Effects of hydroxyapatite and PDGF concentrations on osteoblast growth in a nanohydroxyapatite-polylactic acid (nHA-PLA) composite for guided tissue regeneration

A. Talal[*×], I. J. McKay[‡], K.E.Tanner[*] and F.J. Hughes[‡+]

[*] School of Engineering, University of Glasgow, Glasgow, G12 8QQ, UK.

[×] Now at College of Dentistry, University of Dammam, Ad Dammam, Saudi Arabia.

[‡] Centre for Adult Oral Health, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, Turner Street, LONDON E1 2AD, UK.

[+] Now at Dental Institute, Kings College London, Guys Hospital, LONDON SE1 9RT, UK.

ABSTRACT

The technique of guided tissue regeneration (GTR) has evolved over recent years in an attempt to achieve periodontal tissue regeneration by the use of a barrier membrane. However, there are significant limitations in the currently available membranes and overall outcomes may be limited. A degradable composite material was investigated as a potential GTR membrane material. Polylactic acid (PLA) and nano-hydroxyapatite (nHA) composite was analysed as a potential GTR membrane material. Its bioactive potential and suitability as carrier system for growth factors were assessed. The effect of nHA concentrations and the addition of platelet derived growth factor (PDGF) on osteoblast proliferation and differentiation was investigated. The bioactivity was dependent on the nHA concentration in the films, with more apatite deposited on films containing higher nHA content. Osteoblasts proliferated well on samples containing low nHA content and differentiated on films with higher nHA content. The composite films were able to deliver PDGF and cell proliferation increased on samples that were pre-absorbed with the growth factor. nHA-PLA composite films are able to deliver active PDGF. In addition the bioactivity and cell differentiation was higher on films containing more nHA. The use of a nHA-PLA composite material containing a high concentration of nHA may be a useful material for GTR membrane as it will not only act as a barrier, but may also be able to enhance bone regeneration by delivery of biologically active molecules.

Keywords: Guided tissue regeneration, Polylactic acid, Nano-hydroxyapatite, Platelet Derived Growth Factor, Osteoblasts

1. INTRODUCTION

The two major objectives in the treatment of periodontal disease are firstly to arrest the progression of the disease and secondly to reverse the damage by regenerating lost periodontal tissues and supporting bone [1]. The technique of guided tissue regeneration (GTR) has evolved over recent years in an attempt to achieve periodontal repair [2]. The technique of GTR uses a membrane to cover the periodontal defect, which isolates the defect area from the overlying gingival soft tissue, thus allowing mesenchymal cells from the periodontal ligament and bone to populate the wound and stimulate periodontal tissue regeneration [3, 4]. A range of both resorbable and nonresorbable membrane materials have been used for GTR procedures. In general resorbable membranes are favoured as they do not require removal, as they gradually breakdown and are eliminated from the body by metabolic processes [5, 6]. However, there are significant limitations with the currently used membranes and overall outcomes of these procedures may be limited. Thus there is a need to develop new materials and production methods for resorbable membranes with appropriate mechanical properties to improve the clinical outcomes.

Biodegradable polylactic acid (PLA) has been used in many biomedical applications due to its biocompatibility and resorbable properties. Since the early 1990s PLA has been used clinically in a range of applications including sutures, in drug delivery systems, as scaffolds and as fracture fixation devices [7, 8, 9]. PLA has also been investigated as a controlled delivery material for drugs and bioactive agents [10]. In this study we have investigated a nanohydroxyapatite polylactic acid (nHA-PLA) composite material as a potential membrane material including its ability to release growth factors.

The use of a ceramic such as HA or other calcium phosphates within degradable PLA may confer a number of potential therapeutic advantages on this material, including increased mechanical properties, promotion of cell attachment and osteoconduction and the potential for use as a vehicle for slow delivery of bioactive proteins such as growth factors. HA–PLA composite materials have been shown to be biocompatible, resorbable and have appropriate mechanical properties for bony applications [11, 12].

Various studies have shown the potential of growth factors to enhance regeneration of periodontal tissues. Platelet Derived Growth Factors (PDGF), Bone Morphogenic Proteins (BMPs), Fibroblast Growth Factors (FGFs) and Insulin Like Growth Factors (IGFs) have all been shown to regulate osteogenic cells and may potentially enhance periodontal regeneration [13, 14, 15]. The incorporation of growth factor into a suitable GTR membrane may enhance significantly the outcome of GTR procedures.

In previous studies we have shown that nHA-PLA composite materials can rapidly absorb proteins, which are subsequently released slowly over a number of days [16]. Protein release kinetics were shown to be dependent on both the nHA content and the manufacturing conditions of the material.

The aims of this study were to investigate the proliferation and differentiation of osteoblast cells on nHA-PLA composites with different concentrations of nHA, to assess the potential of this material to act as a carrier system for growth factor delivery and to analyze the bioactivity of these nHA-PLA composites. In addition the nHA and the nHA-PLA composite films produced for the experiments were characterized by Scanning Electron Microscopy (SEM), Fourier Transform Infra Red spectroscopy (FTIR) and X-Ray Diffraction (XRD).

2. MATERIALS AND METHODS

2.1. Film fabrication

PLA (PURAC Biochem, The Netherlands) with an L: DL ratio of 70/30 and nHA were used for the fabrication of nHA-PLA composite films. For the bioactivity study nHA with particle size less than 200 nm was purchased from Sigma (UK). For the cell culture and PDGF studies nHA was synthesised, using an ethanol-based sol–gel method for the synthesis of nHA reported by Feng et al [17]. Calcium nitrate tetrahydrate [$Ca(NO_3)_2.4H_2O$] (0.083M) and sodium hydrogen phosphate [$(NH_4)_2HPO_4$] (0.05M) were mixed and the ratio of calcium and phosphate precursors were maintained at 1.66. $Ca(NO_3)_2.4H_2O$ was mixed in 50ml of ethanol and the solution was stirred and heated to 85°C, on reaching this temperature $(NH_4)_2HPO_4$ which had been dissolved in 50 ml of water was added dropwise. Ammonium hydroxide (NH_4OH) was added to the solution to maintain the pH above 10. The solution was stirred for 5-6 hrs as gel formation occurred (Equation 1). The final gel was then oven aged for 24 hrs at 40°C.

$$10Ca(NO_3)_2\,4H_2O + 6(NH_4)_2HPO_4 + 8NH_4OH \rightarrow \quad Ca_{10}(PO_4)_6(OH)_2 + 20NH_4NO_3 + 6H_2O$$

Equation: 1

The aged sample was then heat treated, the temperature was increased at 10°C min^{-1} to 700°C and held for 1 hour at that temperature, the sample was cooled down to 20°C again at 10°C min^{-1}(Carbolite, Sheffield, UK). The powder was then ball milled for 12 hrs.

To produce the PLA films 25 ml of chloroform was poured into spherical flasks and 0.5 g of PLA was added while stirring. The solution was then left on the stirrer for 24 hrs at room temperature. Then the solution was spread in 120mm Petri dishes and then left overnight to allow slow evaporation of the chloroform. For the production of nHA-PLA composite films nHA powder was added at the required concentration to give 10, 40 and 70 weight (wt) % to the PLA solution after 24 hrs of stirring. These weight contents are equivalent to 4.2, 20.9 and 48.0 volume % using 1.25 Mgm^{-3} and 3.156 Mgm^{-3} for the densities of PLA and HA respectively. These nHA-PLA solutions were then stirred for a further 48 hrs to achieve homogenous nHA dispersion, after which the solution was again spread on 120mm Petri dishes followed by slow evaporation of the chloroform. The same size of Petri dishes was used for all the films to obtain films with the same PLA content. After evaporation of the chloroform, the films were pealed off the dishes and were placed in a vacuum oven for one day. The films were then lightly annealed in the oven to relax any stresses

produced in the films during manufacture, by pressing the films between the bases of two Petri dishes in an oven at 70 ◦C for one hour. The films were then allowed to cool gradually overnight. This annealing prevented the films rolling up in the cell media during cell culture.

The films were cut into 12 mm diameter samples and sterilized by ultraviolet (UV) irradiation.

2.2. nHA powder and film characterization

2.2.1 Fourier Transform Infra red spectroscopy (FTIR)

FTIR spectra of the PLA and nHA-PLA composite films produced using synthesised nHA were collected using a Thermo-Electron Nicolet 8700 FTIR spectrometer. The spectra were obtained at $8cm^{-1}$ resolution and a total of 256 scans were collected.

2.2.2 Scanning Electron Microscope (SEM)

The morphology and the size of synthesised nHA particles and the films were characterised by SEM (FEI Inspect F, EFI Company, The Netherlands) using the field emission technique. For nHA samples 20kV was used and for PLA and nHA-PLA composite films 5kV was used, to prevent damage to the PLA.

2.2.3 X- ray diffraction (XRD)

The nHA powder produced was characterized using XRD, to find chemical composition, crystal state and the crystalline content. A Siemens/Bruker D5000 Diffractometer, with monochromatic Cu Kα radiation (k = 0.15418 nm) was used to obtain the nHA powder XRD. Scans were obtained in the 2θ range = 20˚–60˚, with a step size of $0.02^{\circ-1}$. Commercial nHA (Sigma, UK) was scanned to provide a comparative XRD pattern.

2.3 Analysis of bioactive potential of films

Bioactivity of films was analyzed by immersing 12mm diameter samples of PLA filled with commercial nHA in Simulated Body Fluid (SBF) which was prepared as described by Kokubo et al. [18].

Before soaking in SBF all samples were disinfected using 70% ethanol. Each disinfected sample was immersed in 20 ml SBF for 1, 7, 14, 21 or 28 days at 37°C. The SBF was changed after 14 days. At each time point, samples were removed from the solution and rinsed carefully with distilled water before drying in a vacuum oven at 40°C overnight. The samples were carbon coated and analyzed by SEM and energy dispersion spectroscopy (EDS) (FEI Inspect F, EFI Company, The Netherlands)

2.4 Cell culture

Primary rat calvarial osteoblasts isolated by collagenase digestion were used in the experiments. The cells were cultured and maintained in Minimal Essential Medium (MEM) Alpha medium (Invitrogen, UK) which was supplemented with 10% fetal calf serum (FCS) (Sigma Aldrich, UK), $0.3\mu g\ ml^{-1}$ fungizone (Invitrogen) and $50\mu\ ml^{-1}$ penicillin and $50\mu g\ ml^{-1}$ streptomycin (Invitrogen). The cells were grown in a humidified incubator at 37◦C with 5% CO_2. The medium was replaced twice weekly.

2.5 PDGF addition

The effect of pre-adsorption of PDGF to the films on the proliferation of cells was evaluated. 100ng recombinant rat platelet-derived growth factor – BB (PDGF-BB) (Sigma Aldrich, UK) in 100 µl phosphate buffered saline (PBS) was added to the surface of the specimens placed in 24 well plates. The films were then incubated for three hrs at 37˚C. After this the PBS solution was carefully

removed from the surface of the films and cells were seeded 1×10^4 cells ml^{-1} on to the films. In a second set of films, rather than adding PDGF directly on the films, PDGF was added to the medium in solution. The concentration of PDGF in the medium was 100ng ml^{-1}. 1ml of this PDGF containing medium was then added to each film placed in 24 well plates resulting in the addition of an equal total amount of PDGF in solution to that used for pre-adsorption treatments. Cells in medium without PDGF but with 1 or 10% FCS supplement were used as negative and positive controls respectively. The rationale for this method of soaking the samples in PDGF in phosphate buffered saline and then removing from the solution was that this models the expected clinical usage. The intention is that the dentist soaks the membrane in a PDGF solution at the chair side while preparing the operative site and then places the soaked membrane directly into the defect.

2.6 Cell proliferation

Cell proliferation on the films was assessed by an MTS assay. A solution of MTS (Promega UK Ltd, UK) and phenazine methosulfate (PMS) (Sigma Aldrich, UK) was used to determine the number of viable cells on the films. MTS changes colour on reacting with live cells. The amount of colour change, which is measured using a photospectrometer, is directly proportional to the metabolic activity of living cells.

To evaluate cellular proliferation on different films, rat osteoblasts of 7-9th passage were seeded on PLA films and 10, 40, 70wt% nHA-PLA composite films. 90% confluent cells were trypsinized and cell density was adjusted to 3×10^4 cells ml^{-1}. Sterile films were transferred into 24 well plates, a small dot of silicon was used to stick the films to the base of the wells. The films were seeded with 3×10^4 cells ml^{-1} $well^{-1}$ for 1, 2, 4, 7, 10 and 14 days in triplicates. Wells with no films, that is tissue culture plastic, were used as controls. For experiments in which PDGF was added, the cell density was decreased to 1×10^4 cells ml^{-1} $well^{-1}$ and the medium was supplemented with 1% fetal calf serum instead of 10% fetal calf serum. The films containing cells were placed in a humidified incubator at 37˚C with 5% CO_2. Cell proliferation was analyzed at day three.

2.7 Cell differentiation

Cell differentiation was determined by measuring alkaline phosphatase (ALP) production by the osteoblast cells. 500µl p-nitrophenyl phosphate solution to the films in 24 well plates, the films were then incubated for at 37°C for one hour. Thereafter three 100µl samples were taken from each well and absorbance was measured at 405nm using a photospectrometer.

SEM was used for morphological analysis of the cells on the films. At 14th day of incubation the films containing cells were washed with PBS twice before fixing the cells in 4% formaldehyde for 15 minutes. The cells were then dehydrated by suspending in successive 50, 70, 80, 90 and 100% ethanol solutions for 5 minutes each. The films and cells were finally transferred to hexamethyldisilazane and were allowed to dry at room temperature. The films and cells were gold coated and analyzed under SEM (FEI Inspect F, EFI Ltd, The Netherlands) at 5kV.

2.9 Data Analysis

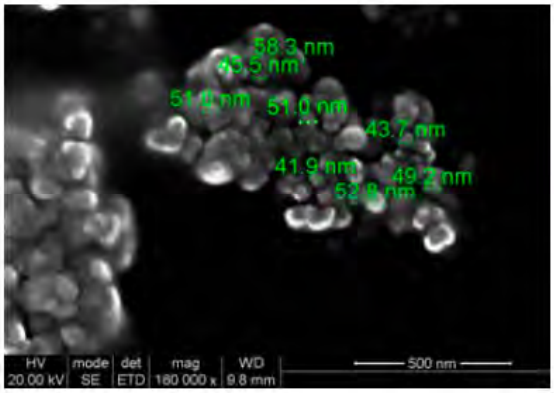
Statistical analyses of cell culture data were calculated by One-way ANOVA with Bonferroni's post-test with $p < 0.05$ was considered significant. Analysis was carried out using GraphPad Prism software.

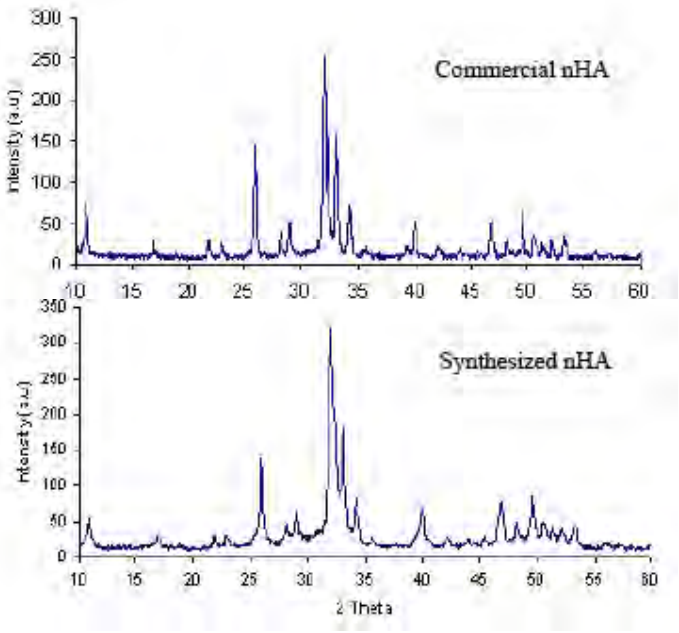
3. RESULTS

3.1. Characterization of synthesized nHA

Figure 1(a) shows the morphology and size of the nHA synthesized. The particles were agglomerates

of spherical nodules with the nodules being 40-60nm across and the agglomerates being 0.5-1.0mm across. XRD patterns of commercial and synthesized nHA are shown in Figure 1(b). Presence of a strong diffraction peak at 31.9° with other peaks at 26˚, 33˚ and 40˚ confirm the phase purity and high crystallinity of the material produced [19]. No non-nHA peaks were observed. The slightly increased width of the peaks for the synthesized nHA indicates lower crystallinity than for the commercial nHA. FTIR spectrum of the synthesized nHA powder is shown in Figure 1(c). The peaks at 3571 cm^{-1} and 631cm^{-1} represents OH groups. The peaks at 1040, 603 and 569 cm^{-1} represents the P-O absorption band. The CO_3 group is represented by the peak at 1473 cm^{-1} [20].





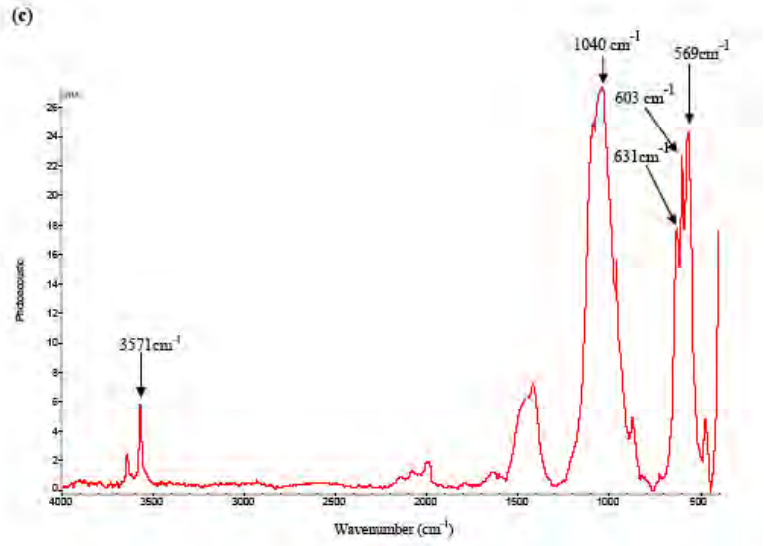


Fig. 1 a SEM of nHA showing nodule size and morphology within the agglomerated granules (scale bar = 500 nm). b XRD patterns of commercial and synthesized nHA. c FTIR spectrum of synthesized nHA powder

3.2. Characterization of films produced

Figure 2(a) shows the morphology of 10, 40 and 70wt% nHA-PLA films, the imaged surface was the air surface during the casting process. The surface of 70wt%nHA-PLA films showed more nHA on the surface compared to 40 and 10wt% nHA-PLA films. The surface of 10wt%nHA-PLA film

appears smooth with the nHA well covered by PLA. Given that 10wt% nHA equates to 4.2 vol% nHA there seem to be good amounts of nHA visible on the surface. With 40wt%nHA again the nHA is well covered with PLA, whereas, the surface of 70wt%nHA-PLA film is rougher, irregular and shows micropores, here only some the nHA appears to be covered with PLA. Given that the nHA and PLA solution were mixed for 48 hours it can be seen that 48 vol% nHA leaves only 52 vol% PLA available to cover the high surface area nHA and that complete coverage has not been obtained.

Figure 2(b) shows the comparative FTIR spectra of (a) PLA, (b) nHA, (c) 10wt%nHA-PLA film, (d) 40wt%nHA-PLA film and (e) 70wt%nHA-PLA film. The spectra of 10wt%nHA-PLA, 40wt%nHA-PLA and 70wt%nHA-PLA composites films show the characteristic peaks of both HA and PLA. As the concentration of nHA increased the intensities of PO^4 peaks at 603 and 569 cm^{-1}, which were attributed to phosphate from HA, increased relative to the intensities of PLA characteristic peaks at 1760 (C=O), 870 (C-COO) and 756 (C=O) cm^{-1}.

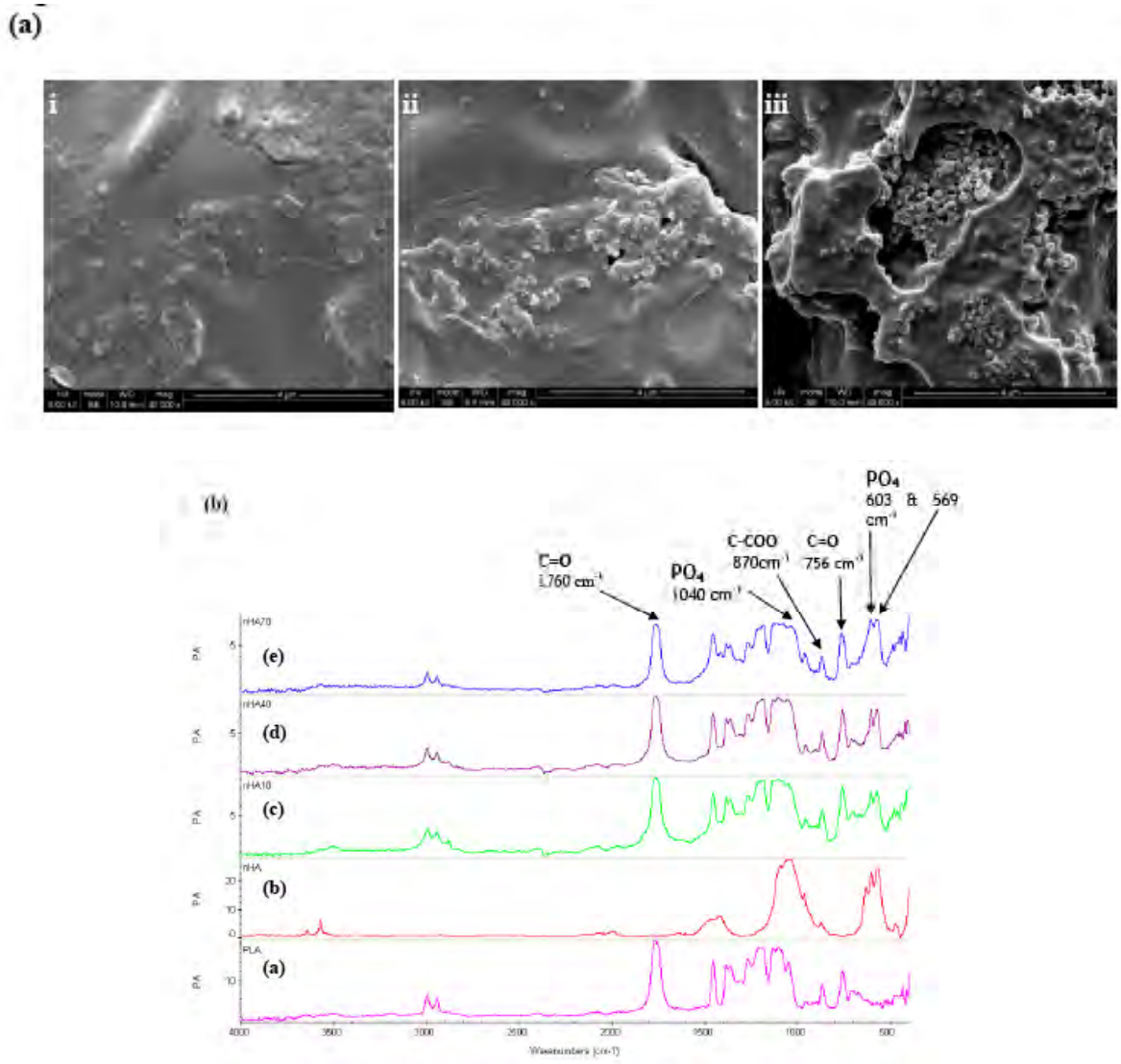


Fig. 2 a SEM images of (i) 10 wt% nHA-PLA, (ii) 40 wt% nHA-PLA and (iii) 70 wt% nHA-PLA films (scale bars = 4 lm). b Comparative FTIR spectra of (a) PLA, (b) nHA, (c) 10 wt% nHA-PLA, (d) 40 wt% nHA-PLA and (e) 70 wt% nHA-PLA films3.3 Bioactivity of the films

The SEM images (Figure 3) show PLA, 10wt%nHA-PLA, 40wt%nHA-PLA and 70wt% nHA-PLA films samples after immersion in SBF for 1, 14 and 28 days all at the same magnification. The amount of apatite formation increases with the increase in nHA content. On plain PLA films there was no apatite formation even after 28 days. On 10wt%nHA-PLA (Figure 3d-f) and 40wt%nHA-

PLA (Figure 3g-i) no apatite layer was visible at day 1, however by day 14 apatite layers were forming and at 28 days the apatite layers covered more than 80% of the film surface. Maximum apatite formation was observed on films containing 70wt% nHA (Figure 3j-l) and even at day 1 there was an apatite layer formed on the film, by 28 days it fully covered the surface and appears much thicker than the apatite layer formed on other films. As the nHA content increased the size of the apatite nodules decreased indicating the growth of the apatite from more individual nucleation points. As the nHA concentration increases the number of potential nucleation points increases thereby allowing smaller apatite nodules to be formed.

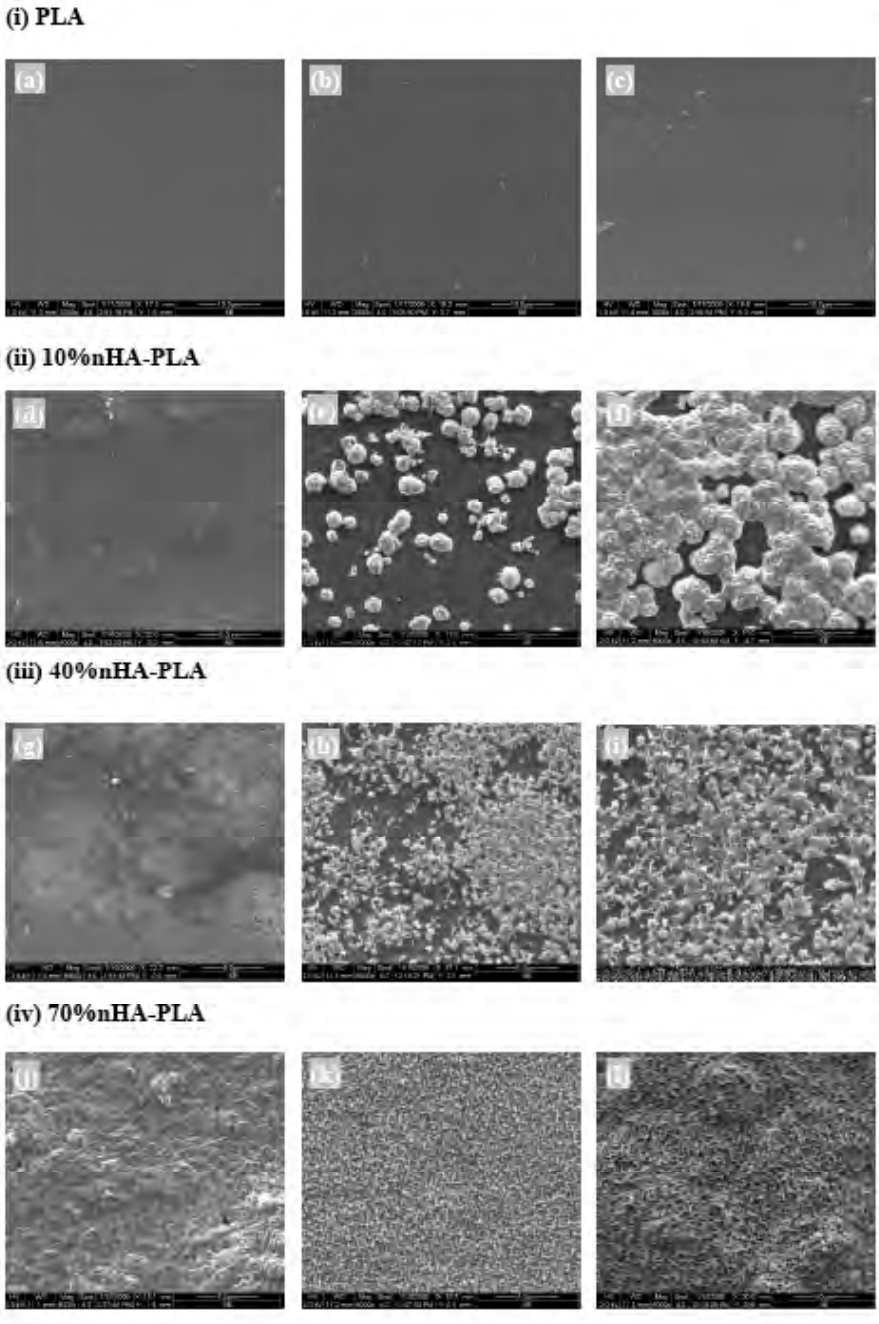


Fig. 3 SEM images of films after immersion in SBF (a)–(c) PLA, (d)–(f) 10 wt% nHAPLA, (g)–(i) 40 wt% nHA-PLA, (j)–(l) 70 wt% nHA-PLA at (a), (d), (g) and(j) 1 day, (b), (e), (h) and (k) 14 days, and (c), (f), (i) and (l) 28 days (all scale bars = 5 μm)

3.4. Cell proliferation on PLA films with 0, 10, 40 and 70 wt % nHA

Osteoblast proliferation on the different films and the tissue culture plastic used as a control is

indicated in Figure 4(a) which shows the MTS signal which is related to cell metabolism. The cells showed highest metabolic rate on 10wt%nHA-PLA films and the cell metabolic rate decreased with the increasing nHA content in the films from 10% to 70%. The MTS signal reached a peak at 7 days, with no significant changes in the level between 7 and 14 days on the control surface, whereas lower peaks were seen at 10 days on the plain PLA with an intermediate level peak at 10 days on 10%nHA-PLA. However for the 40% and 70%nHA-PLA the MTS signals were still increasing at 14 days. 70wt%nHA-PLA films showed lower cell growth when compared to the other composite films, although all the composite films showed higher levels than the plain PLA film. The experiment was repeated five times and the results showed similar trends, although with different absolute values.

Figure 4b shows the SEM images of cells at day 14 after culturing on plain PLA and the three different filled films. The cells are well spread and fully cover the surfaces. The cells on PLA and 10wt%nHA-PLA films are densely packed and forming layers. On the films containing 40 and 70wt% nHA the cells appear to be bridging over the surface depressions and pores formed by nHA particles. Furthermore on these films the cells are rounded and thus appear to still be dividing, compared to the flattened cells on the plain PLA and 10%nHA-PLA films.

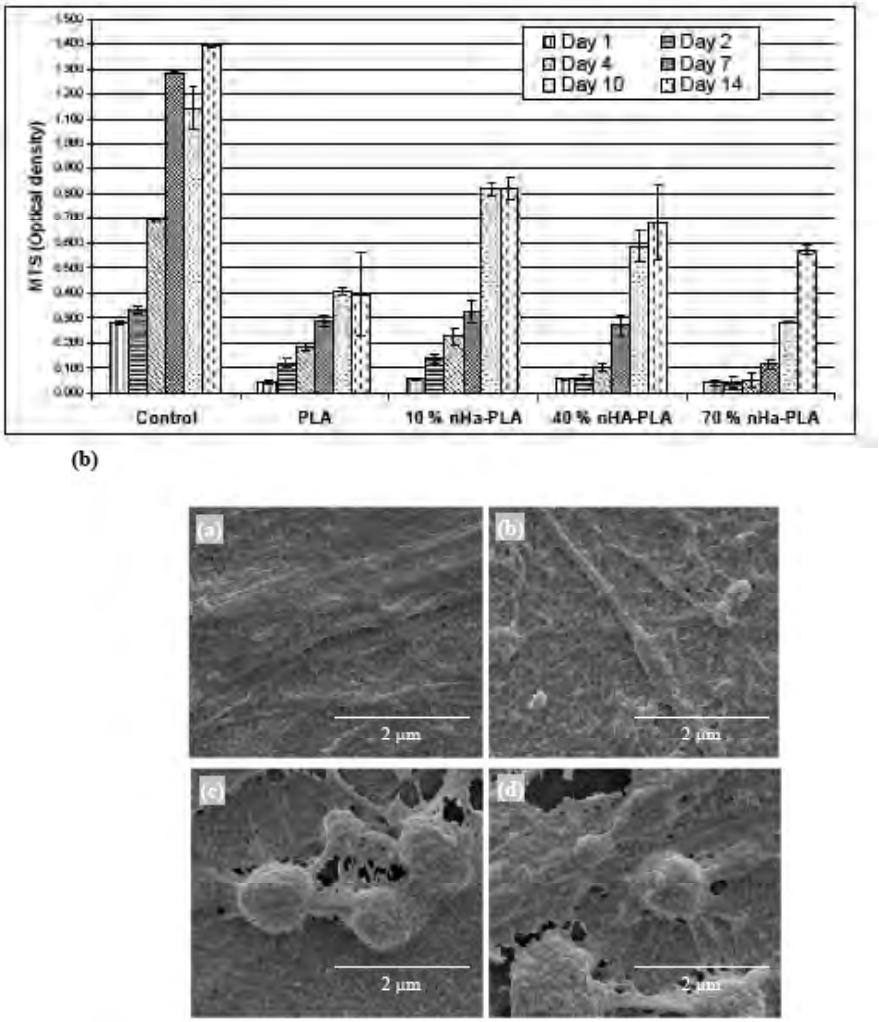


Fig. 4 a Osteoblast proliferation on tissue culture plastic (control), non-filled PLA and PLA filled with 10, 40 or 70 wt% nHA. Mean ± SD n = 3 per material and time. b SEM images of cells cultured to day 14 on (a) PLA, (b) 10 wt% nHA-PLA, (c) 40 wt% nHAPLA and (d) 70 wt% nHA-PLA films (all scale bars = 2 µm)

3.5. Cell proliferation on PLA films with 0, 10, 40 and 70 wt % nHA in response to PDGF

Figure 5 shows the metabolic activity of cells at day 3 on films loaded with PDGF and on films in which PDGF was added in the cell medium. Two controls were used for each film type, culture medium with 1% or 10% FCS but no PDGF. The films on which PDGF was pre-adsorbed showed higher cell metabolic activity when compared to cultures where PDGF was added to the cell medium in solution. There was no difference between cell metabolic activity on positive control films with 10% FCS and films with PDGF in the medium, whereas PDGF adsorbed to films caused increased metabolic activity. The lowest cell metabolic activity was seen on negative control films with 1% FCS and no PDGF added. The experiment was repeated five times and the results showed similar trends although with different absolute values as would be expected with cell culture studies.

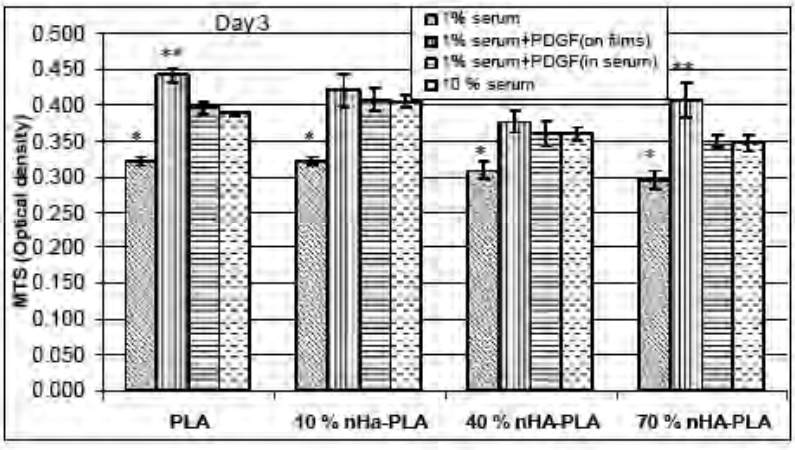


Fig. 5 Osteoblast cell proliferation at day 3 in response to PDGF adsorbed to films or in solution in the medium. 1 and 10 % serum in the medium were used as negative and positive controls, respectively. Mean ± SD n = 3 per material and serum condition. *Significantly less than other groups. **Significantly greater than other groups, P<0.05 one-way ANOVA and Bonferroni post-test

3.6. Alkaline phosphate activity

Figure 6 shows alkaline phosphatase activity of the cells after 14 days culture on various films. The data was normalized for cell number using the MTS assay as measure of cell number. The cells on 70wt% nHA-PLA films showed a significantly significant increase in alkaline activity which indicates cell differentiation, compared to control and 10wt%nHA-PLA films with a non significant increase for 40%nHA-PLA.

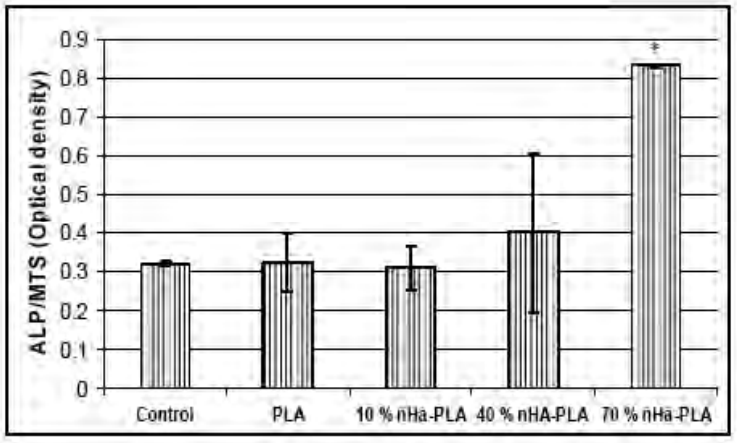


Fig. 6 ALP activity of osteoblasts cells seeded on different films for 14 days (n = 3). Mean ± SD. *Significantly greater than other groups, P<0.05 one-way ANOVA and Bonferroni post-test

4. DISCUSSION

A range of materials are currently used as barrier membranes for guided tissue regeneration (GTR), with various advantages and disadvantages. There is considerable interest in further developments of regenerative techniques to improve outcome and predictability. One potential development is using a composite combining the properties of different materials to enhance the treatment outcome. Furthermore, growth factors can be incorporated into the membranes to encourage their local release to enhance regeneration.

In this study nHA-PLA composite films have been analyzed as potential materials for GTR membranes. The aim was to develop a membrane which can enhance regeneration by combining the properties of resorbable PLA and bioactive nHA. Furthermore, it was designed to also have the ability to deliver bioactive proteins such as growth factors at the defect site, thus further enhancing regeneration. Hydroxyapatite is a known biocompatible and osteoconductive material and has the ability to absorb and release proteins [21]. nHA with particle size between 40-60 nm was successfully produced and SEM (Figure 1) of nHA shows the nanospherical structure of the particles. Nanospherical structured HA is advantageous as it provides greater surface area for cell attachment and more binding sites for protein absorption. The crystallinity and purity of nHA was confirmed by XRD which only showed the peaks associated with HA and no peaks related to tricalcium phosphate or calcium oxide were present. Similarly, the FTIR spectra showed typical HA characteristics [22].

SEM images of films with different nHA content show that as the nHA content increases the surface of film becomes rougher, which again could provide increased surface area for cell attachment [23]. In addition generally an increase in filler content in a composite increases the modulus [24, 25]. However, the proposed application of these films is to form the bone contacting surface of a fibre reinforced GTR membrane and the main purpose of the film is to stimulate bone and periodontal ligament regrowth.

In FTIR spectra of films there was no shift of PLA carbonyl peak at 1760cm^{-1} showing that the mixing of PLA and nHA is purely physical and no chemical bonding has taken place. Phosphate bending peaks of HA at 569cm^{-1} and 603cm^{-1} and their height compared to PLA peaks increase as the nHA content increases from 10 to 70 %.

Bioactive materials, as indicated by the formation of an apatite layer on the implant surface during soaking in SBF [26], favour the growth of osteoblasts. The apatite which is formed on the surface of the bioactive material is carbonate-containing hydroxyapatite with small crystallites and resembles bone mineral in composition and structure. The formation of apatite layer was seen to be proportional to the concentration of nHA in the films, with faster apatite formation on films containing higher nHA concentrations. It has been proposed that osteoblasts preferentially proliferate and differentiate on the apatite layer and produce apatite and collagen [27]. The apatite layer was formed on day 1 on films containing 70 wt% nHA, whereas it did not appear on the plain PLA films even at 28 days. Furthermore, the morphology of the apatite layer formed on the films containing 40 and 70 wt% nHA was different from the layer formed on 10wt%nHA-PLA films. The layer formed on 40 and 70 wt% nHA-PLA films showed a finer structure and a more continuous apatite layer, whereas the apatite layer formed on PLA and 10 wt% nHA-PLA film presented a more globular appearance. This difference may be due to presence of more nucleation sites for apatite formation in films containing increased amounts of nHA.

We tested the effect of nHA content on the proliferation and differentiation of osteoblasts. Both cell proliferation and differentiation were assessed on the same samples so the results can be correlated. Initial cell adhesion to a material is important because it controls the subsequent processes of cell proliferation and differentiation [28, 29]. Osteoblasts showed higher proliferation, as assessed by MTS activity, on films with lower nHA content and lower proliferation on films with higher nHA content. There was minimal difference between the number of cells on PLA and 10wt%nHA-PLA films at day 10 and 14. However, this may be because the cells had already reached confluence at

day 10, whereas the cells on 40 and 70wt%nHA-PLA films were still proliferating at day 10, showing an increase in number between days 10 and 14.

ALP activity can be attributed to the degree of cell differentiation [30]. In general there is a reciprocal relationship between cell proliferation and differentiation in cell culture and as cells start to differentiate, they cease proliferating. In our study increased ALP expression, showing differentiation of cells on films with high nHA content may explain the low number of cells on 40wt%nHA-PLA and 70wt%nHA-PLA film, and the higher number of cells on PLA and 10wt%nHA-PLA films. The highest ALP activity was seen in the cells on 70wt%nHA-PLA films, which is probably due to the differentiation effect of nHA on these cells. Low levels of ALP in cells on PLA and 10wt%nHA films suggest limited differentiation of these cells. So we speculate that with no or low nHA content the cells continually proliferate, whereas with higher nHA content the cells slow growth in order to differentiate. This theory is in agreement with classical osteogenesis timelines and behaviours as shown by Stein and Lian [31]. Our results are also in support of various studies that the presence of HA can cause bone cells to differentiate and even to start to deposit mineral [32, 33, 34]. The presence of the HA should have a further advantage, PLA degradation products are acidic and therefore have been known to cause an adverse biological response. However, the addition of HA or tricalcium phosphate, which are chemically basic, has been shown to limit changes in the pH [12].

PDGF is a heparin-binding polypeptide and has mitogenic, proliferative and chemotactic affect on connective tissue cells [36]. PDGF adsorption into the composite films and its subsequent effect on the proliferation of cells was analyzed. The cells which were treated with PDGF, either by adding PDGF in the serum or by absorption into the films, showed increased proliferation on day 3 as compared to 1% serum negative control samples (Figure 5). Cell proliferation on samples where soluble PDGF was added to the medium and on control samples containing 10% serum in the medium were similar. However the highest cell proliferation was seen on films where PDGF was pre-adsorbed to the films. This result shows that the effect of PDGF on the cells was highest where a carrier was used and also establishes that these films can be used as a carrier for growth factor delivery. The absorption of the PGDF into the HA particles increases the cellular response to the particles. The HA content can be optimized in terms of balancing osteoblast proliferation and differentiation.

5. CONCLUSIONS

All nHA-PLA composite films showed the formation of an apatite layer, however the fastest apatite layer formation was on 70wt%nHA-PLA films. A composite material containing high concentration of nHA may be a very useful material for application as a GTR membrane. Highest proliferation of osteoblasts was achieved on 10wt%nHA-PLA films, whereas highest ALP activity, thus differentiation, was shown by cells cultured on films containing 70wt%nHA. PDGF was shown to enhance the proliferation of cells, more proliferation was shown by cells on films loaded with PDGF which shows the suitability of these composite as a carrier system for growth factor delivery.

Acknowledgements:

Authors would like to acknowledge the help provided by Peter Chung at Department of Geographical and Earth Sciences, University of Glasgow in obtaining the SEM images. Dr I.U. Rehman and Dr A.S. Khan at Department of Materials, Queen Mary University of London for help with FTIR. PURAC Biochem, The Netherlands for providing the PLA.

There are no conflicts of interest related to this study.

References:

[1] Needleman IG, Worthington HV, Giedrys-Leeper E, Tucker RJ. Guided tissue regeneration for periodontal infra-bony defects. *Cochrane Database Systemic Review* (Online) 2006; CD001724.
[2] Dupoirieux L, Pourquier D, Picot MC, Neves M. Comparative study of three different membranes for guided bone regeneration of rat cranial defects. *International Journal of Oral and Maxillofacial Surgery* 2001; **30**:58-62.
[3] Bunyaratavej P, Wang H-L: Collagen membranes. A review. *Journal of Periodontology* 2001; **72**:215-29.
[4] Ueyama Y, Ishikawa K, Mano T, Koyama T, Nagatsuka H, Suzuki K, et al.,. Usefulness as guided bone regeneration membrane of the alginate membrane. Biomaterials 2002; **23**:2027-33.
[5] Aurer A and JorgiÊ-Srdjak K. Membranes for periodontal regeneration. *Acta Stomatologica Croatica* 2005; **39**: 107-112.
[6] Kasaj A, Reichert C, Götz H, Röhrig B, Smeets R and Willershausen B. *In vitro* evaluation of various bioabsorbable and nonresorbable barrier membranes for guided tissue regeneration. . *Head & Face Medicine* 2008; **4**:22.
[7] Cooper ML, Hansbrough JF, Spielvogel RL, Cohen R, Bartel RL, Naughton G. In vivo optimization of a living dermal substitute employing cultured human fibroblasts on a biodegradable polyglycolic acid or polyglactin mesh. *Biomaterials* 1991; **12**:243-48.
[8] Kumar AV, Staffenberg DA, Petronio JA, Wood RJ. Bioabsorbable plates and screws in pediatric craniofacial surgery: A review of 22 cases. *Journal of Craniofacial Surgery* 1997; **8**:97-99.
[9] Rokkanen P, Bostman O, Vainionpaa S, Vihtonen K, Tormala P, Laiho J, et al., Biodegradable implants in fracture fixation: Early results of treatment of fractures of the ankle. *Lancet* 1985;**1**:1422-24
[10] Domb AJ. Polymeric carriers for regional drug therapy. *Molecular Medicine Today* 1995; **1**:134-39
[11] Shikinami Y, Okuno M. Bioresorbable devices made of forged composites of hydroxyapatite (HA) particles and poly-lactide (PLLA): Part I. Basic characteristics. *Biomaterials* 1999; **20**:859-77.
[12] Bleach NC, Nazhat SN, Tanner KE, Kellomäki M, Törmälä P. Effect of Filler Content on Mechanical and Dynamic Mechanical Properties of Particulate Biphasic Calcium Phosphate Polylactide Composites. *Biomaterials*, 2002; **23**:1579-85.
[13] Cho MI, Lin WL, Genco RJ. Platelet-derived growth factor-modulated guided tissue regenerative therapy. *Journal of Periodontology* 1995; **66**:522-30.
[14] Hughes FJ, Turner W, Belibasakis G, Martuscelli G. Effects of growth factors and cytokines on osteoblast differentiation. *Periodontology 2000* 2006; **41**:48-72.
[15] Giannobile WV. Periodontal tissue engineering by growth factors. *Bone* 1996; **19**:S23-S37.
[16] Talal A, Waheed N, Al-Masri M, McKay I J, Tanner KE, Hughes FJ. Absorption and release of protein from hydroxyapatite-polylactic acid (HA-PLA) membranes. *Journal of Dentistry* 2009; **37**: 820-26.
[17] Wang Feng, Li Mu-Sen, Lu Yu-Peng, Qi Yong-Xin. A simple sol-gel technique for preparing hydroxyapatite nanopowders. *Materials Letters* 2005; **59**:916-19.
[18] Kokubo T, Kushitani H, Sakka S, Kitsugi T, Yamamuro T. Solutions able to reproduce in vivo surface-structure changes in bioactive A-W glass-ceramic. *Journal of Biomedical Material Research* 1990; **24**:721-34.
[19] Koutsopoulos S: Synthesis and characterization of hydroxyapatite crystals. A review study on the analytical methods. *Journal of Biomedical Material Research* 2002; **62**:600-12.
[20] Rehman I, Bonfield W. Characterization of hydroxyapatite and carbonated apatite by photo acoustic FTIR spectroscopy. *Journal of Material Science: Materials in Medicine* 1997; **8**:1-4.
[21] Matsumoto T, Okazaki M, Inoue M, Yamaguchi S, Kusunose T, Toyonaga T, et al., Hydroxyapatite particles as a controlled release carrier of protein. *Biomaterials* 2004; **25**:3807-12.

[22] Khan AS, Ahmed Z, Edirisinghe MJ, Wong FS, Rehman IU. Preparation and characterization of a novel bioactive restorative composite based on covalently coupled polyurethane-nanohydroxyapatite fibres. *Acta Biomaterialia* 2008; **4**:1275-87.
[23] Teng SH, Lee EJ, Park CS, Choi WY, Shin DS, Kim HE. Bioactive nanocomposite coatings of collagen/hydroxyapatite on titanium substrates. *Journal of Material Science: Materials in Medicine* 2008; **19**:2453-61.
[24] Kesenci K, Fambri L, Migliaresi C, Piskin E. Preparation and properties of poly(l-lactide)/hydroxyapatite composites. *Journal of Biomaterials Science* 2000; **11**:617-32.
[25] Bonfield W, Wang M, Tanner KE. Interfaces in Analogue Biomaterials. *Acta Materialia*, 1998; **46**: 2509-18.
[26] Pereira MM, Clark AE, Hench LL. Calcium-phosphate formation on sol-gel-derived bioactive glasses in-vitro. *Journal of Biomedical Material Researsh* 1994: **28**: 693-98.
[27] Kokubo T. Apatite formation on surfaces of ceramics, metals and polymers in body environment. *Acta Materialia* 1998; **46**:2519-27.
[28] Dalby MJ, Di Silvio L, Harper EJ, Bonfield W. In vitro adhesion and biocompatability of osteoblast-like cells to poly(methylmethacrylate) and poly(ethylmethacrylate) bone cements. *Journal of Materials Science: Materials in Medicine* 2002; **13**:311-14.
[29] Verrier S, Blaker JJ, Maquet V, Hench LL, Boccaccini AR. PLDLA/Bioglass composites for soft-tissue and hard-tissue engineering: An in vitro cell biology assessment. *Biomaterials* 2004; **25**:3013-21.
[30] Matsuyama T, Lau KH, Wergedal JE. Monolayer cultures of normal human bone cells contain multiple subpopulations of alkaline phosphatase positive cells. *Calcified Tissue International* 1990; **47**:276-83.
[31] Stein, G.S. & Lian, J.B. Molecular mechanisms mediating proliferation/differentiation interrelationships during progressive development of the osteoblast phenotype. *Endocr Rev* 1993; **14**, 424-442.
[32] Hott M, Noel B, Bernache-Assolant D, Rey C, Marie PJ. Proliferation and differentiation of human trabecular osteoblastic cells on hydroxyapatite. *Journal of Biomedical Material Research* 1997; **37**:508-16.
[33] Matsumura K, Hyon SH, Nakajima N, Iwata H, Watazu A, Tsutsumi S. Surface modification of poly(ethylene-co-vinyl alcohol). Hydroxyapatite immobilization and control of periodontal ligament cells differentiation. *Biomaterials* 2004; **25**:4817-24.
[34] Zhang Y, Hao L, Savalani MM, Harris RA, Di Silvio L, Tanner KE. In vitro biocompatibility of hydroxyapatite-reinforced polymeric composites manufactured by selective laser sintering. *Journal of Biomedical Material Research A* 2009 15; **91**:1018-27.
[35] Nevins M, Camelo M, Nevins ML, Schenk RK, Lynch SE. Periodontal regeneration in humans using recombinant human platelet- derived growth factor-bb (RHPDGF-BB) and allogenic bone. *Journal of Periodontology* 2003; **74**:1282-92.