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Gelatin sponges (Gelfoam®) as a scaffold for osteoblasts

Ramin Rohanizadeh · Michael V. Swain · Rebecca S. Mason

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Abstract Gelatine sponge because of its flexibility, biocompatibility, and biodegradability, has the potential to be used as a scaffold to support osteoblasts and to promote bone regeneration in defective areas. This study aimed to determine osteoblast proliferation, differentiation, and integration in modified and un-modified gelatine sponges. Three scaffolds were studied: gelatine sponge (Gelfoam®), gelatin sponge/mineral (hydroxyapatite) composite, and gelatin sponge/polymer (poly-lactide-co-glycolide) composite. 2–D plastic coverslip was used as control. The gelatin sponges were modified using PLGA coating and mineral deposition to increase biodegradation resistance and osteoblast proliferation respectively. The scaffolds were characterized using Scanning Electron Microscopy (SEM) and X-ray diffraction. Cell number (DNA content), cell-replication rate (thymidine assay), and cell differentiation (alkaline phosphatase activity) were measured 24 h, 3 days, and 1, 2, 3 weeks after the osteoblast-like cells were cultured onto the scaffolds. Cell penetration into the sponges was determined using haematoxylin-eosin staining. Both modified and unmodified gelatine sponges demonstrated ability to support cell growth and cells were able to penetrate into the sponge pores. In a comparison of different scaffolds, cell number and cell replication were highest in sponge/hydroxyapatite composite and lowest in sponge/PLGA composite.

R. Rohanizadeh · R. S. Mason
Bone and Skin Research Group, Department of Physiology, University of Sydney, Sydney, NSW, Australia

R. Rohanizadeh (✉)
Advanced Drug Delivery Group, Faculty of Pharmacy, University of Sydney, Sydney, NSW, Australia
e-mail: raminr@pharm.usyd.edu.au

M. V. Swain
Biomaterials Unit, Faculty of Dentistry, University of Sydney, Sydney, NSW, Australia

Introduction

A healthy bone has the ability to regenerate spontaneously if the volume of the defect does not exceed a certain size [1]. In cases involving large defects, or when bone metabolism is not able to properly repair the bone defect (e.g. high bone turnover cases such as osteoporosis or Paget's disease) bone graft biomaterials can be used to both bridge the defects and to facilitate bone formation in the defective areas [2, 3]. Various types of bone graft materials such as autograft (patient bone), allograft (human cadaver bone), xenograft (animal bone), and synthetic biomaterials (*e.g.* ceramics, metals, polymers, and composites) have been tested and used to repair bone defects [2–4]. All of these materials have their associated disadvantages such as limited healthy available bone grafts from patients, additional surgical trauma caused by donor site, and longer operation times for autograft ; risk of host reaction due to genetic differences, high resorption, disease transformation, ethical and religious concerns for allograft and xenograft. Low rate of biodegradability, inadequate architectural properties (lack of interconnected pores), low affinity to matrix macromolecules (resulting in interfacial instability with bone), and lack of mechanical stimulus in the surrounding bone (resulting in a higher bone resorption) are all concerns when using synthetic bone graft materials [2].

The biomaterials used for bone grafts should provide three dimensional support for cell migration, proliferation, differentiation, and thereby act as a scaffold for new bone formation in the defective areas. The scaffolds must possess a wide range of different characteristics. They need to be biocompatible, biodegradable, porous, adequate mechanical properties, for some applications preferably malleable into desired shapes or injectable, and most importantly show compatibility and affinity to osteogenic bone matrix proteins and growth factors [2–4].

Biodegradable polymers derived from natural sources (*e.g.* collagen, gelatin, elastin, fibrin, hyaluronic acid, chitosan/chitin, and alginate) have been studied and used as bone graft or cartilage graft materials to repair defects in various orthopaedic and dental applications [4, 5]. 'Natural-derived' polymers are extracellular matrix proteins of different tissues providing a scaffold for cellular support in body. As bone graft materials, protein-derived materials have superior properties over synthetic materials due to their excellent biocompatibility, and their high affinity to and compatibility with the other matrix proteins.

Gelatin is a thermal-denatured collagen that may be prepared either by acidic (type A) or alkaline (type B) treatments (extraction) of bovine or porcine skin, bone or tendon, followed by heat-treatment in an aqueous environment (hydrolysis) [6, 7]. The final product is separated from bouillon using various methods such as settling, filtering, and centrifuging. Gelatin has a long history of usage in the food industry as a clarification agent, stabilizer and protective coating material, and in the pharmaceutical industry for manufacturing capsules, ointments, cosmetics, tablet coating, and emulsion [6]. Due to its hemostatic properties, gelatin sponge has been widely used in surgery as a wound dressing, adhesive and absorbent pad [8]. The advantage of gelatin over collagen matrix is its ease of extraction and preparation, which results in a cheaper and high quantity production of gelatin matrix. Furthermore, unlike collagen, gelatin does not express any antigenicity in physiological conditions [9]. Gelatin is composed of some 18 different amino acids repeated in particular sequence to form a coil structure (collagen has a triple helix structure). One-third to half of all amino acids in gelatin structure is either glycine or alanine. Gelatin prepared using acid extraction (type A) has a higher quantity of alanine than that prepared by alkaline treatment (type B). The other predominant amino acids in gelatin are either proline or hydroxyproline [6].

Collagen-based materials in different forms and textures (sponge, gel, film, and fibre) or in combination with ceramics or polymers have been widely studied as scaffolds and/or drug carriers for bone regeneration in defective areas, or in vitro as a support for bone and cartilage cells growth [10–14]. Unlike collagen-based scaffolds, few studies have investigated the ability of gelatin-based scaffolds to be used for supporting osteoblast or chondrocyte proliferation and phenotype [15–17]. Due to flexibility in shape, biocompatibility, affinity to proteins, and biodegradability, gelatin-derived sponges may be excellent candidates for bone graft scaffolds in low-load areas or as drug delivery materials.

Gelfoam® is a gelatin-based sponge prepared from purified type A pork skin gelatin which has been widely used to control bleeding and wound dressing, during and after surgical procedure. Ponticiello et al. [15] demonstrated that 21 day cultures of mesenchymal stem cells (MSCs) in Gelfoam® using medium supplemented with TGF-β3 resulted in formation of a cartilage-like extracellular matrix containing cartilage markers such as type-II collagen and glycosaminoglycans. In the same study the gelatin sponges were implanted in osteochondral defects in a rabbit model and the authors reported a very good biocompatibility of gelatin sponge without the presence of any immune response. In an in vivo study Finn et al. applied four different hemostatic agents including Gelfoam® to evaluate their potential to regenerate bone in an iliac crest defect created in a dog model. After two month implantation, bone formation was observed in the presence of Gelfoam® in the defective areas. The residuals of gelatin sponge were incorporated in the new bone, without the presence of any significant foreign-body reaction. In a clinical study of 32 patients, gelatin sponges (Gelfoam®) were also successfully implanted as control groups in the defective areas of jaw after cyst enucleation [18].

Gelfoam® has also been tested as a graft material in cardiovascular application [19–21]. Fetal cardiomyocytes were seeded into the gelatin sponge and then the cell-seeded graft was transplanted into the rat heart muscle [20]. The authors reported spontaneous contraction occurred when the cells were seeded into the gelatin sponge, and the beating continued after the transplantation of sponge into heart and even after the sponge was dissolved [19]. Gelatin sponge alone or in composite with other materials has also been used as a drug delivery device in several studies [7, 22, 23].

The present study aimed to evaluate osteoblast-like cell proliferation, differentiation, attachment, and integration in three different scaffolds: Gelatin sponge (Gelfoam®), gelatin sponge/polymer, and gelatin sponge/mineral composites. One of the major disadvantages of gelatin or collagen sponges as a biomaterial is their high degree of biodegradation due to enzymatic digestion. An inadequate mechanical stability of protein-derived scaffolds will lead to a mismatch between the new bone formation and the scaffold degradation rates. An appropriate ratio between bone formation and scaffold degradation is essential to provide a suitable environment for bone growth in defective areas. To overcome this problem, collagen and gelatin fibres have been cross-linked in different studies using

chemical agents such as various aldehydes (*e.g.* glutaraldehyde, formaldehyde); acyl acid; carbodiimides; hexamethylene-diisocyanate. Cross-linking the structure results in a lower degradation rate, increasing the longevity of materials in the living environment. However, cross-linking also reduces the ability of proteins to bind to the matrix because of a reduction in the number of free amino acid groups in the gelatin or collagen fibres. More importantly, these agents are all cytotoxic and free cross-linking agents (leached from structure) have the potential to damage the surrounding living tissues.

In our study, two groups of gelatin sponges were modified by coating with poly lactic acid-co-poly glycolic acid (PLGA) and by deposition of calcium phosphate apatitic crystals. PLGA is a biocompatible and biodegradable polymer (lower degradation rate than gelatin). PLGA coating was performed to decrease the biodegradability of gelatin and apatite deposition to stimulate cell proliferation and to enhance cell attachment.

Materials and methods

Scaffold preparation

Gelatin-based sponge (Gelfoam®) derived from purified type A pork skin gelatin was purchased from Pharmacia & Upjohn (Kalamazoo, Michigan). Using a hole punch, Gelfoam sheets of 7 mm thickness were cut into cylinders of 3 mm diameter which were then used for cell culture assays. The Gelfoam/PLGA composite (poly lactic acid co poly glycolic acid with the ratio of 70:30, purchased from Sigma) was prepared by immersing the Gelfoam into 0.02 g/mL PLGA dissolved in Chloroform. PLGA solution, 50 μL, was dropped on each side of the Gelfoam and then dried at room temperature. Sponges treated with chloroform were also prepared to determine the effect of chloroform alone on the gelatin sponge.

Gelfoam/mineral samples were prepared by immersing the sponges in 200 mM $CaCl_2$ for 3 min, removing all of the excess solution, and then immersing them in 120 mM Na_2HPO_4 for another 3 min. The pH of the calcification solution was adjusted to 8.5 and the samples were mineralized at 45 °C using a water bath. After mineral deposition, the samples were rinsed in double distilled water to remove all of the calcification solution and dried at 37 °C for 3 h. Over-drying was prevented to minimize sponge shrinkage.

Scaffold characterization

The morphology and pore size of the modified and un-modified sponges as well as the shape, size, distribution and chemical elements of deposited mineral were analyzed using Scanning Electron Microscopy, SEM (Philips-505) working at 20 kV and coupled to a Electron Dispersive Spectroscopy, EDS (Oxford System). In order to evaluate the efficiency of the modifications (PLGA coating and mineral deposition) the cross section views of the samples (sectioned from the middle of sponges) were also analyzed under SEM. SEM was also used to observe the cell morphology and coverage on different scaffolds. For this purpose the sponges seeded with osteoblasts were fixed using 2% glutaraldehyde solution (in PBS) and then dehydrated through different baths of ethanol (70, 80, 90, 95, 100%, 30 min in each bath). Prior to SEM observation, the samples became electron conductive by sputter coating with a thin layer of gold-palladium.

X-ray diffraction (Siemens D5000) using grazing incident angle of 2° was used to determine the crystal structure of deposited mineral into sponges. XRD was carried out using CuK_α radiation working at 40 kV and 40 mA with 0.01°-step size, and 10 s analysis time per step.

Degradation of scaffolds

In order to evaluate the degradation rate of the scaffolds in vitro, six samples ($10 \times 30 \times 7$ mm) from each group of scaffolds were first placed individually in a capped glass vial, weighed (W_O), and then 4 mL 25% papain solution (diluted in PBS) added to each vial. The samples were then incubated at 37°°C for 4 h. The papain solution was prepared by dissolving 25 μg/mL of papain powder (10 units) in a solution containing 50 mM sodium phosphate, 2 mM *N*-acetyl cysteine, and 2 mM EDTA. After the degradation, the samples were washed in distilled water, and air-dried (24 h), and then weighed again (W_t). The percentage of weight loss (ΔW%) was calculated using the formula, $\Delta W\% = (W_O - W_t)/W_O \times 100\%$. The weight loss percentage due to digestion in papain solution indicates the degradation rates of different scaffolds. The higher the weight loss percentage, the greater the degradation rate.

Seeding the osteoblasts

The samples were cold sterilized in 70% ethanol for 30 min and then washed three times with culture medium to remove all of the remaining alcohol. A very thin layer of silicon gel was used to mount the samples on the bottom of the 96-well plate. The human MG-63 osteoblast-like cells were incubated in a 75-cm^2 culture flask in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Dulbecco's Modified Eagle's Medium (DMEM) with Ca^{2+} and supplemented with 10% fetal bovine serum (FBS), ascorbic acid (0.044 g/L), and glutamine (0.292 g/L) was used as culture medium. At confluence (3–4 days), the cells were

detached from the flask using 0.25% trypsin and 200,000 cells/ml (20,000 cells/sample) were seeded on top of each sponge (placed in a 96-well culture plate). This cell density, which was chosen based on our previous optimization work, allows a good plating efficiency, but is low enough to let cells continue to proliferate over several more days, even on plastic wells without sample. The cells were cultured on sponges for 24 h, 3 days, 1, 2, and 3 weeks. Thermanox coverslip was used as a control since osteoblasts can form a 3-dimensional multilayer when cultured on this material [24].

Cell proliferation

At the end of each time-point, osteoblasts proliferation in sponges and on coverslips (as control) was determined by measuring the total dsDNA (double-stranded DNA) content. The culture wells containing the sponges were washed with PBS (3 times, interval 5 min), and then the sponges placed individually into sealed-sterile-DNA-free vials containing 1 mL papain solution and incubated over night at 65 °C to digest the sponges. After the digestion, the vials were placed at –80 °C until all of the samples for different culture periods were ready for DNA measurement using PicoGreen dsDNA quantitation kit (Molecular Probes, Eugene, Oregon). PicoGreen reagent is a fluorescent nucleic acid stain. The fluorescence measurements were made using BMG plate reader with excitation and reading wavelengths of respectively 480 and 520 nm. The dsDNA contents (expressed in nano-gram) of the samples were determined by comparing to those of standard DNA contents.

Cell replication rate

Cell replication rate during the 24 h prior to the end of each time-point was determined by measuring ^{3}H-thymidine incorporation. For the 24 h time point, the osteoblasts were treated for 24 h with culture medium containing 0.5 μCi/mL thymidine, and the treated cells were then seeded onto the sponges. After 24 h culture, the samples were washed with PBS (3 times, 7 min interval) and then digested in papain solution (500 μL papain solution per sample) as mentioned above. For the other time points (3d, 1, 2, 3 weeks), the samples were treated with thymidine for 24 h prior to the end of the time-point, washed with PBS and digested in papain solution. After the sponge digestion, 400 μL of the solution was added to 3 ml scintillation fluid. The beta radiation was measured for each sample using a PerkinElmer beta counter (model 1900). Sponges without any cells but treated with thymidine were used as a background of beta radiation and the results were corrected for the background radiation.

Measuring cell penetration into the sponges

At the end of each time-point the samples were fixed in 10% formalin and dehydrated in a series of ethanol baths and then embedded in paraffin and sectioned at a thickness of 4 μm and stained with haematoxylin-eosin (H&E staining). Cell penetration into the sponges was evaluated for each time-point using light microscopy.

Alkaline phosphatase (ALP) activity

Osteoblastic phenotype was evaluated after a 7-day culture by measuring alkaline phosphatase activity of the cells which were seeded into the different scaffolds. The samples were first washed three times (7 min interval) with PBS and then 200 μL ALP reagent was added in each well and incubated at 37 °C for 1 h. After neutralizing the reaction with 20 μL of 1 M NaOH (in each well), the absorbance of 405 nm wavelength (yellow colour) was measured using BMG plate reader.

Statistical analysis

All the cell culture assays were repeated twice on at least 6 samples per group. The statistical differences between groups were determined using analysis of variance (ANOVA) followed by post hoc multiple comparison test (Tukey test). A p value of less than 0.05 was considered significant.

Results

Scaffold characteristics

Gelfoam demonstrated a very porous structure in which the pores were separated with thin (few μm in thickness) walls (Fig. 1A,B). The pore size varied between 30 and 700 μm. A population of closed pores with flat boundaries between them were also observed under SEM analysis (Fig. 1B). After coating the gelatin sponge with PLGA, the SEM micrographs demonstrated a lower porosity probably due to blocking the small pores of sponge with the PLGA film (Fig. 1C, D). PLGA film did not cover the entire sponge structure and some areas of sponge remained intact and uncovered (Fig. 1C, D). PLGA film was also observed inside the sponge/PLGA scaffolds under the SEM in cross section views. After immersion of the gelatin sponges into calcium and phosphate calcifying solutions (sponge/mineral group), small crystals (~0.5 μm) attached to the sponge walls were observed on the surface and/or in the pores of the sponges (Fig. 1E). These crystals had a flat shape at higher magnification (Fig. 1F) and did not cover

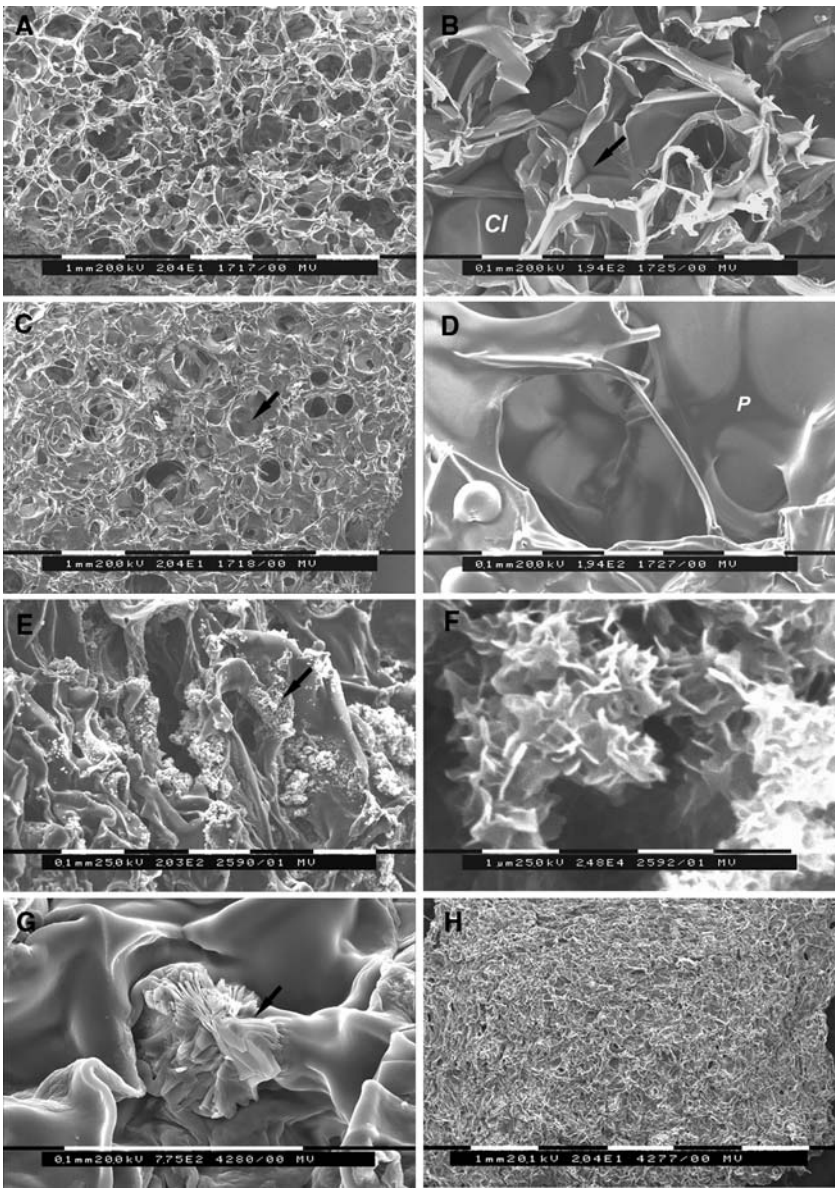

Fig. 1 SEM micrographs of (**A**) gelatin sponge showing a porous structure, (**B**) at higher magnification closed pores (*cl*) and the flat walls between the pores (*black arrow*) were observed in gelatin sponge. (**C**) Gelatin sponge/PLGA composite demonstrated lower porosity than unmodified sponge due to blockage of some of the pores by PLGA film (*black arrow*). (**D**) At higher magnification, PLGA film (*p*) covering the pores' walls of sponge was observed. (**E**) Deposited micro-crystals were observed on the sponge structure after carrying out the mineralization in calcification solution (black arrow). (**F**) The deposited micro-crystals had a flat shape. (**G**) A minor population of another type of crystals (based on the morphology). The crystals grow in the form of radial flat plates from the centre of crystal agglomeration (black arrow). (**H**) The sponge/mineral composite demonstrating a smaller pore size compared to the unmodified sponge (Fig. 1H vs. A)

the entire surface. A minor population of another type of crystals (based on their morphology) was also observed under SEM (Fig. 1G). These crystals were bigger (~30 μm) and grew in the form of radial flat plates from the centre of crystal agglomeration (Fig. 1G). The morphology of these types of crystals was very similar to that of Dicalcium Phosphate Dihydrate (DCPD) prepared using precipitation method in calcium phosphate solution [25]. The sponge/mineral group demonstrated a smaller pore size compared to the unmodified sponge (Fig. 1H vs. Fig. 1A) because of shrinkage of the sponges' structure after the mineral deposition process. The sponges swelled in the calcifying solution and shrank after drying because of dehydration.

X-ray diffraction showed that the majority of deposited crystals had apatetic structure with the highest peak of hydroxyapatite (HA) corresponding to (100) lattice located at $2\theta = 31.44°$ (Fig. 2). The presence of an additional Dicalcium Phosphate Dihydrate (DCDP) phase (the highest peak of DCPD corresponding to (100) lattice was located at $2\theta = 11.69°$) was also revealed by X-ray diffraction.

The atomic ratio of calcium to phosphorous (Ca/P) ratio of the small deposited crystals (corresponding to Fig. 1F) was measured as 1.63 ± 0.05 using EDS. This Ca/P ratio is close to that of stoichiometric hydroxyapatite (Ca/P = 1.67). The Ca/P ratio for the bigger crystals (corresponding to Fig. 1G) was 1.1 ± 0.07, which is close to that of DCPD (Ca/P = 1).

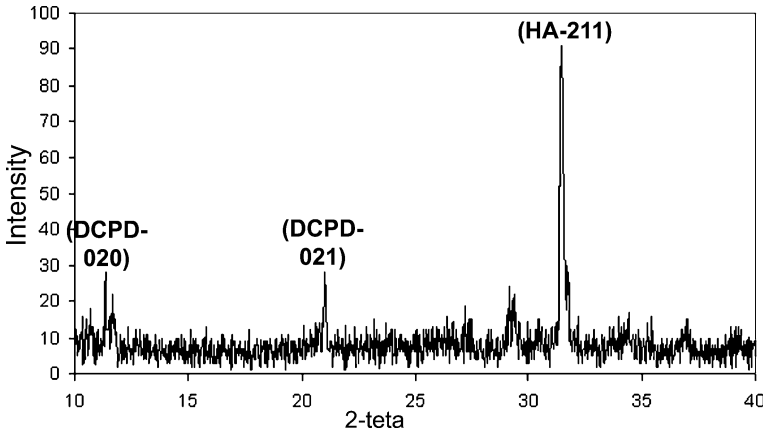

Fig. 2 X-ray diffraction pattern from the deposited crystals on the gelatin sponge. The peak located at $2\theta = 31.44°$ corresponds to the (211) hydroxyapatite lattice plane. An additional calcium phosphate phase identified as Dicalcium Phosphate Dihydrate (DCPD) was also present in diffraction patterns after mineral deposition into the gelatin sponge

The sponges coated with PLGA demonstrated significantly ($p < 0.05$) higher degradation resistance in papain solution compared to the unmodified sponges and sponge/mineral composite groups (Fig. 3). PLGA coating improved by 37% the biodegradation resistance of gelatin sponge. Sponge/PLGA composites lost on average 45% of their weight after degradation in papain solution, while this percentage was higher for unmodified sponge and sponge/mineral composite (72% and 78.8% respectively).

Cell coverage and penetration into the scaffolds

Osteoblasts covered the entire surface of all of the different scaffolds after 1-week of culture (Fig. 4A,B). The morphology of the cells varied depending on the shape of the surface. The cells had a tapered shape on a curved surface (Fig. 4A) and a flat shape on a flat surface (Fig. 4B).

H&E staining also demonstrated the ability of all the scaffolds to allow the cells to penetrate into the interior of the sponge structure. Although the cells invaded the entire sponge structure in all of the tested scaffolds after 1-week culture, the cells on the sponge surface were always present in higher numbers than those on the inside of the sponges (Fig. 5).

Cell proliferation

DNA content of cells cultured in different scaffolds and that of the control group is shown in Fig. 6. The DNA content (representing the cell number) increased constantly in all of the scaffolds as a function of culture period. After the first 24 h of culture, the DNA content was higher on the 2-D plastic coverslip (control group) than the 3-D test scaffolds, though this difference was not significant. Cell proliferation became significantly lower ($p < 0.05$) in the control group compared to that of scaffolds after 1 week of culture. In a comparison of different scaffolds, sponge/mineral composite demonstrated the highest DNA content at 1, 2, and 3 week time-points. However, the difference in DNA content was not significant between sponge/mineral and unmodified sponge groups at these time-points. The DNA content in sponge/PLGA scaffolds was significantly lower than those of unmodified sponge and sponge/mineral after 2 and 3 weeks culture.

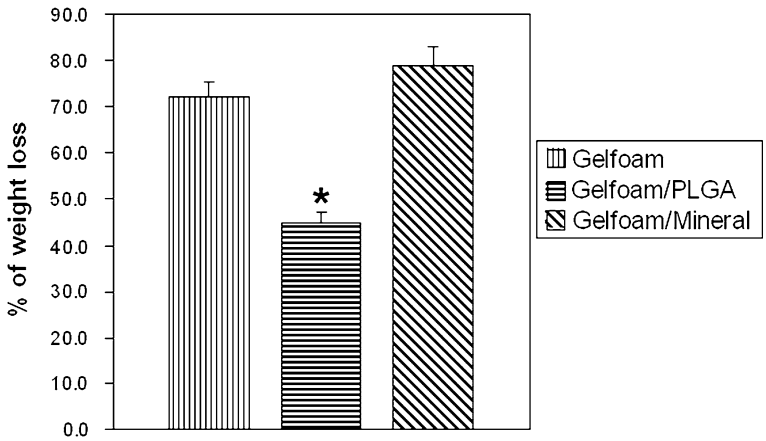

Fig. 3 The weight loss percentage of different scaffolds after degradation in papain solution. Lower weight loss indicates higher resistance to the enzymatic biodegradation. Sponge/PLGA composite demonstrated the highest and sponge/mineral composite demonstrated the least biodegradation resistance. * Significant difference ($p < 0.05$)

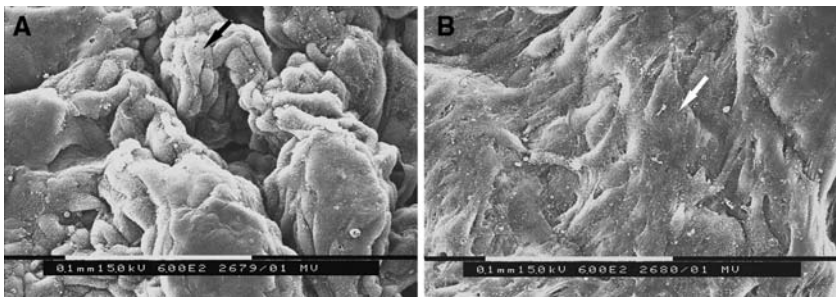

Fig. 4 (**A**) SEM micrograph showing the coverage of the osteoblasts on the surface of gelatin sponge after 1-week culture. The cells were able to cover the entire surface of all the scaffolds after 1 week. (**A**) On a curved surface the cells demonstrated a tapered shape (*black arrow*) and (**B**) on a flat surface they appeared to have a flat shape (*white arrow*)

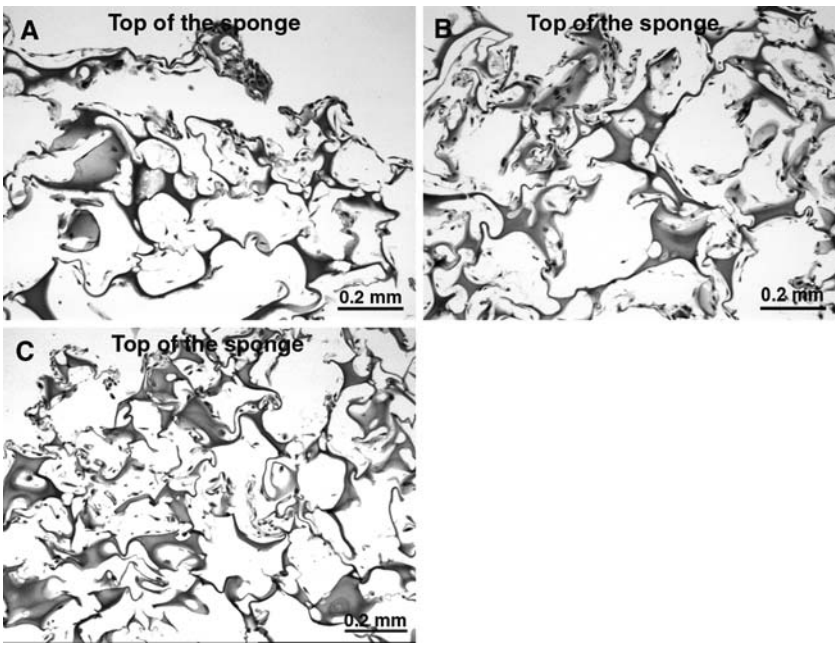

Fig. 5 H&E stained section of the scaffolds after 2-week culture demonstrating the invasion of osteoblasts into the scaffolds' pores. A higher cell number was observed on the top surface of the scaffolds where the cells were seeded. (**A**) gelatin sponge; (**B**) gelatin sponge/mineral composite; (**C**) gelatin sponge/PLGA composite

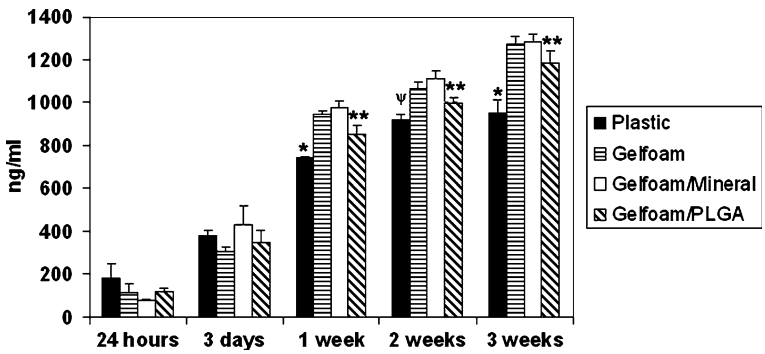

Fig. 6 DNA content of different scaffolds (the plastic is 2D coverslip control group) after culturing osteoblasts for various time-points.
* Significantly low ($p < 0.05$) than all of the groups.
** Significantly lower than gelfoam/mineral composite.
$^{\psi}$ Significantly lower than gelfoam and gelfoam/mineral composite

Thymidine incorporation (representing cell replication rate during 24 h prior to the end of the culture period) was much higher in the 2-D control group than in cells on the scaffolds at the 3 day time-point (Fig. 7). Cell replication rate decreased dramatically in the control group after 1 week of culture, and cells in all of the scaffolds demonstrated a higher replication rate than the control group. At this time, the difference between cell replication rates of control and scaffolds was less significant at 2 weeks of culture and not significant at 3 weeks of culture (Fig. 7). Among the scaf-

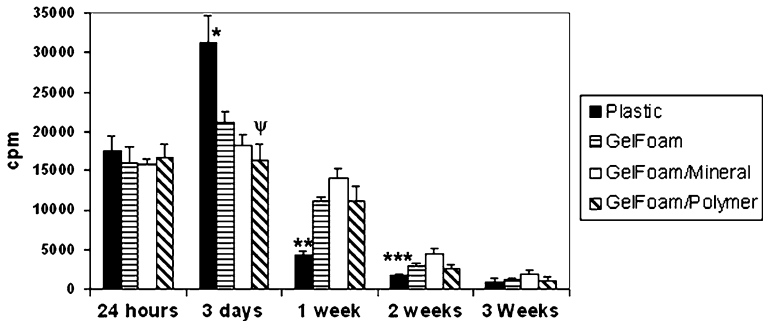

Fig. 7 Cell replication rate measured by ^{3}H-thymadine incorporation during 24 h prior to the end of the time-point. * Significantly higher than all of the groups. $^{\psi}$ Significantly lower than plastic (coverslip) and gelfoam groups. ** Significantly lower than all of groups. *** Significantly lower than gelfoam/mineral group

folds, cells in sponge/mineral composite demonstrated the highest cell replication rate at 1–3 weeks of culture, however these differences were not significant (Fig. 7).

Cell differentiation

Figure 8 shows the alkaline phosphatase (ALP) activity (measured by absorbance of yellow colour) of control and different scaffolds after a 7 day culture period. The ALP activity presented in this graph was corrected for the cell number. The ALP activity was similar for all of the scaffolds and slightly lower than that of the plastic coverslip. ALP activity demonstrated that sponge modifications (PLGA coating and mineral deposition) did not affect the osteoblast phenotype and the cells seeded in all of the scaffolds were able to produce alkaline phosphatase.

Discussion

Natural protein-derived biomaterials in the form of porous blocks, mesh, fibre, or as a coating, or in composite with the other types of material have been the subject of several studies on scaffolds used for osteoblasts and to conduct and promote bone formation in defective areas [5]. As a tissue scaffold, protein-derived biomaterials have excellent compatibility with and affinity to other matrix proteins. However, the lack of mechanical strength and/or high biodegradation rate after implantation limit the clinical application of these materials [26, 27].

The present study investigated the ability of gelatin scaffolds (modified and unmodified gelatin sponges) to be used as a support for osteoblast proliferation and differentiation. All of the tested scaffolds demonstrated the ability to support osteoblast activities and allowed cell proliferation and cell migration into the sponge porosities. Although the surface of Thermanox coverslip (used as control group) is treated by the manufacturer to allow cells to grow in multiple layers [24], cell number (measured by DNA content) was higher in all of the scaffolds (after 1-week culture) than in the control group. Cell replication decreased dramatically in the control group (coverslip) after the 1-week culture, probably because cell confluence was achieved in a shorter time in the 2D plastic coverslip than in the 3D scaffold. This demonstrates that the structure and composition of gelatin sponge allowed osteoblasts to attach to the sponge surface and to invade and penetrate into the porous structure of the sponge. Caution must be advised in extrapolating the results from this study to the clinical situation as the size of the scaffolds used were relatively smaller than clinically-suitable constructs. For bone applications a typical critical size defect is around 5 cm and penetration and survival of cells in such relatively thick scaffolds may not be achieved.

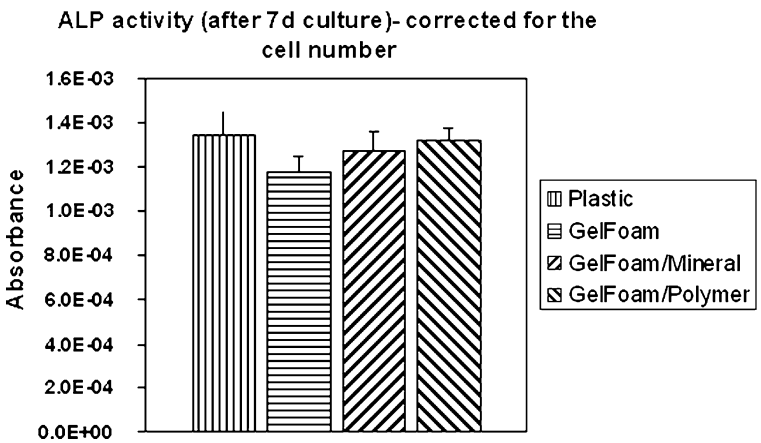

Fig. 8 Alkaline Phosphatase Activity (demonstrating cell differentiation) of osteoblasts in different groups. The ALP activity presenting in this graph is divided by the number of cells in each group. No significant difference was observed among different groups

The ability of gelatin sponge to play the role of scaffold for supporting chondrocyte and osteoblast cells was demonstrated in vitro by Stanton et al. [28]. Stanton et al. reported the formation of cartilaginous matrix in the pores of gelatin sponges after 25 days in culture. In an vitro study Yang et al. [29] reported that a cross-linked gelatin scaffold composed of tricalcium-phosphate provided an excellent porous structure, conductive to osteoblast attachment and differentiation, and that this ability was significantly improved by the incorporation of BMP-4 into the scaffold structure. Osteoinduction property of BMP loaded-gelatin sponges (with different contents of tricalcium phosphate) was also demonstrated in an in vivo study [30].

In the present study, the gelatin scaffold was modified by PLGA coating or by hydroxyapatite deposition to decrease the biodegradability of gelatin sponge and to stimulate osteoblast proliferation, respectively. Although PLGA integration in gelatin sponge decreased the degradation rate of the sponge by 37.5%, it also decreased osteoblast proliferation. Lower cell proliferation in the PLGA coated sponges could be either due to the blockage of some small pores (less available space for cell invasion), and/or due to lower adhesion of osteoblasts to PLGA compared to the gelatin surface. Cell/substrate adhesion is modulated by the binding of cell membrane adhesive receptors (integrins) to extracellular matrix proteins (*e.g.* osteonectin, osteopontin, fibronectin, vitronectin) [2, 31]. Physico-chemical properties of a biomaterial surface such as topography, hydrophobicity, charge, free energy, and the degree of hydroxylation and sulfation, all modify the attachment mechanism of extracellular matrix proteins to biomaterials thereby affecting cell/material adhesion [32, 33]. Integrins attach to specific sequences of amino-acids of extracellular matrix proteins notably RGD sequence (R: arginine; G: glycine; D: aspartic acid) [31]. Cell adhesion motifs such as the RGD sequence existing in gelatin structure could bind directly to the integrins of cell membrane, leading to adhesion of osteoblasts to the gelatin scaffold. PLGA coating of the scaffold covers the gelatin surface and reduces the number of available adhesion motifs to integrins, therefore decreases overall cell/scaffold adhesion.

In this study hydroxyapatite (HA) crystals were deposited into the pores of gelatin sponge by alternative immersions in calcium and then phosphate solutions with adjusted pH and temperature. The results showed that both cell proliferation and differentiation improved in gelatin/mineral (HA) sponges, however the difference was not significant compared to the unmodified sponges. The shrinkage of gelatin sponge due to the mineral deposition process can counteract the positive effects of calcium on cell adhesion and proliferation. A decrease in the total surface area of the sponges leads to less available space for cell invasion. In order to measure the DNA content of the samples (representing the cell number in each scaffold), the scaffolds were digested in papain solution. During scaffold digestion the rate of cell liberation from the scaffold could be dependent on types of scaffold. However, the use of papain (overnight at 65 °C) was chosen to be maximally effective and resulted in apparently complete dissolution of the scaffolds. It has been reported that the incorporation of Ca^{2+} ions into scaffolds stimulates osteoblast proliferation and differentiation in vitro [13, 16, 34]. Kim et al. [34] studied osteoblast activities in gelatin/hydroxyapatite (HA) composite scaffolds, which were prepared by the co-precipitation of HA within a gelatin sol and further freeze-drying process to obtain a porous structure. They reported an improvement in cellular responses in gelatin/HA scaffolds compared to those without HA crystals. The improvement was greater when the composites were prepared by co-precipitation of HA in gelatin sol, compared to those prepared by mixing of gelatin solution with HA powder. Gelatin/siloxane composite in which apatite crystals were deposited by soaking of the composite in Simulated Body Fluid (SBF) for 14 days also demonstrated higher osteoblast proliferation and differentiation in vitro than the composites without mineral [16]. The presence of calcium ions improves cell/substrate adhesion by different mechanisms: (i) calcium activates integrins by binding to divalent-cation domains of integrins located at extracellular parts of α chains [35, 36]; (ii) calcium stimulates both osteoblast proliferation and differentiation [37, 38]; (iii) if the substrate contains calcium, that calcium binds to phosphorylated amino acids or carboxyxylglutamic acids of proteins, and thereby increases protein/substrate adhesion [39]. In our study, the mineral deposited into the sponge after immersion in calcifying solution was determined to be mainly hydroxyapatite (HA). However Dicalcium Phosphate Dihydrate (DCPD) was also precipitated during mineral deposition as an additional calcium phosphate phase. The pH and temperature of calcifying solution was adjusted respectively at 8.5 and 45 °C. LeGeros [25, 40] reported that under these conditions DCPD cannot form in an aqueous solution containing calcium and phosphate ions. The minor DCPD phase presented after mineral deposition was probably formed during the sponge drying stage. The sponges were dried at 37 °C for 3 h and then placed on the lab bench over night. The residue of calcifying solution remaining in the sponge could cause the precipitation of DCPD at room temperature. The complete dehydration of sponges at 37 °C was avoided because that destroys the structure of gelatin sponge. DCPD is biocompatible and bioactive and has a solubility lower than HA [25], ensuring that DCPD would dissolve first when the samples were immersed into the cell culture medium.

In conclusion, gelatin sponge could be a candidate for a bone-scaffold in low-load areas or as a drug delivery carrier to promote bone regeneration in defective areas. PLGA coating of gelatin sponge increased its biodegradation resistance, but also decreased osteoblast proliferation. Although osteoblast proliferation was slightly enhanced by hydroxyapatite (HA) deposition in gelatin sponge, this increase was not significant compared to cell proliferation in the unmodified sponges. Changes in sponge structure (smaller pore size), caused by the drying stage after mineral deposition, may compromise the beneficial effects of mineral Ca (hydroxyapatite) on osteoblast proliferation and adhesion.

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