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A biphasic scaffold design combined with cell sheet technology for simultaneous regeneration of alveolar bone/periodontal ligament complex

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ABSTRACT

This study describes the design of a biphasic scaffold composed of a Fused Deposition Modeling scaffold (bone compartment) and an electrospun membrane (periodontal compartment) for periodontal regeneration. In order to achieve simultaneous alveolar bone and periodontal ligament regeneration a cell-based strategy was carried out by combining osteoblast culture in the bone compartment and placement of multiple periodontal ligament (PDL) cell sheets on the electrospun membrane. In vitro data showed that the osteoblasts formed mineralized matrix in the bone compartment after 21 days in culture and that the PDL cell sheet harvesting did not induce significant cell death. The cell-seeded biphasic scaffolds were placed onto a dentin block and implanted for 8 weeks in an athymic rat subcutaneous model. The scaffolds were analyzed by μCT, immunohistochemistry and histology. In the bone compartment, a more intense ALP staining was obtained following seeding with osteoblasts, confirming the μCT results which showed higher mineralization density for these scaffolds. A thin mineralized cementum-like tissue was deposited on the dentin surface for the scaffolds incorporating the multiple PDL cell sheets, as observed by H&E and Azan staining. These scaffolds also demonstrated better attachment onto the dentin surface compared to no attachment when no cell sheets were used. In addition, immunohistochemistry revealed the presence of CEMP1 protein at the interface with the dentine. These results demonstrated that the combination of multiple PDL cell sheets and a biphasic scaffold allows the simultaneous delivery of the cells necessary for in vivo regeneration of alveolar bone, periodontal ligament and cementum.

1. Introduction

Periodontitis is a common chronic inflammatory disease that results in degradation of the supporting tissues around teeth, which if left untreated, can lead to tooth loss [1]. Periodontal wound healing following conventional therapy results in repair by collagenous scar tissue and is accompanied by the apical migration of gingival epithelium between the gingival connective tissue and the root surface [2,3]. However, periodontal regeneration requires the formation of periodontal ligament fibers and the insertion of these fibers into newly formed cementum on the root surface, as well as reconstitution of the adjacent resorbed alveolar bone. To this end, dedicated surgical techniques have been developed in order to promote periodontal regeneration and the most widely utilized of these is based around the principles of Guided Tissue Regeneration (GTR) [4,5]. This technique utilizes barrier membranes to selectively promote the repopulation of the periodontal defect by cells capable of periodontal attachment regeneration (periodontal ligament cells, osteoblasts) on the root surface at the expense of those that do not (gingival epithelial cells). However, although this approach is conceptually sound and can be successful in ideal clinical scenarios, the clinical results have been unreliable and predictable regeneration remains elusive [2]. It may be hypothesized that a tissue engineering approach could be utilized to optimize the conceptually sound principles of GTR (selective cell population, space maintenance, wound stabilization) through the use of scaffolds that can deliver the various cells required for periodontal attachment formation to the anatomically desirable locations on the periodontal defect.

Akizuki et al. [6] have proposed a strategy whereby various periodontal cell sheets were applied onto the root surface in order to form new cementum and promote periodontal attachment [6–12]. This tissue engineering strategy involved the use of

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a thermoresponsive culture plate in order to harvest the cell sheet without damaging the extracellular matrix formed during the in vitro culture process [13]. The implantation of such fragile tissue also required the support of a non-degradable or a biodegradable membrane (HA membrane [6], fibrin gel [7], non-absorbable Gor-eTex membrane [12], polyglycolic acid [10]) to facilitate the handling and placement of the cell sheet. This strategy, although promising, might be compromised due to inadequate biomechanical stability of the membrane/cell sheet construct during the wound healing process [6].

We hypothesized that the cell sheet technology could be utilized as a component of an optimized scaffold design, incorporating a bone and PDL compartment, which would provide clinically relevant biomechanical stability to the cell sheets. In addition, this design concept would enable the implantation of cell sheets in a controllable and reproducible way and allow for adequate cell sheet attachment onto the root surface. As a result, we have developed a biphasic scaffold, made of a Fused Deposition Modeling (FDM) component (bone compartment) and a micro-fibrous membrane (periodontal ligament compartment) which would not only meet the aforementioned criteria, but would also allow the simultaneous delivery of the two cell types (periodontal ligament cells and osteoblasts) necessary for the regeneration process in the correct anatomical orientation. This is important because the complex structure of the periodontium, consisting of soft (gingiva, periodontal ligament) and hard (bone and cementum) tissues, requires specific co-ordination for regeneration of a functional periodontal attachment complex. This scaffold will also fulfill the principles of GTR while allowing for space maintenance and wound stabilization which are required for successful regeneration to occur.

This paper reports the design rationale, fabrication and characterization of the biphasic scaffold and demonstrates the efficacy of the construct when combined with periodontal ligament fibroblast cell sheets and osteoblasts for the purpose of promoting periodontal regeneration in vivo.

2. Materials and methods

2.1. Biphasic scaffold fabrication

Medical grade polycaprolactone (PCL, Lactel, USA) containing β-tricalcium Phosphate (β-TCP, 20% wt) was utilized to fabricate composite scaffolds via Fused Deposition Modeling (FDM, Osteopore Inc.,Singapore). The scaffolds measured $100 \times 100 \times 2$ mm^3 and had 100% interconnectivity, 70% porosity and a 0/60/120 degrees lay-down pattern. Prior to use the FDM scaffold block were sectioned with a sharp scalpel blade into $5 \times 5 \times 2$ mm^3 specimens.

PCL was electrospun using an in-house solution spinning device. The polymer was first dissolved in a mixture of chloroform and dimethylformamide (9/1 vol/vol) at a concentration of 15% wt/vol. The polymer solution was loaded into a 10 mL syringe and electrospun at a feed rate of 2 mL/h, at 10 kV and at a 20 cm tip to collector distance for 30 min. The biphasic scaffold consisted of an FDM component for the bone compartment and a flexible electrospun membrane for the periodontal compartment. To assemble the biphasic scaffold, the FDM component was placed 1 cm from a hot plate heated to 300 °C for 4 s and then quickly press-fitted for 10 seconds onto a PCL electrospun membrane ($7 \times 9 \times 0.4$ mm^3). This heat treatment partially melted the first layer of the FDM component enabling it to strongly bind to the electrospun scaffold upon cooling and solidification.

2.2. Scaffold morphology

Scanning electron microscopy (SEM) was performed to investigate cohesion between the two components. Scaffolds were immersed in liquid nitrogen for 5–10 min and a sharp razor blade was used to section the structures. The samples were gold coated for 3 min and observed with a FEI Quanta 200 Environmental SEM operating at 10 kV.

2.3. In vitro study

2.3.1. Cell isolation and culture

Osteoblast and periodontal ligament explants were obtained from Merino sheep (ovis aries). Animal ethics approval for this study was granted by the Animal Ethics Committee of Queensland University of Technology.

2.3.1.1. Osteoblasts. Compact bone samples were collected under sterile conditions from the mandible under general anaesthesia with a trephine drill (5 mm diameter), minced, washed with phosphate buffered saline (PBS) and vortexed 5 times. Bone samples were then incubated with 10 mL of 0.25% trypsin/EDTA for 3 min at 37 °C in a 5% CO_2 atmosphere. After trypsin inactivation, samples were washed once again with PBS and transferred in basal culture media into 175 cm^2 tissue culture flasks. Outgrowth of osteoblasts was observed after 5–7 days. Cells were expanded and used at the third passage (P3).

2.3.1.2. Periodontal ligament cells. Two incisors were extracted and placed into a 50 mL tube containing DMEM with 2% penicillin/streptomycin and 4 μg/mL fungizone. The middle third of the periodontal ligament (PDL) was subsequently gently removed from the root surface with a scalpel and further sectioned into approximately 1×1 mm^2 pieces. The PDL tissues were placed into a 25 cm^2 culture flask which was left standing upright in an incubator at 37 °C and 5% CO_2 atmosphere for 30 min to allow tissue adhesion. After this incubation period, 3 mL of DMEM containing 10% FBS, 1% of penicillin/streptomycin and 0.1 μg/mL fungizone were added and the flask was carefully laid down in the incubator. The first media change occurred 4 days post extraction. After one week of culture, cells started migrating outwards from the PDL tissues and generally reached confluence after 2–3 weeks of culture. The cells were passaged using 0.25% trypsin and further expanded until P3.

2.3.2. 2D culture

Osteoblasts and PDL cells were cultured separately in 24-well plates for 14 days in order to assess their mineralization potential. Osteoblasts were seeded at 500 cells/cm^2 whereas the PDL cells were seeded at 250 cells/cm^2 due to early cell sheet contraction at higher cell seeding densities. Both cell lines were cultured under basal or osteogenic induction media (50 μg/mL ascorbate-2-phosphate, 10 mM β-glycerophosphate and 0.1 μM dexamethasone) for 2 weeks. Cell proliferation as measured by DNA content and cell differentiation as measured by ALP activity were evaluated in the same samples at days 7 and 14. The cells were also stained with alizarin Red S in order to assess the deposition of mineralized matrix.

2.3.2.1. DNA content and alkaline phosphatase activity. ALP activity was measured in the cell culture media after a 24 h release period. Briefly, samples were first rinsed in DMEM without phenol red three times and placed back in the incubator for precisely 24 h. ALP activity was measured according to the Sigma*FAST*™ kit. A total of 100 μL of *p*-nitrophenyl phosphate in Tris-base buffer was added to 100 μL of the culture media in a 96-well plate. This was further incubated in the incubator (37 °C and 5% CO_2) for 24 h. At the end of the second incubation period the plate was brought back to ambient temperature (20 °C) for 5 min and the absorbance was read at 405 nm using a plate reader (Benchmark Plus™ microplate spectrophotometer, BIO RAD).

For cellular DNA content analysis, the remaining media was removed from the wells and the samples frozen at −80 °C for at least 48 h. The cell membrane and the extracellular matrix were disrupted in 300 μL of 0.5 mg/mL Proteinase K in Phosphate Buffered EDTA (PBE) at 37 °C overnight and then transferred into 1.5 mL Eppendorf tubes and further incubated for 24 h at 60 °C. A total of 100 μL of the diluted (1/50 in PBE) lysate was aliquoted into black 96-well plates with 100 μL of PicoGreen (P11496, Invitrogen) working solution according to the manufacturer's instructions. After 5 min incubation in the dark the fluorescence (excitation 485 nm, emission 520 nm) was measured using a fluorescence plate reader. A standard curve was also constructed using known concentrations of λ DNA provided with the kit. The standard ranged from 10 ng/ml to 1 μg/mL λ DNA and were used to calculate the final DNA content of the sample.

2.3.2.2. Alizarin Red S staining. A 1% solution alizarin Red S was used to assess the deposition of mineralized matrix. The cells were rinsed twice in PBS and then fixed for 10 min in cold methanol. The cell sheet was then rinsed in ddH_2O and 500 μL of alizarin solution was added for 10 min. The unfixed dye was removed by gently rinsing the stained cell sheet with ddH_2O until a clear solution was obtained. The samples were air dried overnight and stored until use. The alizarin dye was extracted using 300 μL of a 50% acetic acid solution. The plates were placed onto a rocker for 10 min to allow for complete dissolution of the dye. The solution was then placed into 1.5 mL Eppendorf tubes and vortexed for 30 s. A total of 150 μL of 4 M NaOH was added to bring the pH to 4.1. The tubes were then centrifuged at 10,000 rpm for 10 min. Triplicates of 100 μL were placed into a 96-well plate and the absorbance was read at 405 nm.

2.3.3. Biphasic scaffold seeding and culture

Osteoblasts (200,000 cells in 40 μL of media) were seeded onto the FDM component and allowed to adhere for 4 h at 37 °C in a 5% CO_2 atmosphere before the well was filled with media, the biphasic scaffolds were then turned upside down in order to minimize cell infiltration into the electrospun component. Biphasic scaffolds were further cultured for 3 weeks either in osteogenic media (50 μg/mL ascorbate-2-phosphate, 10 mM β-glycerophosphate, 0.1 μm dexamethasone) or in basal media. Osteoblast proliferation was measured by Picogreen assay at 7, 14 and 21 days post seeding according to the procedure previously described in Section 2.3.2.1. Due to the larger volume of the scaffold, 500 μL of proteinase K solution was used. Osteoblast morphology into the bone compartment was also imaged by SEM