Multichannel mould processing of 3D structures from microporous coralline hydroxyapatite granules and chitosan support materials for guided tissue regeneration/engineering

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A three-dimensional composite material was produced from microporous coralline origin hydroxyapatite (HA) microgranules, chitosan fibers and chitosan membrane. Cylindrical HA microgranules were oriented along channel direction within multichannel mould space and aligned particles were supported with fibers and a chitosan membrane. The positive replica of mould channels was clasp fixed to produce thicker scaffolds. Light microphotographs of the developed complex structure showed good adhesion between the HA particles, the fibers and the supporting membrane. The composite material showed 88% (w/w) swelling in one hour and preserved the complex structure of the original material upon long-term incubation in physiological medium. MEM extract test of HA chitosan complex showed no cell growth inhibition and cell viability assay (MTS) indicated over 90% cell viability.

1. Introduction

The new and rapidly developing tissue engineering field is requiring novel processing methods and designs of scaffold and membranes for guided tissue regeneration. The scaffolds have been fabricated using conventional techniques such as fiber bonding, solvent casting, particulate leaching, membrane lamination and melt moulding [1–4]. The disadvantage of the above technologies includes the intensive fabrication process, the limitation to thin structures, and residual particles in the polymer matrix, irregularly shaped pores, and insufficient interconnectivity. Recently, rapid protyping technologies such as fused deposition modelling, laminated object manufacturing, three dimensional (3D) printing and 3D plotting have also been applied to process biodegradable and bioresorbable materials with controllable and reproducible porosity and well-defined 3D microstructures [5–8].

Marine skeletal material is usually made of $CaCO_3$ in the crystalline form of calcite and aragonite and silicated SiO_2 materials combined with $Mg(OH)_2$. Organisms from different species of corals and hydrocorals have a wide variety of skeletal architectures and crystalline structures [9]. Coralline carbonate derivatives, mostly coralline hydroxyapatite, are currently being used mostly as bone fillers or nonweight-bearing areas and orbital implants [10]. The internal structure of some coral species is very similar to the porous structure of bone and replica of porous calcium carbonate can be converted to highly biocompatible and osteogenic tricalcium phoshate and/or hydroyapatite by a hydrothermal process [11, 12].

Chitin and chitosan are biologically renewable, biodegradable, biocompatible, non-toxic and biofunctional [13]. The use of chitosan together with bioceramics such as calcium phosphate as a scaffold material for tissue engineering applications has been reported previously by a number of researchers [14–16]. The incorporation of bioceramics has been shown to increase osteoconductivity and improved mechanical strength greatly [17–18].

Chitosan fibers, which can be easily extended into 3D structures of woven, knitted and nonwoven are widely used in a variety of biomedical applications, including sutures, wound dressing, and artificial hair [19, 20]. This interest is mainly derived from the fact that chitosan is a natural polymer that shows well-known wound acceleration ability and a biological aptitude to stimulate cell proliferation. Depending on the processing conditions, chitosan fibers can present good mechanical properties. Several authors have described the process of improving the mechanical strength of fibers from chitosan [21–23]. Adequate mechanical properties can make chitosan

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based materials suitable for the developing of tissue engineering scaffolds that can be used for bone or cartilage regeneration.

The aim of this study is the development of a composite material from coralline origin hydroxyapatite (HA) particles and chitosan fibers for use in tissue engineering or in guided tissue regeneration. By orienting the cylindrical coral particles (microchannels run parallel along the particle length) in one direction, an anisotropic material similar to biologic structure of bone can be produced. The bone forming cells osteocytes are known to be arranged in concentric layers called lamellae. In turn, lamellae surround central microscopic channels known as haversian canals, through which run capillaries and nerves. Haversian canals and surrounding lamellar structure are oriented parallel to bone structure. It is believed that a scaffold structure mimicking these arrangements can provide a guided bone ingrowth and correct vascularisation of biomaterial inside the body.

Since coralline origin HA granules are prone to damage from mechanical stirring or harsh handling procedure, a new processing method was suggested in order to blend cylinder shaped coralline HA particles with chitosan fibers. In this method, semi-cylinder parallel channels (1 mm in width and deep) were prepared in a mould for holding and orienting the particles in one direction. This composite material was reinforced with chitosan fibers along and right angle to channels and finally, a membrane was used to cover the material in the mould. After obtaining a replica of mould channels over the membrane, thicker scaffolds can be produced by physical clamp fixing of subsequent replicas of coralline HA to each other.

2. Materials and methods

Chitosan (deacetylation degree 87%), acetic acid, and sodium hydroxide were obtained from Sigma Chemical Co. (St. Louis, USA). Methanol was obtained from Merck Chemical Co. (Germany). Sodium sulphate was obtained from Aldrich Chemical Co. All the other chemicals used were of analytical grade.

2.1. Preparation of coralline origin HA reinforced composite material

2.1.1. Chitosan fiber preparation

Chitosan fibers were produced as previously reported [24]. In brief, chitosan was dissolved in aq. 2% (v/v) acetic acid solution in 5% (w/v) concentration by stirring magnetically at room temperature overnight. Methanol was added to dilute the viscous solution for easy injection until reaching 3% (w/v) final concentration. Glycerol was used as a plasticiser (2.5% (w/w)). After filtration with a cloth filter, solution was placed in an ultrasonic bath to remove the air bubbles. The solution was allowed to remain at room temperature overnight for aging. The clear solution was injected into a coagulation bath at 40 °C, (30% 1 N Na_2SO_4, 10% 1 N NaOH and 60% distilled water). The formed fibers were kept in this coagulation medium for one day and then washed several times with distilled water. They were suspended in aqueous 30% methanol for 4–5 h and then in aqueous 50% methanol overnight. The chitosan filaments were wound on the cylindrical support and the fibers were dried at room for one day.

2.1.2. Chitosan membrane preparation

Chitosan membranes were made by a simple solvent casting technique. The chitosan was dissolved in aqueous acetic acid solution (1% (w/v)), and then was casted onto a plastic petri dishes in a ratio of 0.25 g/cm^2. Membranes were then allowed to dry slowly in an incubator at 37 °C for 3 days.

2.1.3. Assembly of coralline origin HA granules, chitosan fibers, and membrane

A chitosan–coralline origin HA slurry was obtained by careful mixing chitosan solution (3%, (w/v)) with prewetted HA microgranules in 1/1 ratio (w/w). The slurry was then placed into channel spaces by means of a gentle smear process (Fig. 1).

Over the coralline origin HA filled spaces, chitosan fibers were aligned as one set parallel and another set right angle to channel direction to produce a chess like pattern (Fig. 2). Finally, a chitosan membrane was placed over the coralline origin HA particles and the fiber network. In order to provide good adhesion between membrane and wet particles, a weight was applied over the membrane and let to act overnight. The assembly with the mould was placed to an oven at 37 °C to dry the material. Special care was given to peel off the composite material from the mould. Released material was stored in desiccator until further assembly.

2.2. Cytotoxicity assays

The cytotoxicity of leachables of the tested material was evaluated by using cell culture methods, namely short MEM (minimum essential medium) extraction test (72 h), according to ISO/EN 109935 guidelines [25].

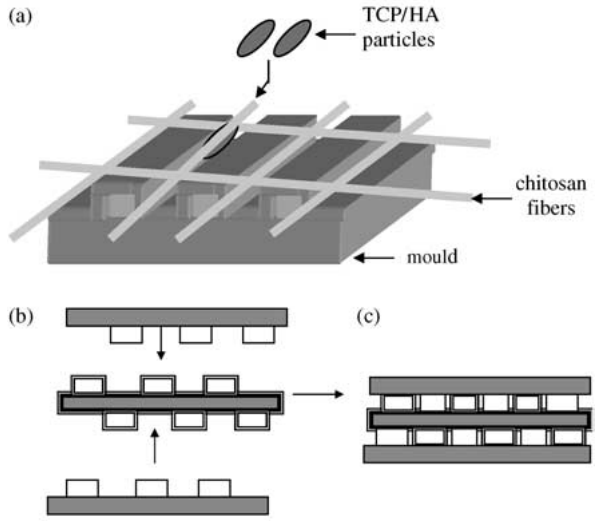

Figure 1 Schematic representation of the preparation method of coralline origin HA-chitosan fiber composite using a multichannel mould (a); Principle of production of thicker scaffolds by clasp fixing of HA replica complexes (b) and (c).

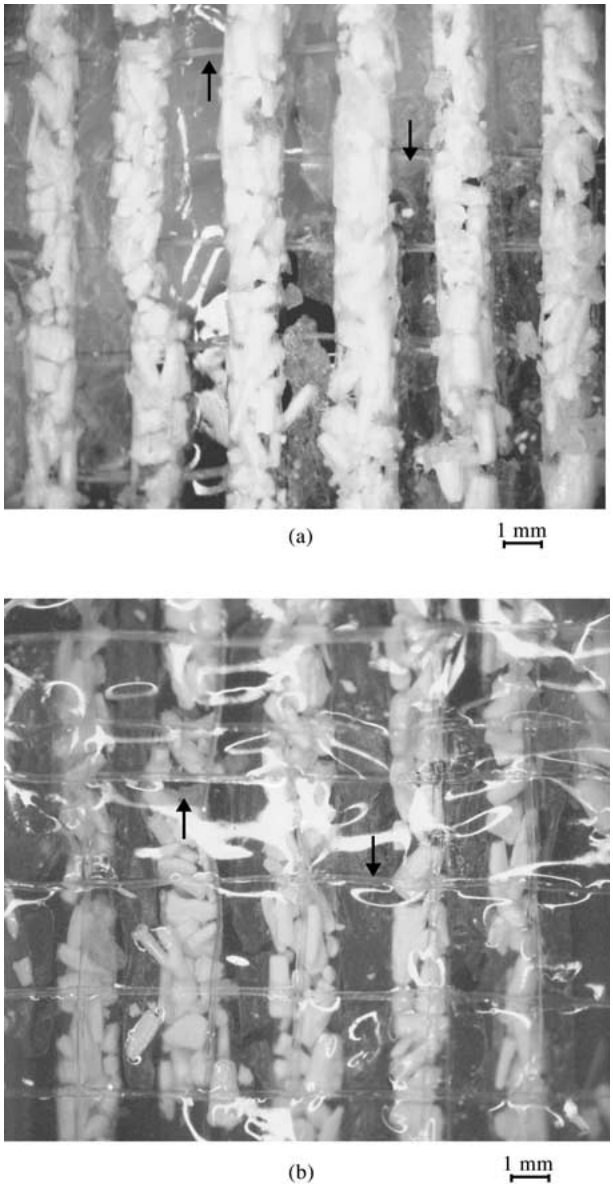

Figure 2 Light transmission microphotographs of HA granules (white cylinder bodies) and chitosan fibers (indicated by arrows) over chitosan membrane. Front view of complex (a) and back view of complex (b).

2.2.1. Cell culture

A cell line of mouse lung fibroblasts (L929) was selected for all cytotoxicity assays. Cells were grown in Dulbecco's modified eagle medium (DMEM, Sigma), supplemented with 10% of foetal bovine serum (Biochrome) and 1% of antibiotics/antimicotics solution (Sigma) until they reach confluency. Twenty-four hours before the cytotoxicity tests start, cells were trypsinized and seeded $(n = 3$ per tested condition$)$ on 24 and 96 well plates using a density of 7×10^4cells/cm^2.

2.2.2. MEM extraction test (72 h)

The materials $(n = 3)$ were incubated in 10 ml of complete culture medium (2.5 cm^2/ml) for 24 h at 37 °C under constant agitation. In the end of the 24 h the extraction fluids were filtered (45 μm of pore size).

2.2.2.1. Morphological evaluation. For morphological evaluation, culture medium present in the 24 well plates was replaced by 2 ml of extraction fluid and incubated for 72 h at 37 °C in a humidified atmosphere containing 5% of CO_2. After 72 h the reaction of the cells to the extracts was evaluated by means of light microscopy and compared to the negative control (cells incubated with standard culture medium) and positive control (latex rubber). A score for confluency of the monolayer, degree of floating cells and change of cellular morphology was then calculated based on the values posted on Table I. Cells were also trypsinised, the percentage of growth inhibition was determined through trypan blue exclusion test and a score was obtained after correction for the value of positive and negative controls. The scores were then combined (four parameters have equal weight) resulting in a final cytotoxic response index ranging from 0 to 8 (Table II).

2.2.2.2. Cell viability assay. After 24 h of cell seeding culture medium was replaced by 200 μl of extraction fluid and incubated for 72 h at 37 °C in a humidified atmosphere containing 5% of CO_2. After 72 h cell viability was assessed by using Cell Titer 96® One solution Cell proliferation Assay kit (Promega, USA). This test is based on the bioreduction of the substrate, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H tetrazolium) (MTS), into a brown formazan product by NADPH or NADP produced by dehydrogenase enzymes in metabolically active cells [26, 27]. For the assay to occur extraction fluids were removed and 100 μl of a mixture of culture medium without FBS and MTS in 5 : 1 ratio was added into each well. Cells were then incubated for three hours at 37 °C in a humidified atmosphere containing 5% of CO_2, in the end of which optical density was read at 490 nm in a plate reader (Molecular Devices, USA). Results are plotted as percentage of negative control.

2.3. Swelling test

In order to test the swelling behaviour, the scaffold was cut into five small pieces and each piece was immersed in 0.154 M NaCl aqueous isotonic saline solution (pH 7.4). The samples were taken out from solution at various time intervals being weighted with an analytical balance. The experiment was continued until reaching of an equilibrium value.

TABLE I Quantitative and qualitative scores used in the cytotoxicity tests

Score	Confluency (%)	Floating cells (%)	Change of cellular morphology	Inhibition of cell growth (%)
0	100	0	No changes during test period	0–10
1	90–100	0–5	Slight changes, few cells affected	10–30
2	60–90	5–10	Mild changes, some cells round/spindle shaped	30–50
3	30–60	10–20	Moderate changes, many cells round/spindle	50–70
4	0–30	> 20	Severe changes, about all cells show morphological changes	70–100

TABLE II Cytotoxicity index

Cytotoxicity index	Reactivity
0–1	None
1–3	Slightly toxic
3–5	Mildly toxic
5–7	Moderately toxic
7–8	Severely toxic

The swelling ratio of the samples was calculated according to the following equation:

$$\%S = (m_w - m_i)/m_i \times 100 \quad (1)$$

where $\%S$ is the percentage of swelling, m_w is the weight of the wet sample after immersion in the NaCl solution. Average values were used to determine the swelling ratio of scaffold for each of the time points.

3. Results and discussion

3.1. Coralline origin HA/chitosan composite material preparation

Light transmission microscopy analysis of the composite material showed that most of coralline origin HA cylinder bodies were aligned along channel direction. Chitosan microfibers are seen to be running along coralline origin HA granules and making chess structure between coralline origin HA replica and chitosan membrane (Fig. 2(b)). Chitosan membrane was observed to hold both granules and fibers securely. The strength of composite materials can be increased greatly with the incorporation of fibers. In this respect, the coralline origin HA filled channels were supported by parallel chitosan fibers. A chess like structure was produced, so that the load can be distributed evenly over the complex.

The parallel distribution of coralline origin HA particles is especially important because, fine microchannel architecture of the cylindrical structures align in longitudinal direction of ceramic structure. By that way, highly oriented channel networks can be created through the composite material. This pattern would mimic the haversian network of natural bone and as a result, a correct remodelling of biomaterial by infiltration of bone tissue and extensive vascularisation at implantation site can be expected. This will be analysed in future *in vivo* studies.

3.2. Cytotoxicity assays

With regard to short-term MEM extract test there was no cell growth inhibition detected after the trypan blue exclusion test (0.00 ± 0.00). Moreover cells revealed similar morphologies and proliferation patterns when compared with the negative control, obtaining a score of 1 in the cytotoxicity index (Table II) showing in this sense that these materials were non-toxic. This type of result is not typical at all for biodegradable polymers.

Regarding cell viability assay (MTS test), cells produced large amounts of the brown formazan product, which indicates a normal metabolism (Fig. 3). L929 cells incubated with composite extracts showed a good incorporation and metabolization of MTS, displaying only a slight inhibition of the metabolism when compared to negative control.

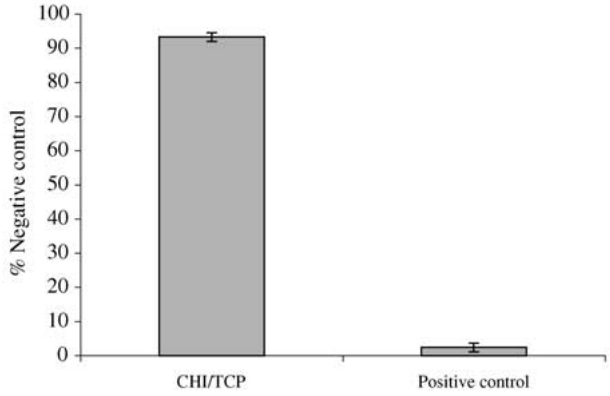

Figure 3 Cell viability of L929 cells after incubation with test and control extracts over a period of 72 h. Results based on optical measurements.

As a result of the cytotoxicity experiments it can be said that the chitosan/coralline origin HA scaffolds are non-toxic and hence can be used for further studies within the biomaterials/tissue engineering field.

3.3. Swelling test

Fig. 4 shows that the composite material was swelled up to 88% and no further swelling was observed afterwards. Upon long-term incubation of scaffold material in a physiological saline solution, no physical alteration or deformation in coralline origin HA replica or fiber alignment was detected. Chitosan material is known to swell in aqueous solutions and sometimes this property can be used to secure biomaterial inside the implantation space [28], taking advantage of a press–fitting mechanism. The developed composite materials maintain their integrity when immersed in physiological media, even though they showed a brief swelling. Consequently, it can be expected that in implantation site it will show no further change in structure due to swelling.

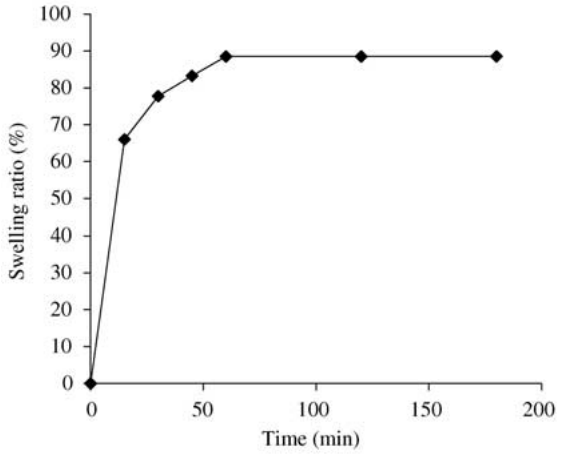

Figure 4 Swelling curve of coralline HA-chitosan composite in physiological saline (pH 7.4).

4. Conclusions

A three-dimensional composite of coralline origin HA microgranules was obtained by using a multichannel mould processing and chitosan as reinforcing material. The composite material showed parallel orientation of cylindrical coralline origin HA bodies along channel direction and good adhesion of particles to chitosan fibers and the outer chitosan membrane. MEM extraction test indicated no cell growth inhibition as determined by the trypan blue exclusion method. MTS test with the composite showed more than 90% cell viability compare to negative controls. The cytotoxicity assays confirmed that the composite material is non-toxic. The swelling test in physiological medium indicated 88% (w/w) swelling in one hour and afterwards no further change was observed. Long-term incubation of the composite in aqueous medium showed no change in integrity of the constituents of the composite material. Consequently, the developed complex architectures presents a range of properties that might allow for their use in guided tissue regeneration and as tissue engineering scaffolds.

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