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Biomaterials 25 (2004) 1049–1058
Biomaterials
www.elsevier.com/locate/biomaterials

Hydroxyapatite–chitin materials as potential tissue engineered bone substitutes

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Received 25 November 2002; accepted 22 July 2003

Abstract

Hydroxyapatite (HA) in 25%, 50% and 75% w/w fractions was incorporated into chitin solutions and processed into air- and freeze-dried materials. These HA–chitin materials were exposed to cell cultures and implanted into the intramusculature of a rat model. The HA–chitin materials were found to be non-cytotoxic and degraded in vivo. The presence of the HA filler enhanced calcification as well as accelerated degradation of the chitin matrix. The freeze-dried HA–chitin matrixes were selected for further cell seeding experiments because of their porous nature. Mesenchymal stem cells harvested from NZW rabbits were induced into osteoblasts in vitro using dexamethasone. These osteoblasts were cultured for 1 week, statically loaded onto the porous HA–chitin matrixes and implanted into bone defects of the rabbit femur for 2 months. Histology of explants showed bone regeneration with biodegradation of the HA–chitin matrix. Similarly, green fluorescence protein (GFP) transfected MSC-induced osteoblasts were also loaded onto porous HA–chitin matrixes and implanted into the rabbit femur. The results from GFP-transfected MSCs showed that loaded MSCs-induced osteoblasts did not only proliferate but also recruited surrounding tissue to grow in. This study demonstrates the potential of HA–chitin matrixes as a good substrate candidate for tissue engineered bone substitute.

Keywords: Chitin; Hydroxyapatite; Mesenchymal stem cells; Tissue engineering; Bone-substitutes; Autologous implants

1. Introduction

Large critical size defects arising from tumor resection, trauma, and skeletal abnormalities require assistance to provide skeletal continuity, mechanical support and eventual regeneration [1]. Periodontal disease that severely affect approximately 10% of the adult population is also characterized by a loss of attachment between the tooth and underlying bone that can lead to bone resorption and ultimately to tooth loss [2]. Presently, materials strategies to address bone defects include the use of autogeneous grafts and flaps, allograft bone, non-degradable bone cement, metals and ceramics [3]. All of these options have their associated problems such as the finite amount of tissue that can be harvested for autografts and the difficulty to shape grafts into the desired form; the potential of transferring pathogens with allografts; and synthetic implants that may result in stress shielding in the surrounding bone or fatigue failure of the implant. Therefore, the search for improved materials for repairing skeletal defects continues.

Bone regeneration is based on the hypothesis that healthy progenitor cells, either recruited or delivered to an injured site, can eventually regenerate lost or damaged bone tissue. Tissue engineering approaches attempt to create tissue replacements by culturing cells onto synthetic three-dimensional polymer matrixes [4]. Three-dimensional porous polymeric matrixes are seen as one approach to enhance bone regeneration by creating and maintaining channels that facilitate progenitor cell migration, proliferation, and differentiation [5]. The requirements that must be satisfied by such matrixes include providing a space with the proper size and shape for tissue development and permitting cells from the surrounding tissue to migrate into the matrix [6]. The surface of the polymer would also ideally promote cell attachment, as many cells are anchorage dependent for survival.

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0142-9612/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0142-9612(03)00612-4

Synthetic biodegradable polymeric matrixes provide a structural framework for selected cells to form new tissues. The open, porous, three-dimensional structure of these polymers has been designed to maximize diffusion parameters and permit vascular in-growth into the implanted structures. The polymer is completely resorbed over time, leaving only newly formed tissue, a chimera of donor cells and recipient mesenchymal elements including blood vessels and connective tissue [7]. Polyesters, specifically polylactic acid (PLA), polyglycolic acid (PGA) and their copolymers (PLGAs) fulfill the necessary criteria of biocompatibility, processability, and controlled degradation and were the first such systems used [8].

The desire to have more choices and ranges of specific polymer properties has provided the stimulus for other polymers to be considered, among which are natural polymers such as collagen, hyaluronic acid and chitosan. Chitin, the precursor of chitosan, is one of the most abundant natural polymers [9]. Chitin has been shown to enhance biological self-defense function in animals, accelerate wound healing, and be used as a biocompatible and absorbable material in both animal and plant tissues [10]. Calcium hydroxyapatite (HA) is the main component of teeth and bones in vertebrates. Sintered HA has sufficiently good mechanical properties with superior biocompatibility to be utilized as bone and tooth implant materials [11]. Therefore, it is reasonable to propose the use of HA–chitin materials as a prospective candidate to form a structural framework for bone regeneration. In this study, HA–chitin materials were prepared and evaluated for general biocompatibility, and subsequently seeded with mesenchymal stem cells harvested from NZW rabbits and evaluated for their performance as potential bone substitutes.

2. Materials and methods

Calcium HA Captal P120 was purchased from Plasma Biotal, UK. Lithium Chloride (LiCl) and *N*,*N*-dimethylacetamide (DMAc) was obtained from J.T. Baker, USA. Chitin was obtained from Sigma, USA and purified in 5% aqueous sodium hydroxide solution for 1 week and in 5% hydrochloric acid for 1 h. All other chemicals were of reagent grade or better.

HA–chitin materials were fabricated according to the method described by Wan et al. with the exception that for freeze-dried samples, lyophilization replaced air-drying [12]. Briefly, HA (25%, 50% and 75% w/w) was first dispersed in the 5% LiCl/DMAc solvent, followed by the addition of chitin and the mixture stirred in a refrigerated shaking incubator (150 rpm) for about 4 days at 10°C. The well dispersed HA in chitin solution was filtered through glass wool and cast into plastic molds and allowed to stand at ambient for 24–36 h to form gels. The HA–chitin gels were immersed in deionized water (with several changes of water) for 1 week to remove residual LiCl and DMAc. The wet materials were air-dried to give thin chitin films or subjected to dry-ice/acetone (−38°C) freezing for 1/2 h followed by lyophilization (Virtis, Advantage) with the condenser temperature set at −50°C and vacuum maintained at about 0.1 Torr for 24 h to give porous HA–chitin matrixes. The pore size for porous HA–chitin matrixes were estimated from SEM photomicrographs obtained using a JEOL JSM-T220A scanning microscope at an acceleration voltage of 5 kV. A fine coat Ion Sputter JFC-1100 coater was used to gold coat samples.

2.1. Preliminary evaluation

2.1.1. Cell culture studies

Mouse fibroblasts (ATCC CCL-1), human fibroblasts (ATCC CCL-186) and human osteoblasts (ATCC, CRL-427) were used to assess the cytotoxicity of the HA–chitin materials by the MTT (3-[4,5-dimethylthiazolyl-2]-2,5-diphenyl tetrazolium bromide (MTT, BDH, England) colorimetric assay methods. Polyethylene (US Pharmacopia) and latex rubber were used as negative and positive controls, respectively.

For the MTT assay, the test materials were cut from large HA–chitin films into $5 \times 5\,mm^2$ samples and sterilized by autoclave at 121°C for 15 min. For the three cell types, cells were grown in $75\,cm^2$ T-flask containing Minimal Essential Medium (MEM, Gibco BRL) modified to contain 2 mM L-glutamine (Gibco BRL), 0.1 mM non-essential amino acids (Gibco BRL), 1.5 g/l sodium bicarbonate (Gibco BRL) and 10% heat-inactivated fetal bovine serum. A confluent cell monolayer was trypsinized with trypsin-EDTA solution 1:1 (Sigma) in phosphate buffered saline (PBS). Cells were suspended in complete growth medium and the cell suspension centrifuged for 10 min at 4°C, 2000 rpm. The pellet was resuspended with complete growth medium and the cell suspension was dispatched in 4 multiwell plates (Nunclon). Thirty-two wells (four rows) of 4 multiwell dishes were filled with 100 μl of 10^5 cell/ml of cell suspension. The sterilized HA–chitin materials were placed in the wells, one row for each sample type (8 replicates for each type of material) with one row maintained free of sample as control. The 4 plates were incubated at 37°C, 5% CO_2 and the percentage of viable cells was determined after 1, 2, 3 and 4 days of incubation. Twenty microliters of MTT solution (5 mg/ml) was added in each well and after 3 h of incubation at 37°C, 5% CO_2 the wells were emptied and 150 μl of DMSO was added to each well and the optical density at 540 nm wavelength measured to determine the percentage of viable cells.

For the colonization of HA–chitin matrixes the CRL-1427 human bone cell suspension was generated in the

same manner as for the MTT assay. Sterilized HA–chitin matrixes were deposited in the bottom of the wells of a multiwell plate. One hundred microliters of the cell suspension was added to each well to cover the materials and the plates were incubated for 2 weeks at 37°C, CO_2. The growth medium was changed every 2 days. After 2 weeks, the cells were fixed with a 2.5% glutaraldehyde-PBS solution and analyzed with an Environmental Scanning Electron Microscopy (XL30, Philips Electron Optics) under normal SEM conditions i.e. non-wet mode and gold coated, but using ESEM detector at 10 kV, spot size 4.

2.2. Intramuscular implantation

Two animal models were used, Sprague-Dawley rats (150–200 g) and male NZW rabbits (2.5–3 kg) obtained from the Laboratory Animals Center, Lorong Chencharu, Sembawang, Singapore. The Ethics Committee, Animal Holding Unit (AHU) of the National University of Singapore (NUS), approved all animal studies.

For the rat study, the animals were anesthetized with an anesthetic cocktail comprising a 1:1:2 mixture of Dormicum, Hypnorm and water supplied by the AHU, NUS. HA–chitin thin films and matrixes were cut into 6 mm × 6 mm implants and sterilized in an autoclave at 121°C for 15 min. The implants were surgically implanted bilaterally into the dorsal-lumbar musculature. A pocket was created deep in the muscle and the implant inserted. Each animal received two implants, one on either side of the midline. The muscle was sutured with 2/0 resorbable sutures and the wound closed with 3/0 black silk-braided non-absorbable sutures (Mersilk®, Johnson & Johnson). The implantation site was covered with topical antibiotic powder (Apex Laboratories) and a moisture vapor permeable spray dressing (Smith & Nephew) to enhance healing. The animals recovered 2 h postoperatively and were housed singly in cages in a 20°C thermostatically controlled environment. The room was artificially illuminated on a time schedule of 12 h of light and 12 h of darkness. Water and rat feed were readily accessible to the animals. After 3 months, the animals were sacrificed by CO_2 asphixiation. Implants were excised together with surrounding muscle tissues, washed in sterile buffered saline and fixed in 10% aqueous buffered formalin solution. Specimens were embedded in paraffin and cut to thin sections, stained with hematoxylin and eosin (H&E) for observation using light microscopy to evaluate the biological response to HA–chitin materials.

The rabbit study is described in a later section.

2.3. Cell-seeding study

The osteoblast culture media comprised Dulbecco's modified eagles medium (DMEM) (GIBCO BRL 11096-054) supplemented with 10% Fetal Bovine Serum (FBS) (GIBCO BRL 11027-098), 2 mM of L-glutamine (GIBCO BRL 25030-081), 50 μg/ml L-ascorbic acid-2-phosphate sesquimagnesium salt (Sigma A8960), 10 mM/ml of β-glycerophosphate disodium salt (G9891, sigma), 10 nM/l of water-soluble Dexamethasone (Dex) (Sigma D-2915) and 100 IU/ml penicillin containing 100 μg/ml streptomycin (Sigma P3539). Serum free media (SFM) comprised the above with the exclusion of FBS. To establish the osteoblasts from mesenchymal stem cells, bone marrow was aspirated from the iliac crest of male adult NZW rabbits weighing ~2.5 kg obtained from the Laboratory Animal Center, NUS. The aspirated fluid was centrifuged (Jouan MR22i) at 1500 rpm for 5 min and the adipocyte and fatty tissues containing supernatant was carefully removed. The remaining cell layer comprising mainly hematopoietic and stromal cells were carefully suspended with SFM to a volume of 20 ml and centrifuged at 2000 rpm for 4 min. The top layer containing fatty tissue was again removed and new SFM media added to retain the volume at 20 ml. The centrifuging and SFM rinsing was repeated 2–3 times until no visible fat was observed. The MSC layer was transferred into 75 cm^2 culture flasks at a density of 5×10^4 cells/cm^2 and replenished to 20 ml with osteoblast culture medium and cultured in an incubator at 37°C with 5% CO_2. The first medium exchange was delayed until day 7. After 10–14 days, 80% of confluence was attained and the cell layer was trypsinized with trypsin-EDTA (GIBCO BRL 25200-056).

Porous HA–chitin matrixes were cut into round pieces (*D*: 8 mm; *t*: 2 mm) and sterilized by λ-irradiation (2.5 Mrad delivered over 50 h). Each matrix was transferred into a labeled well of a 96-well plate and pressed gently to fit the well. One hundred microliters of 10^5 cells/ml of the MSC generated osteoblasts suspension was pipetted into each well containing the matrix. After 24 h the medium was changed. Twenty microliters of the supernatant was retrieved and examined for osteoblast activity by determining the alkaline phosphatase (Alkphase-B 8012, Metra Biosystems, USA) and osteocalcin (NovoCalcin 8002, Metra biosystems, USA) activities after 48 h incubation.

2.4. Green fluorescence protein (GFP)-transfection of mesenchymal stem cells (MSC)

The primary MSCs were transfected with GFP as cell marker using CalPhos™ Mammalian Transfection Kit (K'2051-1, clontach, CA, USA) performed according to user manual. Briefly, solution A (3 μl of plasmid DNA, 12.4 μl of 2 M of calcium solution and sterile water in 100 μl volume) was added to solution B (100 μl of Hank's balanced salt solution) dropwise under slow vortexing and incubated at room temperature for 20 min. Two hundred microliters of the combined

solution was added dropwise to the 35 mm tissue plate with 80% confluence of MSCs. After 10 h incubation, the calcium phosphate-containing medium was replaced with 2 ml of fresh complete osteoblast growth medium and incubated at 37°C until 80% confluence. The GFP-transfected MSCs were trypsinized and statically loaded on matrixes.

2.5. Cell loading onto HA–chitin matrixes for morphological study

HA–chitin matrixes ($4 \times 4 \times 4\,mm^3$) were sterilized by λ irradiation and placed in 96-well plates and pre-wetted with two changes of ethanol and phosphate buffered saline (PBS). MSCs cell suspensions (30 μl) (passage 1, 1.4×10^7 cells/ml) were loaded twice onto each matrix within a 20 min interval and incubated in a 5% CO_2 incubator at 37°C for 3 h after which, 1 ml of medium was added to each well. The medium was changed every 3 days. After predetermined time intervals (1, 2, 3 and 4 weeks), the cell-loaded matrixes were rinsed with PBS and immersed in 10% buffered formalin (Sigma). All samples were processed routinely for histological examination at the Department of pathology, NUS.

SEM was also performed on the 1-week culture samples (JEOL JSM-T220A scanning electron microscope at an acceleration voltage of 5 kV). Critical point drying (CPD) was used to prepare these cell-loaded matrixes for SEM examination. Briefly, the cell-loaded matrixes were prefixed with 2.5% of glutaraldehyde and 2% of formalin at 4–10°C for 2–3 h and washed with cacodylate buffer (pH 7.23, 300 mOs m/l). After dehydration with alcohol in a graded increase of concentrations, CPD was performed using a Tousimis SAMDRI-780 CPD apparatus with CO_2 as the cortical fluid. The alcohol-exchanged specimens were placed in the CPD chamber with the intermediate fluid. The chamber was closed, cooled to 0°C and liquid CO_2 was allowed to fill the chamber. The temperature was maintained between 0°C and 10°C for 15 min, after which the chamber was drained of CO_2. The empty chamber was refilled immediately with CO_2 and drained again after 1/2 h. This process of filling and purging with CO_2 was repeated twice. The chamber was heated to 42°C in 7 min and maintained at this temperature for 4 min. The pressure was subsequently reduced at a rate of about 50 psi per 1/2 min, to give the critical point dried samples.

2.6. Implantation

Cell-loaded HA–chitin matrixes after 1-week's culture were rinsed thoroughly with PBS to remove fetal bovine serum and made ready for implantation. Adult male New Zealand White rabbits (2.5–3 kg) were administered general anesthesia with hypnorm (0.5 ml/kg intramuscularly) and valium (2 mg/kg intravenously), shaved, and the operation site scrubbed with 75% ethanol twice. A 5-cm longitudinal incision was made at the lateral side of the right thigh with the rabbit prone. The *vastus lateralis* and *biceps femoris* were separated bluntly and the insertion of *Glutaeus Maximus* in the third trochanter was peeled off. A 4 mm diameter round bone defect was made in the cortical bone near the third trochanter with a 4 mm drill bit (B. Braun, Germany). The cell-loaded matrix was press-fitted into the bone defect gently with cell-free matrix as control. The incision was closed in two layers; the fascia was closed with interrupted 4-0 cat-gut (B. Braun, Germany) and the skin was closed with interrupted 3-0 silkam (B. Braun, Germany). The rabbits were not immobilized post-operatively and the animals allowed free cage activity. Rabbits were euthanized after 2 months by CO_2 asphyxia. The proximal part of the femur with the implant was explanted without surrounding tissue for histological examination.

2.7. Confocal microscopy of GFP-transfected MSCs explants

The GFP-transfected MSCs were loaded onto the matrixes, cultured and implanted as described above. After 2 months implantation, the explants were retrieved and decalcified with decalcifier II (Hydrochloric acid, EDTA water, Surgipath, USA) for 7 days and fixed in 70% ethanol for 24 h and cryo-cut with crypstat (Leica CM3050S Germany) to 10 μm slice and embedded with cover slips. The slides were examined with a confocal microscope (Leica) at 488 nm.

3. Results and discussion

The development of good biodegradable polymers to perform the role of a temporary matrix is an important factor in the success of cell transplantation therapy. The objective of this study was to evaluate HA–chitin materials as prospective candidate for tissue engineered bone applications by providing the environment for osteoblasts to attach, migrate, proliferate and differentiate. The approach focused on first assuring that no cytotoxicity was imparted in the materials preparation process followed by evaluating the suitability of porous HA–chitin matrixes to permit the migration and growth of host cells in the matrixes that on implantation promotes tissue regeneration.

3.1. Preliminary evaluation

Cell culture tests are a starting point to establish that materials are non-cytotoxic [13]. The tetrazolium-based colorimetric assay (MTT test) is a quantitative assay

method for the biological response of cells to a foreign body while the direct contact test permits qualitative assessment of the osteoblasts' response after exposure to HA–chitin materials [14,15]. The in vitro study was augmented by an in vivo study using the rat model to investigate the suitability of HA–chitin as a biomaterial in vivo.

Table 1 summarizes the overall results of MTT assay with HA–chitin materials. The percentage of viable cells after exposure to various HA–chitin thin-films and matrixes were above 80% in most instances throughout the 4 days of the experiment based on the percentage of viable cell referenced to 100% for the control. The 20% difference with the control was attributed to occupancy of some of the well space by the sample. Therefore, the HA–chitin materials were non-cytotoxic to the mouse fibroblast, human fibroblast or human bone cells.

Direct contact tests give an idea of the response of a cell layer to the contact with the HA–chitin materials. After 3 days of culture, the CRL-1427 osteoblasts started to adhere and attach as isolated cells onto the surface of the HA–chitin thin-films. After 14 days of culture, cells had started to proliferate on the surface of the matrixes, therefore forming clusters of cells as shown in Fig. 1. The ability of the cells to proliferate on the surface of the HA–chitin indicated that the support provided by HA–chitin did not alter the biological activity of the osteoblasts as they grew normally and retained their characteristics. In contrast, non-observance of live cells were obtained for the negative and positive controls.

For the preliminary intramuscular implantation evaluation, after 14 days, a dense and thick fibrous capsule surrounded the HA–chitin thin-film explants (Fig. 2A). The scar tissue was mainly made of fibroblasts that had multiplied upon wound repair with newly formed capillaries. Macrophages and multinucleated giant cells of macrophage origin were present in the vicinity of the HA–chitin thin-film that were slightly degraded and starting to loosen. The paucity of leukocytes, lymphocytes, plasma cells and eosinophils was evidenced as a mild inflammatory response with no allergic response detected. A slight implant calcification was observed (Fig. 2B), either at the periphery or in the middle of the materials. While calcification in soft tissue is normally not a good sign, it must be noted that the HA–chitin was developed to promote calcification, desirable at the intended implant sites.

The results after 14 days of intramuscular implantation indicated that HA–chitin thin-film did not induce any acute inflammatory response in the animal model. These results justified a further implantation study over 90 days to evaluate the biological behavior of HA–chitin materials on a longer-term basis. In addition to HA–chitin thin-films, porous HA–chitin matrixes were also implanted.

After 90 days, the thickness of the fibrous capsule around the HA–chitin materials had decreased (Fig. 2C), due to the organization and the maturation of the fibrous scarring process. The scar tissue still comprised fibroblasts, macrophages and giant cells. Hence, the inflammatory response, which had subsisted after 3 months, had remained mild. The degradation degree was advanced, the implants being completely loosened in comparison to the chitin control and the HA–chitin sample did not form a cohesive matrix any longer with the HA particles released (Fig. 2C). Vascularized tissue in-growth was also noticed for explant sites that had the porous HA–chitin matrix (Fig. 2D). For some explant sites, the in-growth was so advanced that small parts of

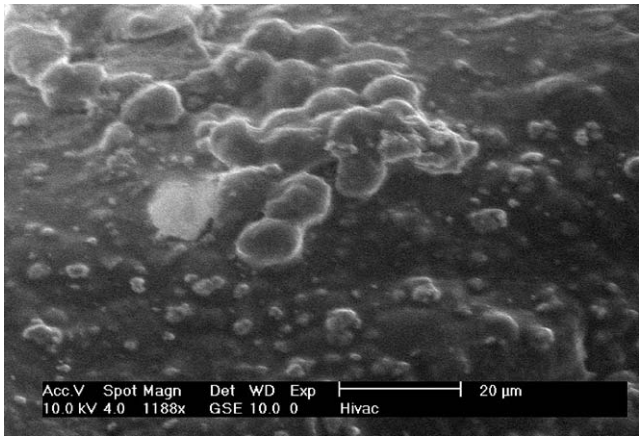

Fig. 1. SEM photomicrograph of osteoblasts proliferating on the surface of a 25% HA–chitin thin-film after 14 days of culture.

Table 1
Percentage of cell viability after 4 days of exposure to HA–chitin materials

Sample	Cell lines		
	Mouse fibroblasts CCL-1 (%)	Human fibroblasts CCL-186 (%)	Human osteoblasts CRL-1427 (%)
25% HA–chitin thin-film	93±6	97±3	87±7
50% HA–chitin thin-film	95±6	94±5	81±6
75% HA–chitin thin-film	106±2	97±4	79±9
25% HA–chitin matrix	84±8	97±12	92±7
50% HA–chitin matrix	76±3	93±10	77±8
75% HA–chitin matrix	85±3	96±9	93±11

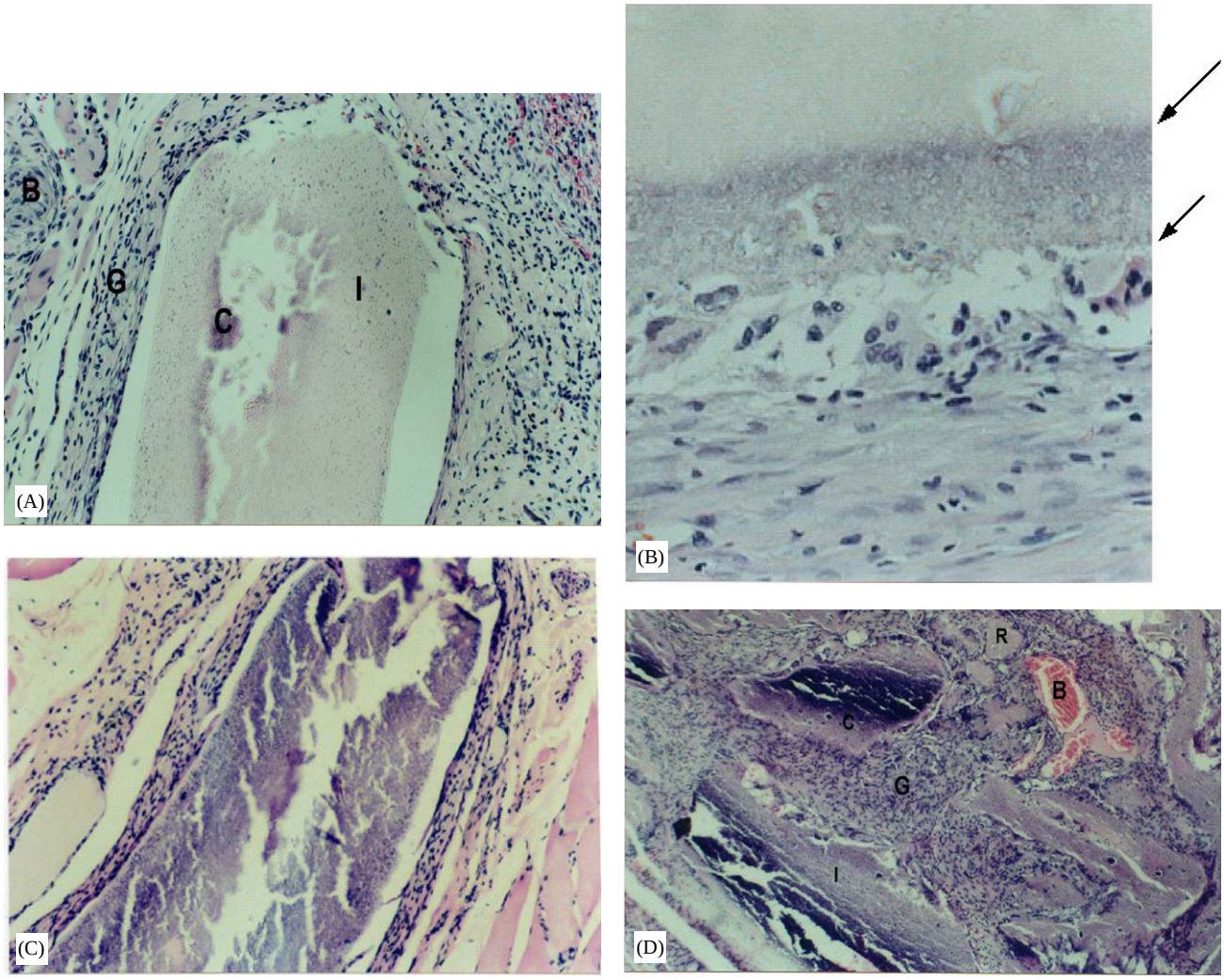

Fig. 2. Morphology of the muscle tissue around HA–chitin implants after 14 days of implantation (Rodent model). (A) 25% HA–chitin sample (100 ×) showing normal fibrous connective tissue (G) and newly formed capillaries filled with red blood cells (B). Calcification (C) is observed in the middle of the implant (I). (B) Interface between a 50% HA–chitin sample (top) and the scar tissue (bottom) (200 ×). A zone of high-calcium concentration is evident by the purple staining of the sample. (C) 75% HA–chitin sample after 3 months implantation showing the breakdown of the implant. (D) Resorption of a 50% HA–chitin matrix after 3 months of implantation. The material was invaded and divided by the granulation tissue (G), leading to the production of remnants (R) of various shapes detached from the main body of the implant (I) (40 ×).

the porous HA–chitin matrix had already been separated from the main body of the implant, forming small remnants inside the scar tissue. In comparison to the HA–chitin thin-films, the tissue in-growth into the porous HA–chitin matrix allowed a faster resorption of the porous HA–chitin matrixes. This can be explained by an increase of material surface in contact with phagocytic cells due to the porous nature of the porous HA–chitin matrixes.

The promotion of further calcification by the presence of HA in the HA–chitin thin-films and porous matrixes supports previous observations that the presence of HA nucleators enhanced further accumulation of calcium, due to low-activation-energy pathways provided by the presence of HA particles [16]. In summary, the HA–chitin materials were found to be non-toxic, promote calcification and degraded in vivo and can be good candidates for temporary bone healing devices.

It is noted that the degradation of the chitin-HA materials in the preliminary study was markedly rapid and could present a dilemma commonly encountered between maintaining strong mechanical properties against rapid resorbability [17]. This could be a potential limitation in the application of HA–chitin materials to load bearing situations. Therefore, it is apparent that these HA–chitin materials would find utility in non-load bearing situations, such as a bone substitute exemplified by the following cell-seeding work where the porous nature of HA–chitin matrix were required for cell proliferation into the matrix bulk that is necessary for tissue engineering applications.

3.2. Cell-seeding onto porous HA–chitin matrix

The importance of preparing the porous matrix right is of importance for cell-seeding and its subsequent in vivo performance and is elaborated briefly. In this work, lyophilization was used to fabricate porous HA–chitin matrixes having pore size ranging from 200 to 400 μm with a porosity of 69% as previously reported [12]. The presence of HA in chitin does not appear to affect the final quality or the pore sizes of the matrix. Fig. 3 shows a scanning electron photomicrograph (SEM) depicting the porous nature of the HA–chitin matrix. One of the criteria for an ideal matrix is for the porosity to be at least 90% to provide a high surface area for cell-polymer interactions, sufficient space for extracellular matrix (ECM) regeneration, and minimal diffusion constraints during in vitro culture. Mooney et al have commented on the advantage of a construct having a large surface area to volume ratio to provide a conduit for uniform cell delivery to give high cell density with the optimal pore size depending on the intended application of the matrix [18]. Compared with PLA/PGA matrixes presently used, the porosity of 69% for the HA–chitin matrixes is below the ideal criteria but may be advantageous as slow degradation is better facilitated, and the maintenance of the original construct shape can be prolonged over a longer regeneration period. Even though differences in matrix pore size within a range of 150–710 μm do not significantly affect osteoblast proliferation or function in vitro it has been suggested that the pore-size range of 200–400 μm is preferred by osteoblasts because it provides the optimum compression and tension on the osteoblast's mechanoreceptors [19]. Therefore, the HA–chitin matrixes produced in this study meets this requirement well.

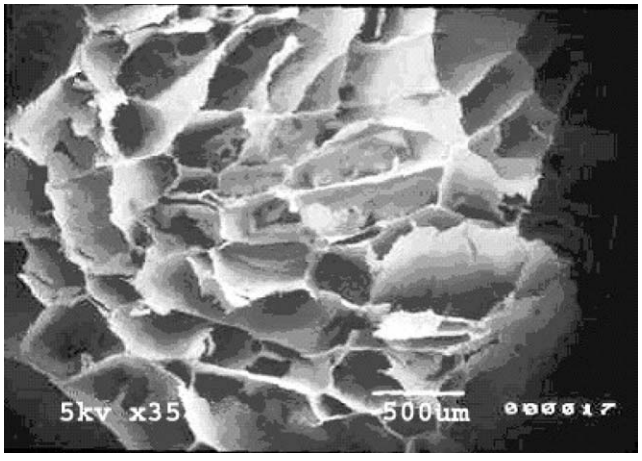

Fig. 3. SEM photomicrograph showing the porous structure of the HA–chitin matrix.

3.3. Induction of osteblasts from mesenchymal stem cells

Four maturational states in osteoblast development have been identified for bone in situ, the preosteoblast, osteoblast, osteocyte and bone-lining cell. Osteoblasts are postproliferative, cuboidal, strongly alkaline phosphatase-positive cells lining bone matrix at sites of active matrix production. The mature osteoblast phenotype is characterized by their ability to synthesize membrane-associated alkaline phosphatase, bone matrix molecules including collagen type I and non-collagenous proteins such as osteocalcin, bone sialoprotein and osteopontin, proteoglycans, hormonal and other growth factor receptors [20]. Osteocalcin is widely recognized as a bone-specific protein or an exclusive product of the osteoblast/osteocyte. It accounts for 10–20% of the non-collagenous protein in bone. While the in vivo function of osteocalcin is unknown, its affinity for bone mineral constituents implies a role in bone formation. Osteocalcin appears coincident with the onset or only after the onset of mineralization. In this work, alkaline phosphatase (AP) and osteocalcin were used as indicative markers for osteoblasts.

The MSCs were first induced to osteoblasts with dexamethasone in vitro [21]. The induced osteoblasts showed typical cuboidal and basophilic appearance under Masson stain (Fig. 4). The measured alkaline phosphatase activities of 12.4 ± 0.3 for MSCs indicate that inducement to osteoblasts was successful.

3.4. Seeding of osteoblasts onto porous HA–chitin matrixes

Osteoblasts were seeded onto porous HA–chitin matrixes using a static seeding method to verify that they would adhere to these matrixes. Histological

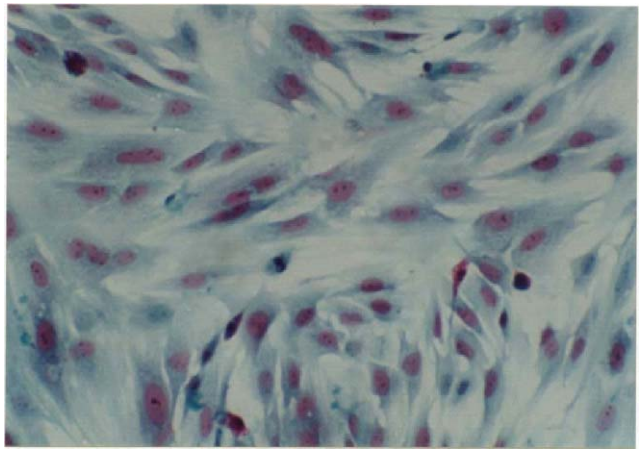

Fig. 4. Photomicrograph showing osteoblast cells derived from mesenchymal stem cells (MSC) of New Zealand White rabbit model (Masson staining, 200 ×).

sections indicated that cells were adhered, spread, and formed a monolayer on the surfaces of the matrixes, confirming that cell proliferation occurred. After 2 weeks, there were more and larger clusters of cells in the matrixes that subsequently proliferated to form a three-dimensional like tissue. Most of the cells had organized into single layers within the matrixes although not all spaces were covered. The efficiency of cell adhesion to matrixes was not examined, but was estimated via histological assessment to be around 50%. Cells located at the innermost of the clusters usually did not survive.

Table 2 summarizes the alkaline phosphatase and osteocalcin activities after loading onto the HA–chitin matrixes. For alkaline phosphatase, the levels were found to be comparable to the MSC reference, although a slight decrease was observed for the 50% HA–chitin matrix. These results indicate that the loaded osteoblasts retained their phenotype and were able to proliferate in the porous HA–chitin matrix [22]. The osteocalcin levels on the other hand were approximately 1/3 lower for all HA–chitin matrixes, with a similar slight dip for the 50% HA–chitin matrix. This may be a consequence of the constraining effects of the HA–chitin matrix in the flow of materials in and out of the matrix bulk or accelerated proliferation of osteoblasts that led to an increased amount of premature osteoblasts. Overall, the detection of osteocalcin activity implied that the osteoblasts retained the properties of bone formation and mineralization.

The optimal matrix should support the movement, proliferation and differentiation of specific cells. From the results, the porous HA–chitin matrixes appeared to provide a good environment for osteoblasts. However, after 2 weeks' culture in vitro, although more cell clusters were found, necrosis of cells in the inner regions of the matrix was observed. This could be attributed to the static incubation system employed that did not promote homogeneous cell distribution throughout the matrixes. Furthermore, when cell clusters formed, the diffusion of nutrients to remote inner regions of the matrix and that of waste products in the counter direction became difficult, limiting cell survival. The loading efficiency of cells onto HA–chitin matrixes was visually estimated to be about 50%, similar to other reports using polymer matrixes. Even though an attempt to improve the cell loading efficiency was made by using a higher density of cell loading (1.4×10^7 cell/ml), the loaded cells did not necessarily use all available space in the matrixes. The higher density of cell suspension rendered it too viscous to distribute well in the matrixes. It is reasonable to surmise that the present porosity found in these HA–chitin matrixes supported enough cell proliferation.

Table 2
Alkaline phosphatase and osteocalcin activities for cell-seeded HA–chitin matrixes after 1 week

Sample	Alkaline phosphatase activity (U/ml)	Osteocalcin activity (U/ml)
Mesenchymal stem cells reference	12.4±0.3	15.7±2.0
25% HA–chitin matrix	12.6±0.3	9.5±0.4
50% HA–chitin matrix	11.4±0.4	9.3±0.3
75% HA–chitin matrix	12.7±0.6	9.8±0.3

3.5. *In vivo model studies*

There are 3 general methods for in vivo osteoinductiveness. These are the segmental or calvarial bone defect; subcutaneous implantation of demineralized bone matrix/material and the diffusion chamber implantation model. In this study, the bone defect models in femurs of NZW rabbits were chosen to evaluate the osteoconductiveness [23].

After 2 months' implantation, the inflammatory response was mild as testified by the paucity of inflammatory cells (leukocytes, lymphocytes, plasma cells and eosinophils). New tissue had grown into the matrix residues and was more prominent for cell-loaded matrixes (Fig. 5) compared to cell-free matrixes where most of the in-growth tissues were dominated by macrophages (Fig. 6). The foreign body responses were similar, regardless of the HA-content of the HA–chitin matrixes. There was a creeping substitution of the implant by the in-growth of granulation and macrophage tissues. For porous matrixes, the degradation rate was accelerated because the surface of materials in contact with the phagocytic cells and body fluid increased. The presence of newly formed capillaries and osteoids around the degraded matrixes indicated

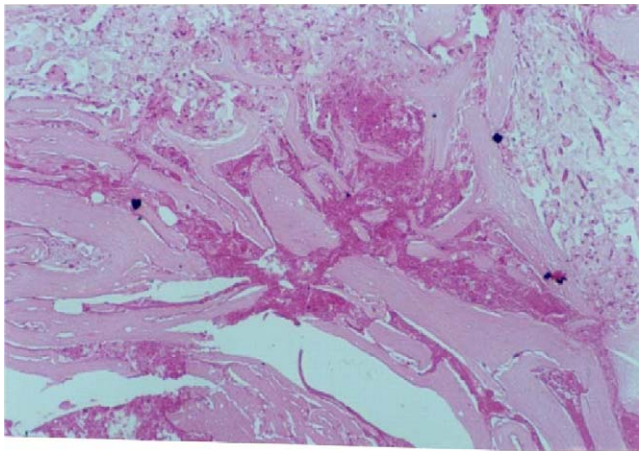

Fig. 5. Photomicrograph of histological section of explant of MSC induced osteoblast loaded porous HA–chitin scaffold after 2 months implantation (Rabbit femur model).

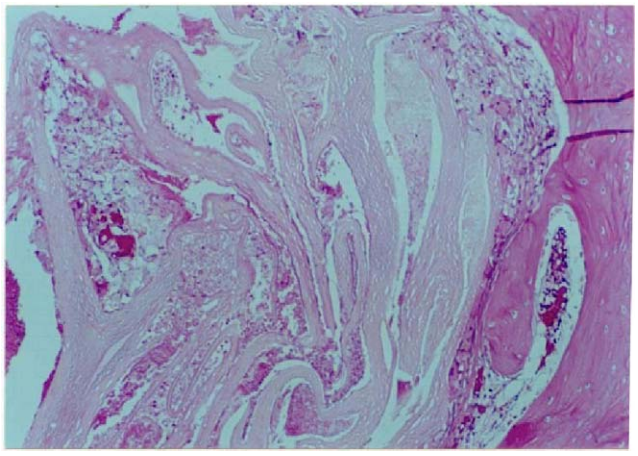

Fig. 6. Photomicrograph of histological section of explant of cell-free porous HA–chitin matrix after 2 months implantation (Rabbit femur model).

that the HA–chitin matrixes did not interfere with normal bone regeneration.

The results from the 2 months' implantation suggest that both cell-free and cell-loaded porous HA–chitin matrixes promoted the in-growth of surrounding tissues, with the cell-loaded HA–chitin matrixes being the better performer. The in-growth tissues were composed mainly of macrophages and osteoblasts and the matrixes had begun to lose their original porous structure, with slivers of the degrading matrix surrounded mostly by macrophages and fibrous tissues. Surrounding osteoprogenitors and osteoblasts invaded the matrix forming new osteoids adjacent to the in-growth of macrophages. The results from GFP-transfected MSC induced osteoblast loaded matrixes showed that the osteoblast did not only survive and migrate when implanted in vivo, but continually proliferated in the matrixes or degraded matrixes as evidenced by the strong green fluorescence observed under the confocal microscope.

The past 30 years has seen a staggering array of biomaterials proposed as "ideal" scaffolds for cell growth yet few have reached clinical efficacy. Regardless of the source or type of biomaterials, i.e. permanent or biodegradable, naturally occurring or synthetic, they have to be biocompatible, ideally osteoinductive, osteoconductive, integrative, and mechanically compatible with native bone to fulfill their desired role in bone tissue engineering. These materials must provide cell anchorage sites, mechanical stability, and structural guidance and within an in vivo milieu, provide the interface to respond to physiological and biological changes and to remodel the ECM in order to integrate with the surrounding native tissue [24]. We have demonstrated that the HA–chitin matrixes developed here can fulfill many of the requirements required of prospective candidates for bone tissue engineering.

Acknowledgements

The authors are grateful to the National University of Singapore and National Science and Technology Board, Singapore, for financial sponsorship (R-143-000-014-112). GZ and SB are recipients of National University of Singapore graduate scholarships.

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