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Fabrication of 3D chitosan–hydroxyapatite scaffolds using a robotic dispensing system

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Abstract

A new robotic desktop rapid prototyping (RP) system was designed to fabricate scaffolds for tissue engineering applications. The experimental setup consists of a computer-guided desktop robot and a one-component pneumatic dispenser. The dispensing material (chitosan and chitosan–hydroxyapatite (HA) dissolved in acetic acid) was stored in a 30-ml barrel and forced out through a small Teflon-lined nozzle into a dispensing medium (sodium hydroxide–ethanol in ratio of 7:3). Layer-by-layer, the chitosan was fabricated with a preprogramed lay-down pattern. Neutralization of the chitosan forms a gel-like precipitate, and the hydrostatic pressure in the sodium hydroxide (NaOH) solution keeps the cuboid scaffold in shape. Comparison of the freeze-dried scaffold to the wet one showed linear and volumetric shrinkage of about 31% and 62%, respectively. A good attachment between layers allowed the chitosan matrix to form a fully interconnected channel architecture. Results of in vitro cell culture studies revealed the scaffold biocompatibility. The results of this preliminary study using the rapid prototyping robotic dispensing (RPBOD) system demonstrated its potential in fabricating three-dimensional (3D) scaffolds with regular and reproducible macropore architecture.

Keywords: Scaffold; Chitosan; Rapid prototyping; Tissue engineering

1. Introduction

The physical characteristics required of scaffolds for tissue engineering necessitate the application of novel processing techniques for its design and fabrication. Scaffolds have been studied and fabricated using conventional techniques such as fiber bonding, solvent casting, particulate leaching, membrane lamination and melt molding [1]. However, the pore geometry in these techniques is usually not easily controllable and some of them do not have interconnected channels.

Various rapid prototyping (RP) technologies such as fused deposition modeling (FDM) [1,2], laminated object manufacturing (LOM) [3], three-dimensional printing (3DP) [4], multiphase jet solidification (MJS) [5] and 3D plotting [6] have been applied to process biodegradable and bioresorbable materials into three-dimensional (3D) polymeric scaffolds with controllable and reproducible porosity and well-defined 3D microstructures.

The author's group uses a fabrication process that resembles the 3D plotting technology reported by Landers and Mülhaupt [6]. The rapid prototyping robotic dispensing (RPBOD) system setup, as shown in Fig. 1, consists of a computer-guided desktop robot (Robokids, Sony) and a one-component pneumatic dispenser. The dispenser is made up of the air filter, regulator and lubricator set, a solenoid valve and a syringe assembly.

The fabrication technique offered by the RPBOD system allows chitosan to be used in a variety of compositions and concentrations while our developed RPBOD software (made up of a slicing and dispensing program) complements the system by allowing users to generate geometrical data of 3D scaffolds through user-friendly interfaces. The geometrical data can be generated automatically either by using the slicing program, written in-house, to segment the 3D model

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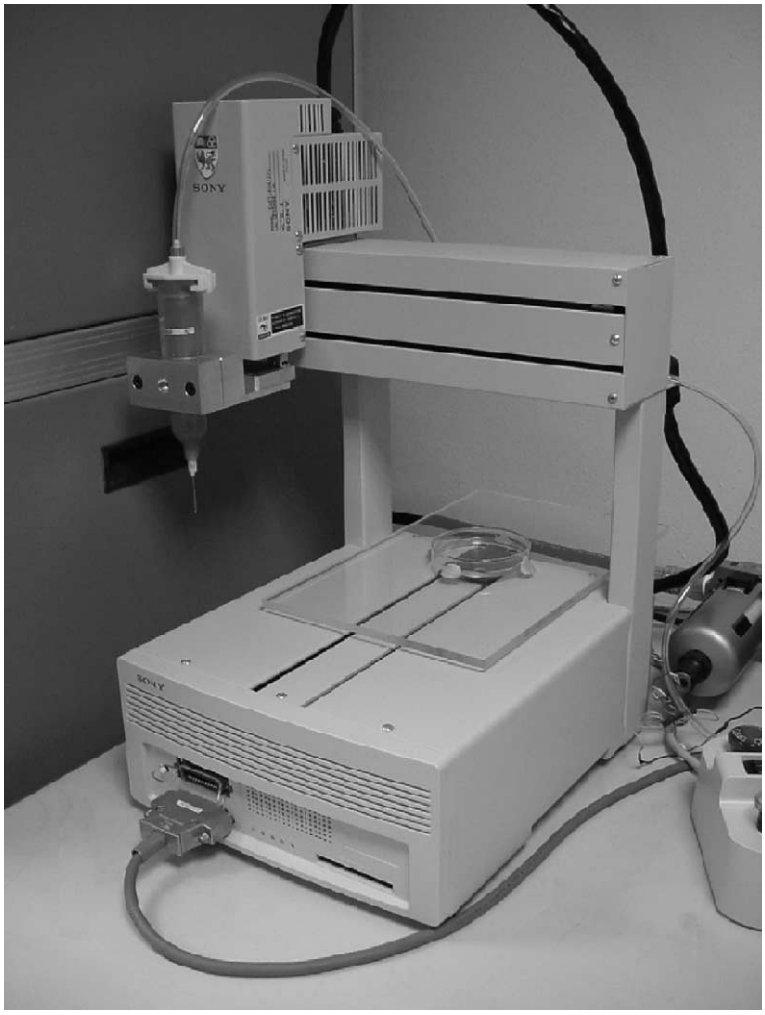

Fig. 1. Rapid prototyping robotic dispensing (RPBOD) system.

in stereolithography (STL) part file format (a de facto standard for the interface between computer-aided design and RP) into two-dimensional (2D) layers or by specifying the required control parameters.

The use of chitosan as a scaffolding material for tissue engineering applications has been previously reported by a number of research groups [7,8]. Chitosan, a naturally occurring amino-polysaccharide, is biodegradable, biocompatible and nontoxic [9]. Chitosan is normally insoluble in aqueous solutions above pH 7. However, in dilute acids, chitosan becomes fully soluble due to the protonation of the free amino groups. The incorporation of bioceramics such as hydroxyapatite (HA) into chitosan scaffolds has also been reported [10,11]. Chitosan–HA has been shown to increase osteoconductivity and biodegradability with significant enhancement of mechanical strength.

The paper investigates the design and fabrication of 3D chitosan and chitosan–HA scaffolds using the rapid prototyping robotic dispensing (RPBOD) system with preliminary in vitro culture studies performed to assess the feasibility of the scaffolds in tissue engineering applications.

2. Materials and methods

2.1. Materials

The materials used are:

- chitosan FM-200 powder form (Lot No. 1225-12) obtained from Koyo Chemical, Japan;
- hydroxyapatite (HA), produced in-house with particle size of about 3–10 μm;
- acetic acid and ethanol (both of analytical grade) purchased from Merck; and
- sodium hydroxide (analytical grade) purchased from JT Baker.

2.2. Methods

2.2.1. Preparation of dispensing material

The dispensing material was prepared by dissolving 3% w/v chitosan (with 0%, 20% and 40% of the chitosan replaced by HA) in 2% v/v acetic acid to form a hydrogel.

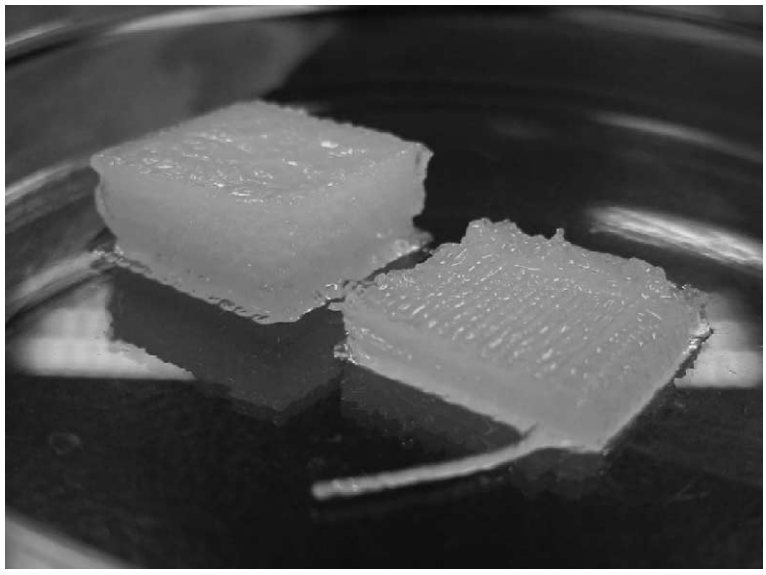

Fig. 2. Effect of line pitch on the number of layers that can be fabricated by using the RPBOD in combination with chitosan. The chitosan scaffolds on the left (21 layers) and right (10 layers) were fabricated with line pitches of 0.8 and 0.9 mm, respectively.

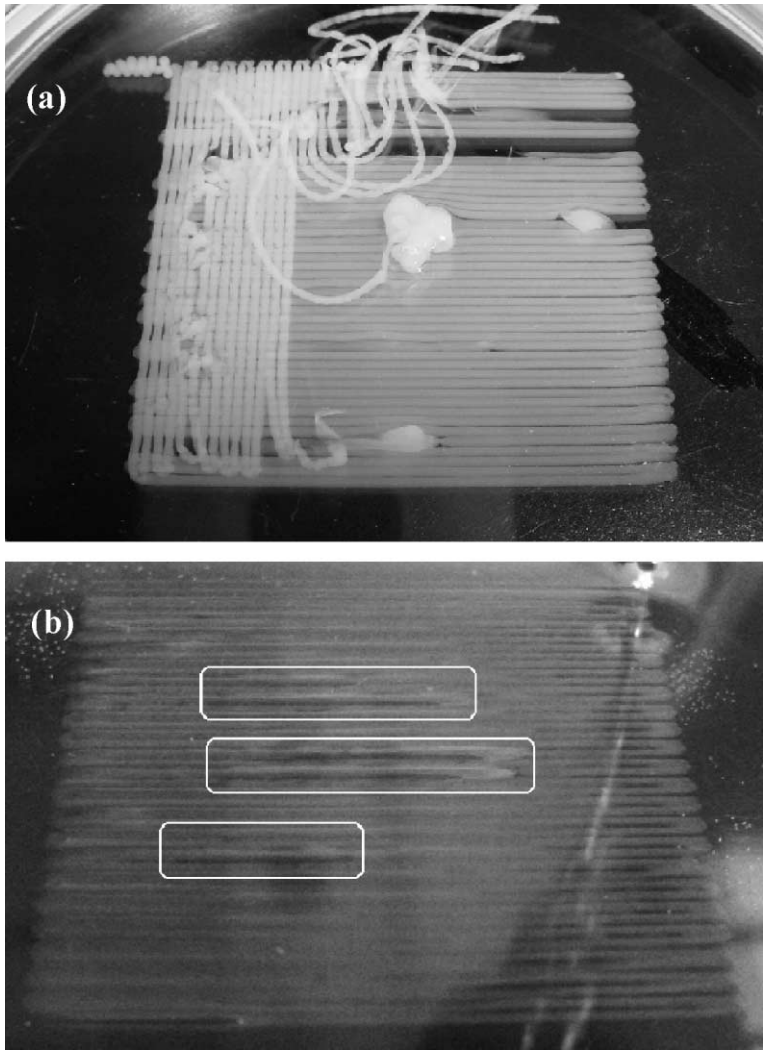

Fig. 3. Effect of NaOH on the scaffold processing. (a) When the NaOH concentration is too high (i.e. 5% v/v NaOH used), there is no adhesion and attachment between the layers. (b) Dragging of the dispensed material from preceding strands will occur if the NaOH concentration is too low (< 0.5% v/v NaOH used).

The mixture was magnetically stirred at room temperature for 2 h and transferred to a vacuum oven (preset at 37 °C) to remove all the air bubbles.

2.2.2. Preparation of dispensing medium

In this study, viscous solutions of chitosan and chitosan–HA are extruded into a bath mixture of sodium hydroxide solution and ethanol to form a hydrated gel-like precipitate.

The hydrogel was extruded into the dispensing medium of varying % w/v NaOH and 100% high-grade ethanol mixed in a ratio of 7:3. In this study, the optimal concentration of NaOH was determined for 2% v/v acetic acid.

2.2.3. Determination of optimal parameters

The Teflon-lined nozzle has an internal diameter (ID) of 150 μm. Before fabricating a 3D scaffold, experiments were carried out to understand the influence of dispensing pressure, the speed of dispensing and the initial height of dispensing on the output quality of the processed scaffold. These three primary parameters, together with the experimented values in brackets, include: (i) the dispensing pressure, *P* (i.e. 3, 4 and 5 bars); (ii) the speed of dispensing, *S* (i.e. 3, 6 and 9 mm/s); and (iii) the initial height of dispensing (i.e. initial distance between the tip and the surface), *H* (i.e. 0.1, 0.2, 0.3 and 0.4 mm).

Based on single-layered scaffolds, macroscopic analysis (i.e. visual examination) of the digitally captured images was performed to select the potential sets of dispensing parameters. Since the primary concern was to produce consistent and uniform strands, the spreading tendency (standard deviation) of the line width and thickness of the strands produced by the potential sets were then measured and compared to pick out an optimized set of parameters. The line pitch (i.e. center-to-center distance between two strands) was another parameter of concern and was also experimentally determined.

2.2.4. Scaffold development

The dispensing materials (i.e. chitosan, chitosan–20% HA and chitosan–40% HA) were poured into three separate 30-ml barrel. Each was extruded through a small Teflon-lined nozzle into the dispensing medium (i.e. NaOH–ethanol) contained in a flat-based polystyrene petri dish to form a gel-like precipitate using the RPBOD system (Fig. 1).

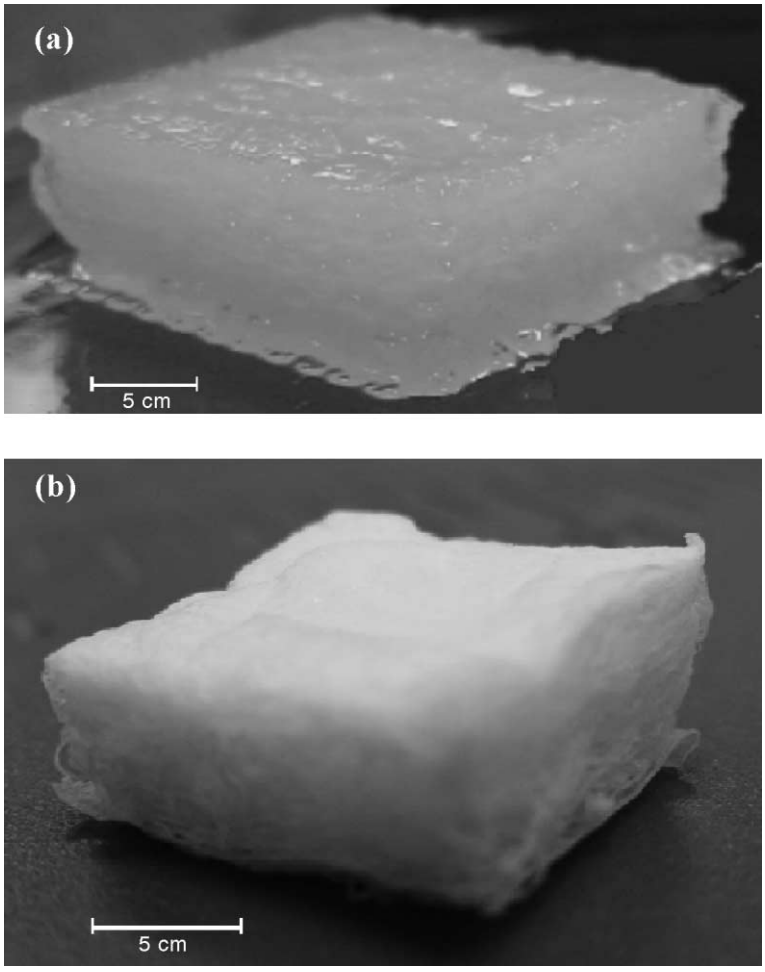

Fig. 4. Chitosan scaffolds (a) before (approximately 20 × 20 × 8.3 mm) and (b) after (approximately 14.8 × 15 × 5.8 mm) freeze-drying. It was evaluated that 3*P*6*S*0.2*H* and the effective line pitch of 0.8 mm are the optimal parameters when 0.75–1.5% v/v NaOH concentration was used.

The process involved dispensing continuous strands of material to produce two-dimensional (2D) layers with alternating 0°/90° lay-down patterns of finite thickness and then building the 3D scaffold up layer-by-layer.

The extruded 3D scaffolds were immersed in 100%, 70% and 50% ethanol sequentially for 5 min each and subsequently washed three times with distilled water. The scaffolds were then rapidly transferred to a freezer at a preset temperature of −20 °C to solidify the solvent so as to induce a solid–liquid phase separation as described in the fabrication method of Zhang and Zhang [8]. The frozen scaffolds were maintained at − 20 °C for at least 8 h and then freeze-dried at − 56 °C for 2 days to remove the residual solvent. The freeze-dried chitosan scaffolds were observed under the scanning electron microscope (SEM) to assess the gross morphology and microstructures.

2.2.5. Preliminary in vitro culture studies

The M199 medium (Gibco, NY) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Gibco) was used throughout the culture period. Osteoblasts were isolated from human calvarial bone chips and cultures were expanded to passage three and used for the in vitro studies.

The scaffolds were washed three times in sterile phosphate-buffered saline (PBS) for 15 min each [7]. They were then sterilized by immersing in absolute ethanol for 1 h, and sequentially in 70% and 50% ethanol for 30 min each. After another three times of 15-min wash in sterile PBS, the scaffolds were freeze-dried for 24 h and rehydrated in complete culture medium for 2 h before cell seeding.

The cell compatibility of the fabricated scaffolds was evaluated preliminarily in in vitro studies. Each scaffold measuring $5 \times 5 \times 3$ mm was seeded with 2×10^5 cells. The three types of scaffolds, namely chitosan, chitosan–20% HA and chitosan–40% HA scaffolds, were each seeded with fibrin glue (Baxter Hyland Immuno, Vienna, Austria). The seeded scaffolds were maintained in 1 ml culture medium, in a self-sterilizable incubator (maintained at 37 °C, 95% air and 5% carbon dioxide), throughout the 4 weeks of culture, with the medium being changed every 3 days. Adhesion of cells and their distribution was studied via the SEM (JEOL JSM-5800LV) at the third week of cell culture. The morphological and proliferation characteristics were also monitored with light microscopy throughout the culture period.

3. Results and discussion

Though studies have shown that RP techniques as well as the conventional ones have the potential to produce scaffolds for tissue engineering applications, each has its shortcomings [1]. Three-dimensional printing and LOM, for example, require post processing to improve the mechanical properties of the scaffold. FDM, on the other hand, allows only the application of thermoplastic polymers. This prevents the implementation and application of biological agents and natural polymers as temperature induces protein inactivation. The range of materials that can be applied to these RP techniques is also limited. Advantages that are

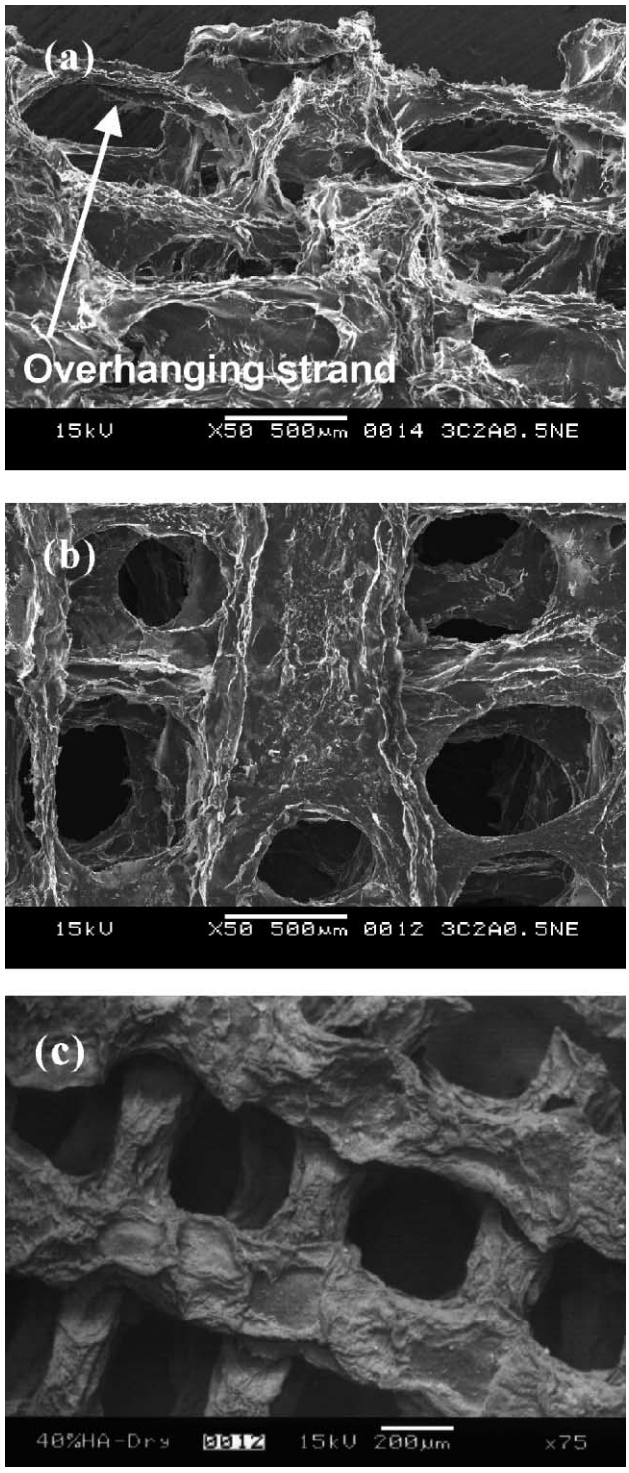

Fig. 5. SEM images showing the (a) top view of the chitosan scaffold at an angle, (b) top view of the chitosan scaffold and (c) top view of the chitosan–40% HA scaffold.

envisioned with the 3D plotting approach includes the following [6].

(1) It can apply a wide variety of polymers, for example, thermoplastic as well as hydrogels and pastes. Hence, it becomes possible to control the solidification process and the properties of the resulting scaffold by using multiple material combinations.

(2) It does not require heating and can apply reactive components as well as thermally sensitive bio-components into the fabrication process. For tissue engineering, this means that the cells and growth factors may be integrated into the scaffold processing, enabling precise control and optimal conditions for homogeneous cell distribution right from the start of fabrication.

3.1. System parameters and optimization of NaOH concentration

A Teflon-lined nozzle was used to prevent the dispensing material from adhering and accumulating around its tip during dispensing in the NaOH–ethanol solution. Each layer was fabricated with one continuous strand because better contact was observed between the extruded strand and the surface of the petri dish when compared with a fabricated layer using many discontinuous strands.

The air regulator was used to vary and set the applied pressure for dispensing while the dispensing software was used to control the RPBOD system to achieve the required dispensing speed and initial height of dispensing. Dispensing pressure (P) of 3 bars, speed of dispensing (S) of 6 mm/s and initial height of dispensing (H) of 0.2 mm, henceforth abbreviated to the form 3*P*6*S*0.2*H*, were evaluated to be optimal for fabricating pure chitosan scaffolds. It was also found that 3*P*4*S*0.2*H* and 1.5*P*4*S*0.2*H* could be used to fabricate chitosan–20% HA and chitosan–40% HA scaffolds, respectively. These parameters are expected to differ when varying compositions and concentrations of chitosan are used, as a result of the difference in viscosity of the hydrogel.

Establishing the parameters to achieve the desired quality of each deposited layer is insufficient to ensure the output quality of the final scaffold because the quality of the bond between the layers are equally important. Experimental results revealed that a smaller line pitch could increase the number of layers that could be built (see Fig. 2). The increase in the number of layers built could be due to providing more contact points (larger contact area) for added diffusion of chitosan molecules to take place between two adjoining layers to result in better establishment of bonding between all layers of the scaffold.

The optimal working range of NaOH concentration was found to be between 0.75% and 1.5% v/v. Rapid precipitation was observed when a higher NaOH concentration was used. When the gel-like precipitate was formed too quickly, there was very little or no mobility of chitosan molecules at the contact points of strands between each layer as every strand behaved as a unique, separate bar. As such, there was little or no attachment between the layers. Conversely, the use of a very low NaOH concentration lowered the rate of precipitation so much that the dispensed strand was not precipitated fast enough to hold its shape. This had undesirably resulted in the connection of adjacent strands and the possible dragging of material because the dispensed material spread into the path of the subsequent parallel strands that were to be built. The NaOH concentration, therefore, was an important factor that affected the mobility of chitosan molecules, as reflected in Fig. 3a and b.

3.2. Macroscopic and microscopic analysis

Since chitosan and chitosan–HA scaffolds were macroscopically similar, only the chitosan scaffolds before and after freeze-drying are shown in Fig. 4a and b, respectively. Slight depression at the center of the freeze-dried scaffold

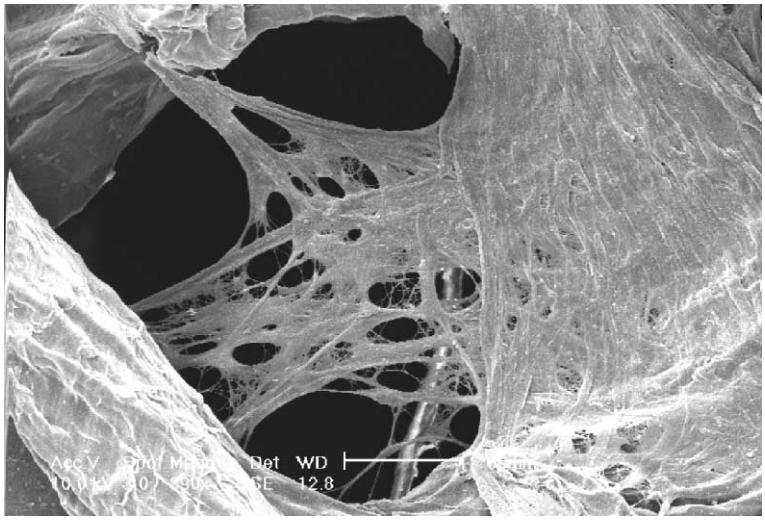

Fig. 6. SEM image shows good cell attachment and proliferation in the third week of the in vitro culture.

and the curling of its edges were observed in both the chitosan and chitosan–HA scaffolds. This would have a slight effect on the uniformity of the scaffolds produced, as it was a result of unavoidable differential shrinkage where the edges dried faster due to different rates of solvent evaporation.

Fig. 5a shows the presence of attachment between layers of the chitosan scaffold, allowing the chitosan matrix to form a fully interconnected channel architecture for the flow of culture medium through the entire scaffold. Similar interconnectivity was observed for the chitosan–HA scaffolds. Macropore diameters of 400–1000 μm were observed for pure chitosan scaffolds (an area is illustrated in Fig. 5b), whereas a more uniform macropore size of about 200–400 μm was observed for the chitosan–HA scaffolds (Fig. 5c). The chitosan–HA scaffolds were able to maintain square macropores and the uniformity of the macropores was more homogeneous than the chitosan scaffold. This is likely due to the enhancement of mechanical strength through the reinforcement by the HA fillers that allowed the scaffolds to hold their shape during the free shrinkage that occurred during freeze-drying.

SEM images showed that the chitosan and chitosan–HA strands were able to suspend themselves as overhanging strips with little or no slack (Fig. 5a shows the SEM image of pure chitosan scaffold). The reason could be the compensation of gravitational force acting on the overhanging part (weight) of the strands by the buoyancy force provided by the dispensing medium.

3.3. Preliminary in vitro culture studies

Similar results of the in vitro culture studies were observed for the chitosan and chitosan–HA scaffolds. The cells that were seeded with fibrin glue remained viable and proliferated throughout the culture period (i.e. 4 weeks). The SEM image (Fig. 6) shows the adhesion and distribution of cells at week 3 on the chitosan–40% HA scaffold.

The cells in the scaffolds seeded with fibrin glue exhibited healthy morphology and strong proliferative ability throughout the culture period for all the scaffolds cultured and the positive result could be observed from the representative light microscopy picture taken of the chitosan–40% HA scaffold (Fig. 7) at week 1.

4. Conclusion

The study began with an investigation into the criteria for building 2D scaffolds followed by the fabrication of 3D scaffolds with the rapid prototyping robotic dispensing (RPBOD) system. Through a series of experiments, the dispensing parameters that yield effective results were identified to be 3*P*6*S*0.2*H*, 3*P*4*S*0.2*H* and 1.5*P*4*S*0.2*H* for the fabrication of pure chitosan, chitosan–20% HA and chitosan–40% HA scaffolds, respectively. The optimal working range of NaOH concentration was found to be between 0.75% and 1.5% v/v. Neutralizing the chitosan formed a gel-like precipitate, and the hydrostatic pressure in the NaOH solution kept the cuboid scaffold in shape. Stepwise material build-up requires strong adhesion between layers which could be achieved by using a small line pitch so that each adjacent strand was placed closer together to increase the area of contact. A low concentration of the dispensing medium was used to control the rate of precipitation so that homogenous diffusion took place. A good attachment between layers allowed the chitosan matrix to form a fully interconnected channel architecture. Results of in vitro studies revealed the scaffold cell biocompatibility. The results of this preliminary study using the RPBOD system

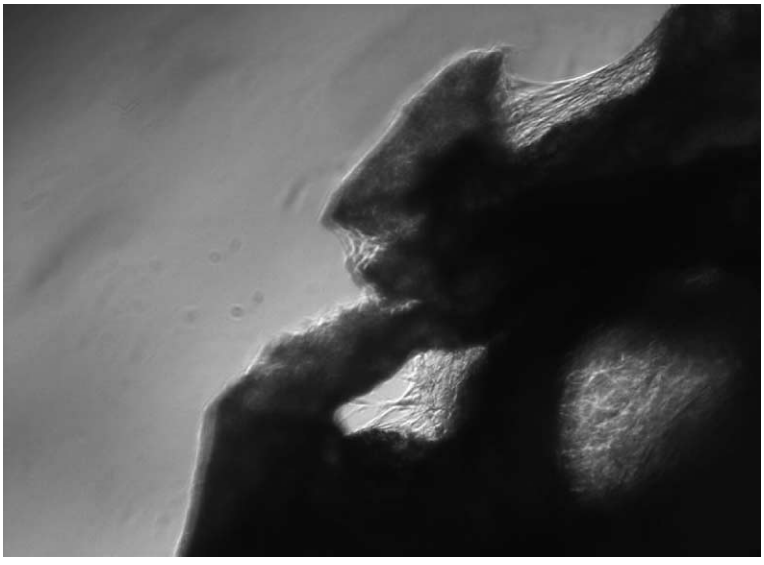

Fig. 7. Light microscopy images showing cell proliferation at week 1 of primary human osteoblast-like cells.

demonstrated its potential in fabricating 3D scaffolds with regular and reproducible macropore architecture.

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