completely filled the defect area and restored the continuity of the radial bone (arrows). A, lateral view, B, medial view

The assessment of the rabbit radial bone was done at the end of 8th week. The subsequent radiographs at 4 and 8 weeks post-implantation showed the smooth unambiguous union evidence (Figure 12). Thus we decided that the 8th week group would be timely and adequate in giving the information of this new scaffold which produced as a bone substitute for repairing bone process. The duration of complete radial bone union and healing in large defect of 1.5 cm was evident at 8th week post-implantation (Figure 13).

Radiographic findings of the new bone formation of the radial bone implant presented at the margin and centre of the defect implanted with seeded scaffolds was diffuse at the centre and margin region of the implanted scaffold indicating the osteogenesis. The newly formed bone image in the defect implanted with seeded scaffold was visible completely bridging the radial bone defect, and the bone formation was faster than the previous studies for the tissue engineering of bone repair (Li and Li, 2005). The results of this study indicate that the bioactivity, stability and osteoconductivity of the scaffolds are present in repairing the radial bone defect.

The ALP and Ca^{2+} was evaluated in this study from the blood sample taken during the period of experiment. It seems that ALP concentration for both scaffolds of group A and B give indication that the secretion of ALP comes from the active osteoblast cells. ALP is an enzyme membrane of osteoblasts and its activity increases with time as the osteoblast differentiation and maturation proceed. Previous study has showed that when osteoblast cells were implanted, the ALP activity appeared after 2 weeks and Ca^{2+} was detected after 3 weeks (Kessel, 1989). Elevated serum ALP may be due to the rapid growth of bones, since it is produced by bone forming cells called osteoblasts. The level of ALP of group A during the 4-8 weeks post-implantation was maintained high as compared to the level of ALP in control. The high Ca^{2+} concentration stimulates the osteoblasts to secrete ALP, which increases the local concentration of PO_4^- ions. The high PO_4^- concentration stimulates further increases in Ca^{2+} concentration where mineralization initiated. At this stage of high extracellular Ca^{2+} and PO_4^- concentration, the osteoblasts release small matrix vesicles contain ALP and pyrophosphatase that cleave PO_4^- ions from other molecules of the matrix (Kessel, 1989; Ross and Pawlina, 2006). The ALP analysis also demonstrated a high osteogenic capacity from 1 week post-implantation. The results obtained at weeks 4 and 8 post-implantation indicated that heated and freeze dryer scaffolds was able to promote osteoblastic activity by seeding the osteoblast cells into the porous scaffolds, so the achievement of early bone formation should enable the rapid repair of bone defect, thereby allowing for early recovery and return to daily activities (Ross and Pawlina, 2006). This experiment shows that there was no significant difference (P >0.05) between the seeded scaffolds and non seeded scaffold (control) in group B. However, there was a significant difference (p> 0.05) between the seeded scaffolds and non seeded scaffold (control) in group A. This indicated that the scaffold of group A is better than those in group B, since the concentration of the ALP was generally higher in group A.

The Ca^{2+} concentration as showed in Figures 14 can be mobilized to enter an adjacent capillary or it can be removed from the blood and deposited in the new bone matrix as needed. Ca^{2+} accumulation by matrix vesicles (MV) was produced by osteoblasts that increase in correlation with the level of bone regeneration. The matrix vesicles that accumulate Ca^{2+} and cleave PO_4^- ions cause the local isoelectric point to increase, which result in crystallization of $CaPO_4$ in the surrounding matrix vesicles. Thus, the $CaPO_4$

crystals initiate matrix mineralization by formation and deposition of $[Ca_{10}(PO_4)_6(OH)_2]$ (hydroxyapatite) crystals in the matrix surrounding the osteoblasts that was showing in period of bone formation during the level of Ca^{2+} was elevated (Kessel, 1989; Ross and Pawlina, 2006).
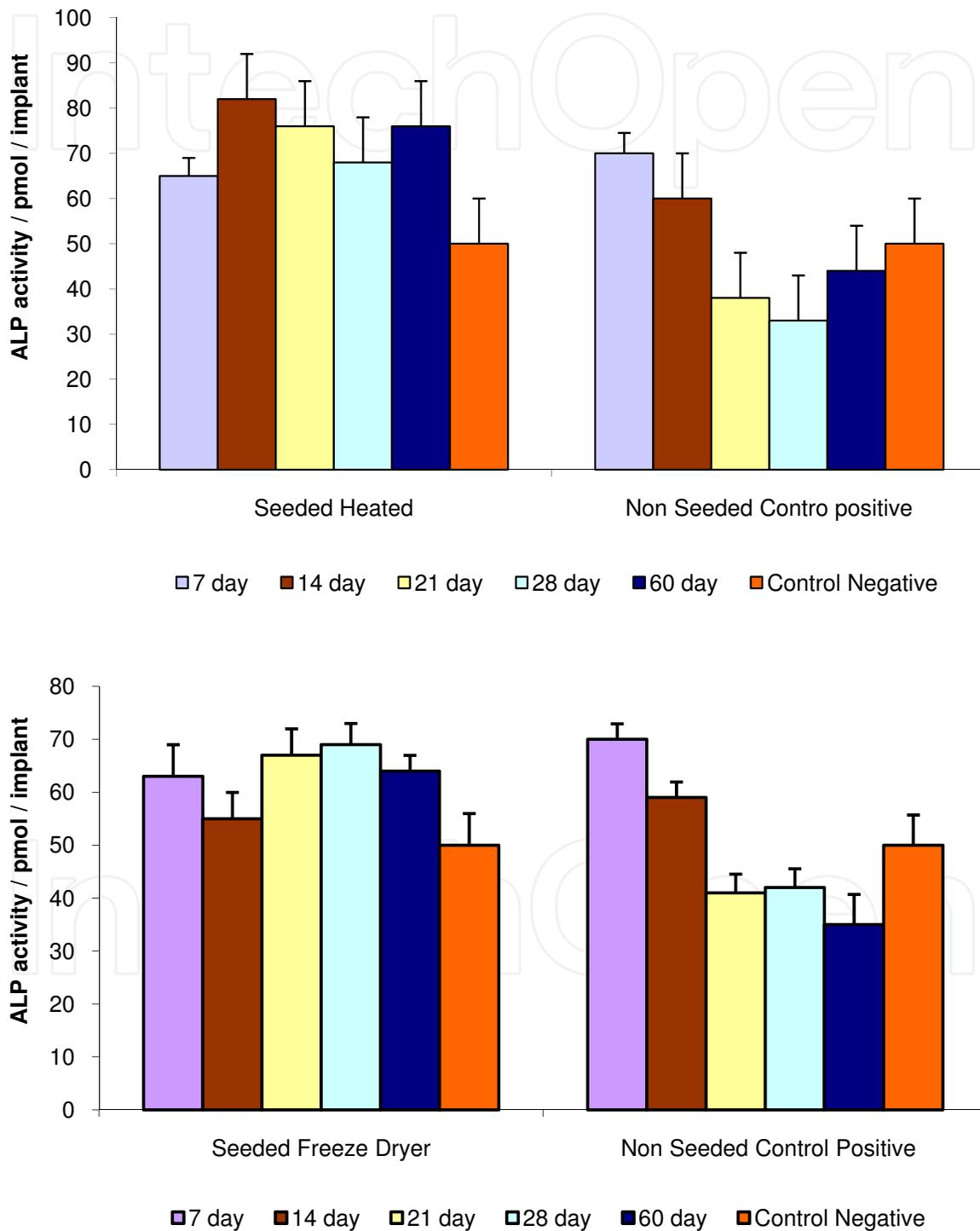

Fig. 14. The graphs show the value of total alkaline phosphatase (ALP) in both groups A and B at different time intervals post-implantation
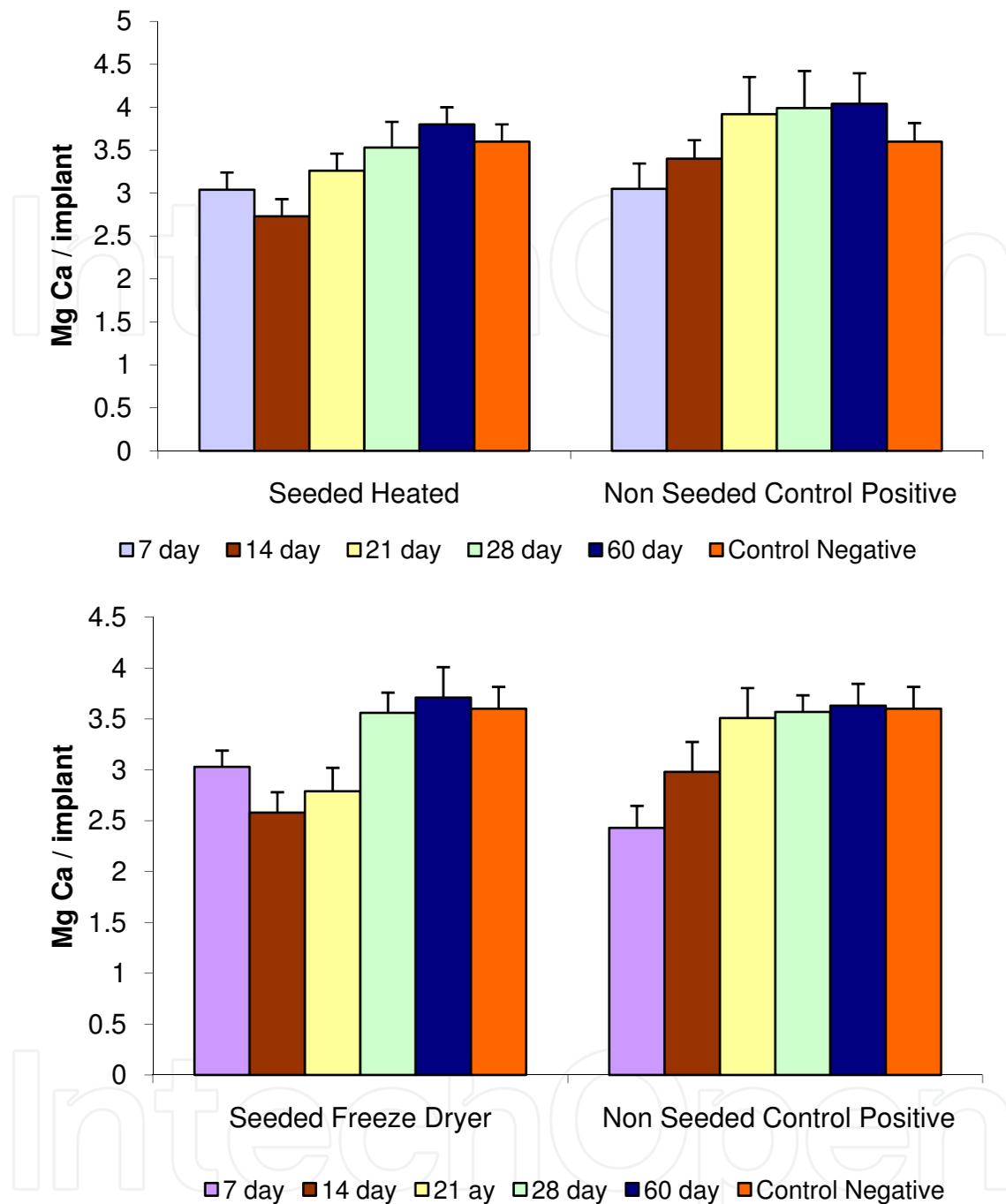
Fig. 15. The grafts show the value of total calcium in both group A and B at different time intervals post-implantation

Histological sections were assessed qualitatively by evaluating the bone formation at the defect area implanted with composite scaffolds. The histological analysis is widely used for examination of bone formation qualitatively. In this study, histological examination revealed complete new bone formation at the defect area replacing the seeded implant scaffolds in both groups A and B. However, in the control animals, it was observed that the bone formation was not complete and left empty defect space at the middle of the defect area, and there was no osteogenesis in the centre of implant. In the animals implanted with

seeded scaffolds, the new bone formation present in the centre of implant. The seeded scaffold were completely resorbed by 8 weeks post-implantation and there were complete new bone formation replacing the implant, and mineralization of new bone and remodeling process was observed at this stage as demonstrated by the presence of osteoclast cells. From the observation, we thus infer that the rapid new bone formation in animals implanted with seeded scaffolds was due to the present of marrow-derived osteoblasts in the scaffolds. These observations showed that osteoconduction, osteoinduction and osteogenesis occur simultaneously in the animals implanted with seeded scaffolds, while in animals implanted with non seeded scaffolds, only osteoconduction was present, thus the new bone formation was slow. These results agree with Li and Li (2005). Previous studies on the new bone formation on scaffolds post-implantation using different types of scaffolds have demonstrated different time of new bone formation. The study performed by Martin *et al*. (1993) who placed blocks of HA in the cortical defects of the humerus and radius in dog revealed that interposition of bone in pores is from 52% at 16 weeks to 74% at 1 year (amount of bone relative to the pore space) post-implantation. The spongy bone regrowth was 38% after 4 weeks, and increased another 17% at 1 year, yet the pore spaces of HA rarely completely filled with bone during this period (Rosen *et al*. 1990). Vuola *et al*. (1995) who used the coral implant reported that after 6 weeks of implantation, the bone does not actually invade the pores of the sample of coral, but replaces the matrix. Yoshikawa (2000) founds that HA modified implants required a large amount of fresh cells from bone marrow and the method would be difficult to apply clinically. In addition, the bone forming capacity of these transplants have not been solved after discontinuation of immunosuppressive. The surface of chitosan modified PDLLA scaffolds study was conducted by Cai *et al*. (2007) after implantation for 12 weeks. A lot of new bone was formed within the scaffolds at this stage with a mild inflammatory reaction. A little new bone even connected them to form circles around the scaffolding, which had not yet completely adsorbed. The previous results demonstrated quite significantly different from the current results on the time taken for the defect to heal completely. The current results showed that at 8 weeks post-implantation, the defect area was completely bridged by the new bone. Thus, the new biocomposite scaffolds developed in this study seeded with osteoblast before implantation is better.
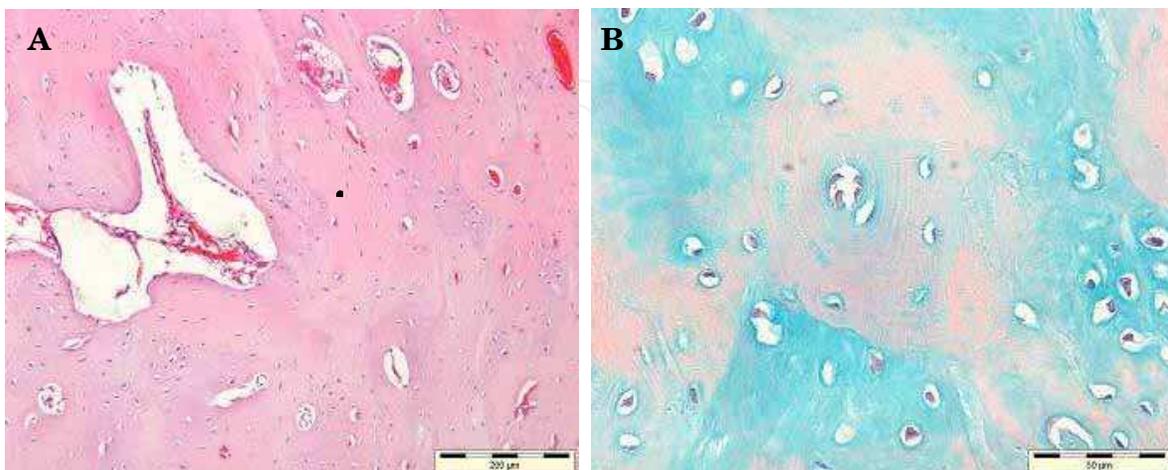

Fig. 16. Microphotographs of the decalcified specimen stained with H&E (A, x100) and Masson's trichrome (B, x400) show the mature bone. Note the osteon with concentric lamellae
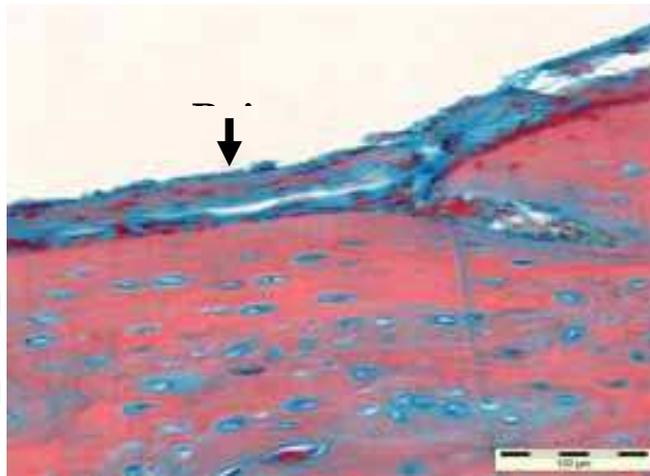
Fig. 17. Microphotograph of the cross section of the bone stained with Masson's trichrome (x200) show the complete bone formation with the present of periosteum (arrow)

5. Conclusion

Our observations indicate that the tissue engineering bone implant constructed by autogenous bone marrow derived osteoblasts and our scaffolds has the capability of osteogenesis, osteoconduction, and osteoinduction with a better osteogenetic effect and quality than scaffolds implant alone. Due to the availability of bone marrow and this scaffold, along with the bone regeneration potential, the tissue engineering bone might be an ideal scaffold for bone defect repair. The osteoinductive scaffold which is needed for the treatment of large bone defects are to be resorbed in a suitable time frame in order to minimize the amount of fibrous tissue and not to interfere with the remodelling of the new bone. In general, for observations in this study, the tissue engineering bone resulted in a higher quality and quantity of new bone formation when compared with the implanting of different types of scaffolds in previous studies, specially the HA, $CaPO_4$ bioceramic, TGF-ß, auto, allograft bone and scaffold alone at the same period within 8 weeks. These results cannot absolutely exclude the possibility that the scaffolds alone give the same result by 12 or 16 weeks. Without doubt, if the scaffolds alone give the same result by 12 or 16 weeks, the cost effectiveness of osteoblast seeding may not be worthwhile. This is an interesting question requiring further investigation due to its importance in the practical application of this tissue engineering bone graft.

This study has revealed a new approach to design and fabrication of scaffolds for tissue engineering by using new materials in combination with natural polymer and water. Scaffolds were characterized for their physical and chemical properties. The analysis and tests used in this study demonstrated that the porous 3D scaffolds created by a new blend of materials through the described procedures are achievable.

This study also provides the fundamental information for researchers and engineers working in advanced composite industry, to open a new approach to the development of bioengineered composites. Cross-disciplinary research efforts are definitely needed in bridging expertise from bio-, nano and advanced composite areas, to work closely along this new scientific and engineering direction.

The work described in this thesis is heavily focused on developing and using biomaterials as tissue engineering matrices. Tissue engineering has been described as "the principles and

methods of engineering, materials science and cell and molecular biology to the development of viable substitutes that restore, maintain or improve the function of humans' bone". Advances in new biocompatible materials, both osteoconductive and osteoinductive, will also aid in the treatment of bone diseases, while new diagnostic methods will help identify those at risk of disease before symptoms are obvious. A better understanding of how bone perceives and responds to mechanical signals will certainly help accelerate the healing of fractures, increasing the osseointegration of implants, and help to ensure that diseases such as osteoporosis can be treated properly. These are undoubtedly exciting times for everyone working in the bone field, and the interdisciplinary nature that has brought us tremendous insight into bone's complexity on a structural and functional level hence serve us well in the future.

6. Acknowledgement

7. References

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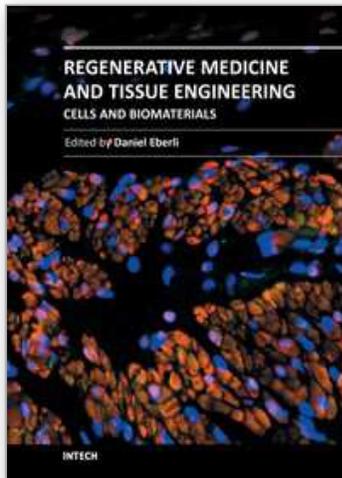
Regenerative Medicine and Tissue Engineering - Cells and Biomaterials

Edited by Prof. Daniel Eberli

ISBN 978-953-307-663-8

Hard cover, 588 pages

Publisher InTech

Published online 29, August, 2011

Published in print edition August, 2011

Tissue Engineering may offer new treatment alternatives for organ replacement or repair deteriorated organs. Among the clinical applications of Tissue Engineering are the production of artificial skin for burn patients, tissue engineered trachea, cartilage for knee-replacement procedures, urinary bladder replacement, urethra substitutes and cellular therapies for the treatment of urinary incontinence. The Tissue Engineering approach has major advantages over traditional organ transplantation and circumvents the problem of organ shortage. Tissues reconstructed from readily available biopsy material induce only minimal or no immunogenicity when reimplanted in the patient. This book is aimed at anyone interested in the application of Tissue Engineering in different organ systems. It offers insights into a wide variety of strategies applying the principles of Tissue Engineering to tissue and organ regeneration.

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