Rapid prototyping of anatomically shaped, tissue-engineered implants for restoring congruent articulating surfaces in small joints

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Abstract

Background: Preliminary studies investigated advanced scaffold design and tissue engineering approaches towards restoring congruent articulating surfaces in small joints.

Materials and methods: Anatomical femoral and tibial cartilage constructs, fabricated by three-dimensional fibre deposition (3DF) or compression moulding/particulate leaching (CM), were evaluated *in vitro* and *in vivo* in an autologous rabbit model. Effects of scaffold pore architecture on rabbit chondrocyte differentiation and mechanical properties were evaluated following *in vitro* culture and subcutaneous implantation in nude mice. After femoral and tibial osteotomy and autologous implantation of tissue-engineered constructs in rabbit knee joints, implant fixation and joint articulation were evaluated.

Results: Rapid prototyping of 3DF architectures with 100% interconnecting pores promoted homogeneous distribution of viable cells, glycosaminoglycan (GAG) and collagen type II; significantly greater GAG content and differentiation capacity (GAG/DNA) *in vitro* compared to CM architectures; and higher mechanical equilibrium modulus and dynamic stiffness (at 0.1 Hz). Six weeks after implantation, femoral and tibial constructs had integrated with rabbit bone and knee flexion/extension and partial load bearing were regained. Histology demonstrated

articulating surfaces between femoral and tibial constructs for CM and 3DF architectures; however, repair tissue appeared fibrocartilage-like and did not resemble implanted cartilage.

Conclusions: Anatomically shaped, tissue-engineered constructs with designed mechanical properties and internal pore architectures may offer alternatives for reconstruction or restoration of congruent articulating surfaces in small joints.

Introduction

Traumatic or degenerative articular cartilage lesions are common clinical problems (1,2). Damaged adult articular cartilage has limited regenerative capacity, which results in joint pain, loss of mobility and osteoarthritis (1). Few current treatment protocols are capable of consistently restoring long-term function to damaged articular cartilage (3–5). Tissue engineering approaches, adopting three-dimensional (3D) scaffold conduits, offer considerable promise as alternative repair strategies (6–9). Current repair protocols typically focus on repairing chondral and/or osteochondral defects using cylindrical single-phase or biphasic scaffolds (10–13). In practice, this requires the repaired cartilage to articulate with native cartilage of the opposing joint, yet natural curvature or congruency of articulating surfaces is often not taken into account in scaffold design. Moreover, there are instances where both articulating surfaces are damaged, thereby requiring reconstruction or restoration of both congruent articulating cartilage surfaces. Motivation for this study was feasibility of developing anatomically shaped, tissue-engineered cartilage implants for reconstruction and/or restoration of congruent articulating surfaces in damaged small joints in an *in vitro* and *in vivo* model (Fig. 1). Previous attempts have been made to tissue engineer various cartilaginous,

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Figure 1. Process for developing anatomically shaped, tissue-engineered constructs with congruent articulating surfaces. (1) Harvest of cartilage biopsy; (2a) development of three-dimensional (3D) model by computed tomography (CT) scans; (2b) cell isolation and expansion to obtain sufficient cell numbers; (3) 3D computer-aided design of desired anatomical shape from CT scan; (4) manipulation and rendering of 3D scaffold model in suitable format ready for rapid prototyping (RP); (5) processing of scaffold by RP, using computer numerical-controlled (CNC) milling of PEGT/PBT foam or 3D fibre deposition (3DF) of molten PEGT/PBT; (6) seeding and dynamic culture of expanded cells from (2a) in anatomically shaped RP scaffolds; and (7) autologous implantation of the anatomically shaped, tissue-engineered construct *in vivo*.

bone and tendon components in the form of a finger, or metacarpophalangeal joint (14–16); however, function has never been evaluated in a load bearing environment *in vivo*.

Materials and methods

Materials

Biodegradable poly(ethylene glycol)-terephthalate/poly-(butylene terephthalate) (PEGT/PBT) block co-polymers were obtained from IsoTis Orthobiologics (Bilthoven, The Netherlands). PEGT/PBT co-polymer composition was defined by a PEG molecular weight of 300 g/mol, and a PEGT : PBT weight percent (wt%) ratio of 55 : 45 (denoted as 300/55/45). This composition was selected based on a number of previous studies that demonstrate its applicability to an extensive range of processing techniques for producing dense and porous 3D architectures (17–20),

including selected rapid prototyping techniques (21–24) and suitable thermal and mechanical stability (22,25), as well as ability to promote chondrocyte attachment and cartilaginous matrix deposition (22,26–29).

Design and rapid prototyping of anatomic scaffolds

Computer-aided design model. 3D computed tomography (CT) scans (Siemens, Berlin, Germany) of distal femur and proximal tibia of 6-month-old New Zealand white rabbits were taken (0.5 mm slice thickness) and rendered to form an anatomical surface model of the knee joint (Fig. 2a). CT surface models were imported (in STL format) into CAD software (Rhinoceros, Seattle, WA, USA) and geometries converted to solid 3D CAD models (DXF format). The CAD models were manipulated to isolate femoral and tibial surfaces (Fig. 2b) and integrate anatomical positioning of the stem for medullary fixation. The 15-mm-long stem was designed to allow axial press

Figure 2. Computer modelling and rapid prototyping (RP) of porous, anatomically shaped femoral and tibial scaffolds. (a) 3D CT surface model of rabbit knee (dotted lines illustrate approximate surgical cutting planes for osteotomy); (b) solid 3D CAD model redesigned to isolate articulating surfaces and integrate anatomical positioning of stem for medullary fixation; (c) solid 3D femoral and tibial models; (d–f) rapid prototyping of porous scaffolds using computer numerical-controlled (CNC) milling of compression moulded (CM) block of PEGT/PBT foam; and (g–i) 3D fibre deposition (3DF) of molten PEGT/PBT. Separate RP-specific models (d,g) were generated from which the raw scaffold could be manufactured (e, h). After removal of support structures, identical anatomically shaped CM (f) and 3DF (i) scaffolds were produced but with distinctly different internal pore architectures.

fixation within the medullary cavity (Fig. 2c). Preliminary osteotomy studies in cadaver rabbits indicated average diameter of the medullary canal to be 7 mm. To ease implantation and press fixation, the stem was tapered from 7 mm to 5 mm along its length. Two rapid prototyping techniques, computer numerical-controlled (CNC) milling and 3D fibre deposition (3DF), were then used to produce anatomically shaped scaffolds with different pore architectures, as described below. For each technique, rapid prototyping-specific CAD models (in STL and DXF format) for femoral and tibial scaffolds, including support

structures, were developed (Fig. 2d,g), allowing rapid prototyping-specific tool paths to be generated.

CNC milling. This process required prefabrication of 25-mm-thick blocks of porous compression moulded/particle leached (CM) foam, produced by compressing a mixture of PEGT/PBT co-polymer and salt crystals (75% by volume and 400–600 μm in size) under heat and pressure, as described previously (30). Tool paths were generated from a rapid prototyping-specific CAD model (Fig. 2d) using DeskProto CNC software (Delft Spline Systems, Utrecht,

The Netherlands). The desired 3D scaffolds were then 'cut' from the CM foam using a CNC milling machine (CPM 4030, illustrated in Fig. 1) and a 2-mm ball ended cutter in a two-sided process (Fig. 2e,f). We have previously described a novel technique for producing 3D fibre-(3DF) deposited scaffolds by successive layering of molten PEGT/PBT fibres in a computer-controlled process (21,22,27). Using similar principles on a 3D BioPlotter™ machine (EnvisionTec, Gladbach, Germany, illustrated in Fig. 1) (31), more complex anatomically shaped PEGT/PBT scaffolds were constructed for this study. Briefly, rapid prototyping-specific femoral and tibial models (Fig. 2g) and fibre deposition criteria were used to generate deposition paths from BioPlotter™ software (e.g. temperature, 200 °C; nitrogen pressure, 5 bar; x-y-z axis speed, 210 mm/min; fibre spacing, 0.6 mm; fibre orientation, 0°–90°; layer thickness, 0.15 mm; nozzle diameter, 250 µm). In a layer-by-layer process, the desired anatomical femoral and tibial scaffolds were produced in a two-part process while having complete control over internal pore structure (Fig. 2h,i). Cylindrical scaffolds (7 × 4 mm) for use in the preliminary scaffold evaluation study *in vitro* (Experiment 1) were cored from simple rectangular blocks (4 mm thick) of CM and 3DF material, produced using identical processing techniques as described above.

To promote adhesion and homogeneous cell distribution throughout the scaffolds (26,32), porous CM and 3DF femoral and tibial implants were gas plasma-treated under argon plasma (0.1–0.2 mbar) for 30 min. However, to prevent cell adhesion to the stem component, and thereby to localize tissue formation only within the articulating regions of femoral and tibial implants, polyvinyl chloride tubing was placed over the entire length of the stem of each scaffold. Scaffolds including tubing were then autoclave sterilized (121 °C for 30 min) prior to use in tissue culture experiments

Biopsy and joint preparation

Ethical approval for use of a total of four 6-month-old New Zealand white rabbits was obtained, for preliminary implantation studies, in accordance with animal study guidelines of the University Hospital Zurich. Although a greater number of animals was requested, the actual number granted was on the basis that a larger follow-up study would proceed following the current preliminary investigation.

Rabbits were operated on under general anaesthesia and with use of a respirator. During the first surgery, a lateral incision (~4 cm) was made in the left knee and the joint capsule was exposed longitudinally. Care was taken to preserve the patella and medial quadriceps tendon and patella ligament by pushing structures medially.

Both posterior and anterior cruciate ligaments were disconnected and medial and lateral menisci were removed. Osteotomy of femoral and tibial articulating surfaces (~2 mm in height) was performed and the medullary canal bored open and freed of marrow tissue. Cutting planes were determined prior to surgery based on 3D CT models used to develop the 3D anatomical scaffolds (Fig. 2a). Femoral and tibial biopsies for each rabbit were labelled and immediately processed for autologous cell isolation and tissue culture (described below). In accordance with ethical approval, a temporary silicon prosthesis (Swanson, Wright Medical, Arlington, TN, USA) was placed within the femoral and tibial medullary cavities to stabilize the knee for a 5-week period while tissue culture was taking place. The medial quadriceps tendon and patella ligament were repositioned, joint capsule, subcutaneous layer and skin were closed with resorbable Polyglactin (Vicryl 3/0) sutures, and spray plaster was applied.

Tissue culture

Rabbit chondrocytes for autologous use were isolated from femoral and tibial cartilage biopsies by collagenase digestion (collagenase type II, Worthington, Lakewood, NJ). Cells were culture expanded (3500 cells/cm²) for approximately 2 weeks until passage 2, in HEPES-buffered Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 20 mM ascorbic acid, 0.4 mM proline (Sigma Aldrich, St. Louis, MO, USA), 0.1 mM non-essential amino acids (Sigma Aldrich), 100 units/ml penicillin (Invitrogen), 100 µg/ml streptomycin (Invitrogen), and 10% foetal bovine serum (Sigma Aldrich). Pairs of femoral and tibial scaffolds were placed on sterile needles and seeded (20 × 10⁶ cells/scaffold) in spinner flasks (50 ml working volume, 60 r.p.m.) for 3 days; they then remained in dynamic spinner flask culture *in vitro* for 21 days (40 r.p.m.), with medium replacement every 3 days.

For the preliminary *in vitro* study, cylindrical CM and 3DF scaffolds (Ø7 × 4 mm) were seeded at a density of 8 × 10⁶ cells per scaffold and were also cultured *in vitro* for 21 days, as described above. Constructs were subsequently implanted in subcutaneous pockets of 6-week-old nude mice (HdCpb:NMRI-nu, Harlan, Horst, The Netherlands). Mice were killed 28 days after implantation and constructs were processed histologically and biochemically, as described below.

Autologous implantation in vivo

After 5-week cell expansion and tissue culture period, tissue-engineered femoral and tibial constructs were implanted into rabbits autologously (Fig. 1). This second

operation was performed in an identical manner to the first osteotomy procedure described above. The left knee joint was exposed and the Swanson prosthesis and any visible scar tissue was carefully removed. A fresh bone bed was prepared by additional osteotomy (~1 mm thick) of bone from the remaining distal femur and proximal tibial surfaces. Tissue-engineered femoral and tibial implants were press fitted into medullary cavities with additional resorbable suture fixation (Vicryl 3/0) between the border of the construct and adjacent periosteum. After verifying secure positioning and articulation between implants, the joint was closed as described above. Rabbits were monitored for a 6-week period for signs of infection or inflammation. Plaster casts were initially to be used to stabilize the joint within the first 2 weeks post-surgery; however, the rabbits naturally maintained a full extension of the knee during this period and casts were not used. Rabbits had freedom to move and their progress in terms of mobility and joint function was monitored daily. The animals were allowed to naturally initiate joint motion and partial load bearing on operated joints as soon as functionally comfortable (typically 2–4 weeks). After 6 weeks, rabbits were sacrificed, and operated (left) and control (right) joints harvested for observation and histological analysis as described below.

Evaluation – in vitro study (Experiment 1)

Scaffold characterization. Methods for characterizing porosity, average pore size, pore geometry, and accessible pore volume of CM and 3DF scaffolds have been described previously (22,28) using techniques such as micro-computed tomography (μ CT) and scanning electron microscopy (SEM), as well as calculations from mass/volume measurements and fibre deposition geometries.

Biochemical analysis. CM and 3DF constructs ($n = 4$ per time point) were placed in a solution containing proteinase K (1 mg/ml), pepstatin A (10 μ g/ml) and iodoacetamide (185 μ g/ml) (Sigma-Aldrich), overnight at 56 °C, to digest the cells and extracellular matrix (ECM). Glycosaminoglycan (GAG) was quantitatively determined by reaction with dimethyl-methylene blue dye (Sigma-Aldrich) (33). Intensity of colour change was measured immediately using a microplate reader (EL 312e, Bio-TEK Instruments, Winooski, VT), at absorbance of 520 nm. Values were compared to those from a standard of chondroitin sulphate B (Sigma-Aldrich). GAG content was normalized to total DNA, which was measured using a Cyquant dye kit (Molecular Probes, Invitrogen, Breda, The Netherlands) *per* manufacturer's instructions, using a fluorescent plate reader (PerkinElmer, Waltham, MA, USA).

Cell viability. A live/dead assay (Molecular Probes) was used to determine cell viability, as described previously (22). Briefly, constructs were harvested and 1-mm-thick sections were incubated in phosphate-buffered saline solution containing 6 μ mol/l ethidium bromide homodimer and 2 μ mol/l calcein AM for 30 min at 37 °C, and immediately examined using an inverted fluorescence microscope (Nikon Eclipse E400, Nikon Corp., Tokyo, Japan) with a FITC Texas Red filter (Nikon Corp., Tokyo, Japan).

Histological analysis. CM and 3DF constructs were fixed overnight in 0.14 M cacodylate buffer (pH = 7.2–7.4) containing 0.25% glutaraldehyde (Merck, Darmstadt, Germany). Samples were then dehydrated in a series of sequential ethanols, embedded in glycol-methacrylate (Merck) and cut using a microtome to yield 5- μ m-thick sections. These were stained with haematoxylin (Sigma-Aldrich) and fast green (Merck) to visualize cells/cell nuclei, and safranin O (Sigma-Aldrich) to visualize sulphated extracellular GAG.

SEM. To examine scaffold architecture and cell morphology, scaffolds were fixed and dehydrated as described above, and critical point dried from liquid carbon dioxide using a CPD 030 Critical Point Dryer (BAL-TEC AG, Balzers, Liechtenstein). Samples were then sputter-coated (Cressington Scientific Instruments, Watford, UK) with a thin gold layer and studied using a Philips XL30 (Philips, Eindhoven, The Netherlands) Environmental Scanning Electron Microscope.

Immunohistochemistry. Collagen types I and II were immunolocalized using an Animal Research Kit (Dako, Glostrup, Denmark) in combination with collagen types I (1 : 1000, Ab-1, Calbiochem, EMD Biosciences, Calbiochem/Merck, Darmstadt, Germany) and II antibodies (1 : 200, II-II6B3, Developmental Studies Hybridoma Bank) as described previously (34). Staining was visualized using DAB solution (Dako) for 5 min in addition to counterstaining with haematoxylin (Sigma-Aldrich).

Mechanical properties. Static and dynamic unconfined compression tests were performed on hydrated, cylindrical CM and 3DF scaffolds (that is, cell-free scaffold), as well as on tissue-engineered constructs (that is, cells/ECM plus scaffold) after 21 days *in vitro* culture and 28 days subcutaneous implantation ($n = 4$ per time point). Constructs were allowed to equilibrate for 3 h in phosphate-buffered saline prior to testing. Equilibrium modulus (20% strain) and dynamic stiffness (15% strain deformation at 0.1 Hz) were determined as described previously (22), and were compared to native articular cartilage properties, obtained from the literature (22,35).

Evaluation – in vivo study (Experiment 2)

Prior to implantation. Anatomical femoral and tibial implants cultured *in vitro* for 21 days were analysed qualitatively for cell viability as well as distribution and composition of ECM components, prior to implantation. Cell viability, histological analysis, SEM and immunohistochemistry were performed as described above.

In vivo analysis. General observations of operated knees were made prior to joint harvesting and histological analysis. Operated joints were checked for range of movement. In addition, a small region of joint capsule was exposed and observations relating to articulation between femoral and tibial implants, implant fixation, and synovial fluid appearance were made. Entire joints, containing intact femoral and tibial implants, were harvested and evaluated histologically as described below.

Histological analysis. Histology was performed on non-decalcified bone and cartilage samples from both operated and control knee joints as described previously (36). Briefly, entire joints were fixed in 4% buffered paraformaldehyde for 2–3 weeks, dehydrated in increasing grades of ethanol, defatted and cleared with xylene, and embedded in acrylic resin (HistoDur®, Leica, Basel, Switzerland; 500 ml methyl methacrylate base, 30 ml phthalic acid dibutyl ester plasticizer, and 5 × 1 g activator). From the polymerized blocks, 200-µm sections were cut using a sawing microtome (Leica SP 1600). These sections were then mounted on acrylic Plexiglas slides (Wachendorf Perspex GS Acrylicglas Opal 1013, Wachendorf AG, Basel, Switzerland), and polished. Sections were etched for 5 min and surfaces stained with basic fuchsin (5 min) and methylene blue (5 min), to visualize bone and soft tissues, respectively. Alternatively, thionine (10 min) was used to visualize soft tissue as well as extracellular GAG.

Statistics

All data are presented as mean ± standard deviations. To test levels of significance in GAG/DNA content and mechanical properties, one-way analysis of variance was performed using SigmaStat statistical software (SPSS Inc., Chicago, IL, USA) with Student–Newman–Keuls post-hoc testing ($P < 0.05$).

Results

In vitro study (Experiment 1)

Scaffold characterization. Previous characterization studies carried out by our group on CM and 3DF 300/55/45

PEGT/PBT scaffolds (27,28) has demonstrated that designed 3DF scaffolds contain a simple, highly accessible pore volume due to large interconnecting pores, whereas as a result of the more random particle-leaching production process, CM scaffolds contained a more tortuous, less accessible pore volume due to smaller interconnecting pores (28). Based on fibre deposition geometries (175 µm fibre diameter and 0.6 mm fibre spacing) and mass volume techniques, 3DF scaffolds used in this study were approximately 76% porous, compared with 80% in CM scaffolds. This indicated that similar pore volumes were available in both scaffold architectures to compare cell infiltration, tissue formation and nutrient/waste exchange. However, 3DF scaffolds had a considerably larger average pore size (415 µm) than CM scaffolds (182 µm) (28). Surface area-to-volume ratio in 3DF and CM scaffolds was 5.5 mm⁻¹ and 10.0 mm⁻¹, respectively.

Biochemical analysis. To determine effects of scaffold architecture on cartilage tissue formation, quantitative assays for GAG and DNA were performed. Significantly higher GAG *per wet weight* was measured in 3DF compared to CM scaffolds, irrespective of *in vitro* culture or subcutaneous implantation *in vivo* (Fig. 3a). However, *in vivo* samples contained significantly higher GAG *per wet weight* than *in vitro* samples of the same architecture. When normalized to cell number, greater GAG/DNA was measured in 3DF compared with CM scaffolds at both 21 days *in vitro* and 28 days subcutaneous implantation *in vivo* (Fig. 3b). This suggests that for expanded rabbit chondrocytes, porous 3DF architectures provide conditions that stimulate chondrogenic ECM formation.

Mechanical properties. Irrespective of culture duration, mechanical testing (Fig. 3c,d) showed that 3DF scaffolds had significantly greater equilibrium modulus and dynamic stiffness (at 0.1 Hz) compared to CM scaffolds of the same porosity (≈ 80%). This was likely to be due to the organized network of PEGT/PBT fibres in 3DF scaffolds compared to the more random architecture in CM scaffolds (28). *In vitro*-cultured 3DF scaffolds had a similar dynamic stiffness to that of natural bovine cartilage prior to implantation (22) and human articular cartilage at 0.1 Hz (35), whereas CM scaffolds were approximately 50% less stiff. Steadily increasing GAG content and ECM formation during *in vitro* and *in vivo* culture (Fig. 3a,b) resulted in synergistic increase in mechanical properties in both CM and 3DF constructs with time, over non-cell-seeded scaffolds (Fig. 3c,d). However, 3DF scaffolds cultured *in vivo* had a significantly greater dynamic stiffness than 3DF scaffolds cultured *in vitro* (Fig. 3d). Due to the limited thickness of cartilage biopsies (approximately 200 µm)

Figure 3. (a) Glycosaminoglycan (GAG) per wet weight and (b) GAG per DNA data for compression moulded (CM) and 3D fibre deposited (3DF) scaffolds after 21 days *in vitro* culture and 28 days subcutaneous implantation. (c) Static (equilibrium modulus) and (d) cyclic (dynamic stiffness) compressive mechanical properties of CM and 3DF scaffolds compared to native bovine and human articular cartilage. Significant differences denoted by: a = between CM, b = between other time points for the same architecture (# from Woodfield *et al.* (22); * from Treppo *et al.* (35)).

and resolution of the test apparatus, mechanical properties of rabbit articular cartilage could not be determined using the same techniques described for constructs. However, previous studies have demonstrated that similar properties exist between distal femoral cartilage in rabbit, human and bovine species (37,38).

Histological analysis and cell viability. Dynamic seeding of expanded (passage 2) rabbit chondrocytes resulted in homogeneous distribution of cells throughout cylindrical CM and 3DF scaffolds (not shown). After 21 days of spinner flask culture, SEM images demonstrated considerable ECM formation (Fig. 4a,b), and after 28 days subcutaneous implantation, cartilage-like tissue formation was observed in both CM and 3DF scaffolds, as indicated by positive safranin O staining for GAG (Fig. 4e,f) and collagen type II staining (not shown). However, tissue formation and fluorescent staining for live (green) cells was limited to peripheral regions of CM scaffolds (Fig. 4c,e), whereas in 3DF scaffolds, positive safranin O staining, collagen type II expression and viable cells were more predominantly observed throughout the highly accessible and interconnected pore volume (Fig. 4d,f).

In vivo study (Experiment 2)

Prior to implantation. Due to the large size of femoral/tibial scaffolds (Figs 2h,i and 5a,b), and limited number of expanded rabbit chondrocytes available for seeding in both implantation and control experiments (20×10^6 cells/scaffold), obtaining a homogeneous distribution of cells and ECM formation was challenging. Nonetheless, after 21 days spinner flask culture, tissue formation was observed within CM and 3DF femoral and tibial implants with a translucent, glossy gross appearance (Fig. 5a,b). Furthermore, after *in vitro* culture, femoral and tibial implants had maintained their original anatomical shape.

Scanning electron microscopy. SEM analysis of sectioned implants prior to implantation demonstrated that even in these large implants, interconnecting pore volume in 3DF scaffolds was nearly completely filled with ECM prior to implantation (Fig. 5d). While cells and ECM were observed within central regions of CM scaffolds, most ECM formation occurred predominantly at the periphery (Fig. 5c).

Figure 4. Tissue formation in cylindrical compression moulded (CM) (a, c, e) and 3D fibre deposited (3DF) (b, d, f) scaffolds illustrated in SEM images (a, b) after 21 days *in vitro* culture as well as live (green)/dead (red) fluorescence staining (c, d) and safranin O staining (e, f) after 28 days subcutaneous implantation *in vivo* in nude mice (scale bar = 500 µm).

Figure 5. Gross appearance of tissue-engineered tibial implants (a, b), and cross-sectional SEM (c, d), safranin O plus cell viability (inset) staining (e, f), and collagen type I/II immunostaining images (g, h) after 21 days *in vitro* culture. (a, c, e, g) Computer numerical-controlled milled compression moulded (CM) scaffold; (b, d, f, h) 3D fibre deposited (3DF) scaffold.

Histological analysis and cell viability. Prior to implantation, safranin O staining revealed cartilage-like tissue formation and GAG staining in both CM (Fig. 5e) and 3DF scaffolds (Fig. 5f). Consistent with tissue distribution results from SEM images, GAG staining (Fig. 5e) and collagen type II (Fig. 5g) immunostaining were limited to superficial regions of CM scaffolds, but were observed more homogeneously throughout the interconnected pore volume of 3DF

scaffolds (Fig. 5f,h). Negligible amounts of collagen type I staining were observed in CM and 3DF scaffolds (Fig. 5g,h). Nutrient diffusion limitation related to implant size, did not appear to limit cell viability in 3DF scaffolds, with live (green-labelled) cells observed throughout the thickness of the implant (Fig. 5f inset). In CM implants, live cells were observed within a 1–2 mm peripheral border whereas central regions were almost acellular

rather than containing large numbers of dead (red) cells (Fig. 5e inset).

In vivo analysis. All surgery for joint osteotomy and implantation of tissue-engineered femoral and tibial implantation occurred without incident. After 6 weeks, rabbits had regained limited function in operated joints. Knee stability and range of motion were sufficient to allow partial load bearing with hopping, and voluntary grooming movements and use of the operated (left) leg was observed. At explanation, there were no indications of joint infection nor inflammation, other than expected scar tissue formation following osteotomy and temporary Swanson prosthesis placement. Joint capsules were healed at 6 weeks and synovia appeared clear.

Histology. Histological evaluation of control and operated joints showed that a significant proportion of articulating cartilage surfaces and underlying cancellous bone were removed during osteotomy. Nonetheless, the precise anatomical design of the articulating surfaces of constructs, stem assisted press-fit implantation and re-establishment of articulating surfaces between femur and tibia (Fig. 6b–d). Although rabbits evaluated in this feasibility study had resumed limited function with operated knees, the engineered cartilage-like tissue that was evident within *in vitro* cultured CM and 3DF femoral and tibial implants prior to implantation (Fig. 5) was not maintained after 6 weeks implantation *in vivo*. Instead, tissue filling pores throughout the articulating regions in 3DF implants contained a mixture of rounded and flattened cells and little to no staining for GAG (Fig. 6b,c), indicative of a predominantly fibrocartilage-like tissue repair. Limited amounts of tissue were observed in pores within the articulating regions of CM implants, but it was similar in appearance to that seen in 3DF implants. However, at the articulating surface of both CM and 3DF implants, a dense layer of tissue, aligned tangentially to the implant surface, was observed (Fig. 6b,c arrows), allowing the implants to facilitate joint articulation. 3DF implants appeared to have maintained their original shape and had not deformed nor fragmented after 6 weeks of implantation (Fig. 6c,d,f), and was likely due to their desirable mechanical stiffness. While bulk shape was maintained in general for CM implants, certain regions appeared to be less resistant to deformation (not shown). This may have been related to lower dynamic stiffness compared to 3DF implants, although differences in tissue formation, animal variability and joint use may also have had an effect.

With regard to construct fixation, ingrowth of cancellous bone was observed distally in the stem of 3DF implants after 6 weeks, bridging the entire joint space and rigidly fixing the tapered stem of the implant to the cortical bone

wall (Fig. 6h). Bone formation was observed in the stem of CM implants (Fig. 6g), but more proximally and to a lesser extent than 3DF implants. This may have been due to the more interconnected pore architecture within 3DF scaffolds, allowing more rapid vascularization and bone ingrowth.

Discussion

In this study, we investigated the feasibility of designing anatomically shaped, tissue-engineered implants and their ability to restore congruent articulating surfaces in small articulating joints, with short-term follow-up in a rabbit knee model, aimed at providing preliminary data that would form the basis for larger future animal studies. Resurfacing articulating cartilage in larger joints, such as the human knee or hip, represents a challenging problem (5,39), which was clearly beyond the scope of this study. Previous research has attempted to tissue engineer finger joints *in vitro* using poly-glycolic and poly-L-lactic acid co-polymer non-woven mesh scaffolds and selective placement of periosteum, chondrocytes and tenocytes (14,15). While individual cartilage, bone and tenocapsule components, with similar-structured human phalanges, were observed following subcutaneous implantation in mice, the scaffold components were sutured together and no functional evaluation under physiological loading *in vitro* or *in vivo* was performed. In other studies, inflexible moulding techniques were used, with limited control over mechanical properties of implants (that is, compressive modulus 12.5% that of native bovine cartilage) (40–43). Other approaches for repairing osteochondral defects have developed porous, biphasic scaffolds with optimized properties for promoting tissue formation and integration in cartilage and bone compartments (10–13,44).

To our knowledge, few *in vitro* and *in vivo* studies describing development of two tissue-engineered constructs, whose anatomically shaped surfaces are congruent and can articulate against one another under load bearing, have been described previously. We chose an autologous rabbit knee model in this study based on similar dimensions to the human metacarpophalangeal joint, and extensive use for focal articular cartilage and osteochondral defect repair models (4). Although the knee presents a significantly more complex joint than the finger in terms of anatomy and joint loading, it was important for this study to evaluate mechanical properties of implants in a load bearing capacity prior to moving to a larger study with long-term follow-up.

The novel aspect of this study is that with CT imaging and RP techniques described, we were not only able to design and engineer the anatomical external shape of PEGT/PBT scaffolds, but also engineer the internal pore

Figure 6. Histological sections of control (a) and operated joints (b–h) at 6-week follow-up using anatomically shaped compression moulded (CM) (c, e, g) and 3D fibre deposited (3DF) implants (b, d, f, h). Example of 3DF femoral and tibial implants shown in (b). Samples were stained with thionine (a–f) and basic fuchsin/methylene blue (g, h). Arrows indicate articulating surfaces (a–d); C, articular cartilage; M, meniscus; BM, bone marrow; CB, cortical bone; CBI, cancellous bone ingrowth.

architecture concurrently. RP techniques based on 3D fibre deposition, which directly allow control of complex shape as well pore architecture, offer greater flexibility and control (45–47), particularly in the design of porous scaffolds to meet specific anatomical and functional mechanical requirements (22). Based on *in vitro* data from the preliminary *in vitro* study (Experiment 1), we observed that large interconnecting pores and the highly accessible pore volume in 3DF scaffolds enhanced

cartilage tissue formation resulting in significantly greater GAG/wet weight (Fig. 3a) and GAG/DNA content (Fig. 3b), with more homogeneous cell and ECM distribution (Fig. 4), compared to CM scaffolds. This was consistent with our previous studies evaluating cartilage tissue formation *in vitro* and *in vivo* on 6×4 mm CM and 3DF scaffolds using bovine (28,48) and expanded human nasal chondrocytes (27), indicating significantly greater GAG/DNA content and chondrocyte re-differentiation potential

(as measured by collagen type II mRNA) of 3DF scaffolds. Furthermore, by measuring nutrient diffusion (that is, using an oxygen probe) within tissue-engineered CM and 3DF architectures (48), we demonstrated that enhanced cartilage formation and cell/ECM distribution following subcutaneous implantation in nude mice, was due to less pronounced oxygen gradients in 3DF compared to CM scaffolds. These data suggest that, for the design of large, anatomically shaped implants of clinically relevant size, interconnecting 3DF scaffold architecture provides a porous 3D environment that promotes chondrocyte attachment and ECM synthesis with minimal nutrient limitations, and was likely to be the reason that we observed homogenous distribution of viable cells throughout femoral and tibial 3DF implants compared with CM implants in this study.

3DF constructs also exhibited significantly higher equilibrium modulus and dynamic stiffness properties to CM scaffolds, which increased with *in vitro* and *in vivo* culture time. Based on a previous study (22), we designed 3D fibre deposition patterns to produce interconnecting porous architectures with similar dynamic stiffness (at 0.1 Hz) to native articular cartilage, thereby providing suitable mechanical stability to allow early implantation and load bearing, of an immature tissue-engineered cartilage construct. In adopting this early implantation strategy, extended *in vitro* culture periods and mechanical pre-conditioning of the tissue-engineered implant to achieve sufficient mechanical properties, were avoided. We preferred to adopt this strategy, given that previous studies have shown that implantation of immature (2-week-old) tissue-engineered cartilage constructs promoted better integration to host tissue than more mature (8-week-old) constructs (49).

Following implantation of *in vitro* cultured femoral and tibial implants in the rabbit osteotomy model (Experiment 2) and a short follow-up after 6 weeks, we observed that limited joint function and weight bearing had been regained. This was a positive result given that the main aim of the study was to evaluate implant fixation, stability and, more importantly, restoration of congruent articulating surfaces. Histological analysis of operated joints, however, indicated that the repair tissue present within femoral and tibial CM and 3DF implants did not resemble the level of cartilage-like tissue present prior to implantation, and was predominantly fibrous-like. Collagen types I and II immunohistochemistry was not evaluated for *in vivo* samples. Nevertheless, implant geometry had been maintained and a functional articulating layer, albeit fibrous-like, was observed. Moreover, the tapered stem and press-fit design of the implants, particularly in 3DF constructs, provided a suitable environment for rapid cancellous bone growth, providing sufficient implant fixation after 6 weeks. Although a suitable outcome in terms of implant fixation

and function in the short-term was obtained, there are several possible explanations for not observing a hyaline-like repaired tissue. First, we did not investigate cell viability nor cell retention in the tissue-engineered constructs after implantation. Although an autologous approach was adopted, it is possible that cells did not survive the implantation procedure, and that due to excessive infiltration of blood- and/or marrow-derived host cells and the associated wound healing response, maintenance of the tissue-engineered cartilage present at implantation was compromised (50). Recent *in situ* hybridization studies have demonstrated that tissue growth in various regions of a tissue-engineered human phalanges model implanted in nude mice for 20 days resulted from the original chondrocytes and osteoblasts seeded within the construct *in vitro*, rather than infiltration and/or differentiation of host cells once implanted (51). However, in osteochondral cartilage repair experiments in rabbits, Ostrander *et al.* (52) demonstrated that tissue-engineered implants are rapidly infiltrated with host cells after implantation, thereby affecting repair responses. In that study, only 14% of the original number of cells implanted were observed within the defect after 28 days implantation *in vivo*. Furthermore, in the current study, the rabbit osteotomy model required surgery twice, thereby subjecting the tissue-engineered implants to a severely degenerated joint environment at the second surgery. It is unlikely that re-establishment of the synovial capsule occurred soon after either operation, and subsequent nutrients supplied by the synovial fluid necessary for maintaining chondrocyte function were limited.

Conclusions

Development of anatomically shaped implants based on 3D CT data is a feasible process. In future, MRI scans may provide even greater anatomical detail. Using advanced RP techniques, we describe in this study the ability to tissue engineer *in vitro* anatomically shaped cartilage constructs to serve as osteochondral implants. Porous, interconnected 3DF scaffold architectures enhanced chondrocyte attachment and re-differentiation capacity while exhibiting mechanical properties similar to native articular cartilage explants.

Restoring congruent articulation in a rabbit knee at 6 weeks following femoral and tibial osteotomy is possible, although the poor quality of repaired cartilage needs to be addressed in future studies.

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