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Integrated Bi-Layered Scaffold for Osteochondral Tissue Engineering

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Abstract

Osteochondral tissue engineering poses the challenge of combining both cartilage and bone tissue engineering fundamentals. In this study, a sphere-templating technique was applied to fabricate an integrated bi-layered scaffold based on degradable poly(hydroxyethyl methacrylate) hydrogel. One layer of the integrated scaffold was designed with a single defined, monodispersed pore size of 38 μm and pore surfaces coated with hydroxyapatite particles to promote regrowth of subchondral bone while the second layer had 200 μm pores with surfaces decorated with hyaluronan for articular cartilage regeneration. Mechanical properties of the construct as well as cyto-compatibility of the scaffold and its degradation products were elucidated. To examine the potential of the biphasic scaffold for regeneration of osteochondral tissue the designated cartilage and bone layers of the integrated bi-layered scaffold were seeded with chondrocytes differentiated from human mesenchymal stem cells and primary human mesenchymal stem cells, respectively. Both types of cells were co-cultured within the scaffold in standard medium without soluble growth/differentiation factors over four weeks. The ability of the integrated bi-layered scaffold to support simultaneous matrix deposition and adequate cell growth of two distinct cell lineages in each layer during four weeks of co-culture in vitro in the absence of soluble growth factors was demonstrated.

1. Introduction

Osteochondral defects, typically caused by traumatic injury or disease, are associated with damage of both articular cartilage and underlying subchondral bone.^[1] While chondral defects exhibit very poor regeneration capacity due to the avascular nature of articular cartilage, osteochondral defects allow for the recruitment of progenitor cells from the localized bone marrow to assist in regeneration of the cartilage and underlying bone structure.^[1,2] Unfortunately, the result is the formation of fibrocartilage in the defect void that has inferior mechanical properties in comparison to native articular cartilage. This scenario leads to further degeneration of both repaired and adjacent native tissues, inducing a decline toward osteoarthritic conditions.^[1–3] Currently, osteochondral defects are mostly treated by autologous osteochondral grafts (i.e., mosaicplasty technique), where the autograft is harvested from a non-load-bearing area of a healthy joint and implanted into the defect.^[4,5] Despite the encouraging results reported, the mosaic-plasty technique suffers from several limitations, such as the amount of material available, difficulty matching the topology of the graft with the injured site, and donor site morbidity.^[1,6] Osteochondral tissue engineering (OTE) offers an alternative with the potential to overcome the above challenges by utilizing three dimensional (3D) scaffolds that promote hybrid regeneration of both bone and cartilage tissues.^[7–10] In particular, one of the recent approaches in OTE uses bi-layered scaffolds that promote individual growth of cartilage and bone tissues simultaneously. The reported bi-layered scaffolds for OTE can be classified into three main types: (1) cartilage tissue on bone scaffold, where chondrocytes are seeded directly onto a bone scaffold base; (2) assembled bi-layered scaffold, where two distinctly different cartilage and bone scaffolds have been assembled either before or during surgical implantation; and 3) *integrated* bi-layered scaffold that consists of two different scaffold segments that have been amalgamated together via the integration of a mutual material common to both layers.^[7,9] The advantage of an integrated bi-layered scaffold is that it provides a complete transition between the bone and the cartilage scaffold layers without requiring a joining procedure during implantation thus simplifying clinical application.

The fabrication of *integrated* bi-layered scaffolds for OTE is challenging.^[7,8] Each layer of the scaffold must be engineered to exhibit tissue-specific biophysical conditions and environments to support a unique cell type leading to the regeneration of two distinct, but integrated tissue types: articular cartilage and subchondral bone. There are only few reports in the literature to date where various fabrication techniques have been applied to obtain integrated bi-layered scaffolds for OTE that are composed of natural and/or synthetic polymers.^[11–17] To enhance bone regeneration, ceramic materials, such as hydroxyapatite (HAp) or β -tricalcium phosphate (TCP), are often incorporated into the portion of the bi-layered scaffold that would generate new bone. For instance, Sherwood et al. reports constructing integrated bi-layered scaffolds using a 3D printing process and salt leaching technique, to produce cartilage and bone regions composed of poly(lactic-co-glycolic acid)/poly(lactic acid) (PLGA/PLA) and PLGA/TCP, respectively.^[11] Holland et al. reported composing an integrated bi-layered scaffold using a two stage crosslinking procedure, where bone and cartilage layers were composed of oligo(poly(ethylene glycol) fumarate) (OPF) and OPF/gelatin microparticles, respectively.^[12] Chen et al. reported developing a bi-layered scaffold using a combination of solvent casting, salt leaching and freeze-drying techniques, where collagen and PLGA/collagen comprised the cartilage and bone layers, respectively.^[13] Oliveira et al. and Malafaya et al. report fabricating chitosan/HAp-chitosan integrated bi-layered scaffolds utilizing sintering/freeze-drying techniques and particle aggregation methodology, respectively.^[14,15]

The aforementioned fabrications for integrated bi-layered scaffolds do not provide precise control over pore size, microstructure, or pore interconnectivity. Our research group has

developed a sphere-templating technique to fabricate porous template scaffolds (PTSs) with interconnected, controllable microstructure where every pore is the same size, and pore interconnects are also uniform in size, with both parameters being adjustable.^[18–22] PTSs based on various materials, such as poly(2-hydroxyethyl methacrylate) (polyHEMA) hydrogels, poly(N-isopropyl acrylamide) (polyNIPAM), silicon elastomer, fibrin, and polyurethane, were fabricated, in pore sizes ranging from 20–200 μm . We have studied the healing of PTSs in multiple in vivo environments (mouse subcutaneous, rat heart, mouse percutaneous) and have determined that a pore size between 30 and 40 μm is optimal for bio-integration of the scaffold into host tissue through enhanced angiogenesis and reduced fibrosis.^[20,21,23–29]

Atom transfer radical polymerization (ATRP) is a controlled radical polymerization (CRP) that allows for the synthesis of polymers with well-defined macromolecular structure, predictable molecular weight (Mw), a narrow polydispersity index (PDI) and tunable properties.^[30–35] Applying ATRP, our research group has developed a method of synthesizing degradable poly-HEMA and polyNIPAM hydrogels with controlled Mw of the parent polymer and its degradation products.^[36,22]

Hyaluronic acid (HAc), a basic component of native articular cartilage, is an important factor in enhancing chondrogenesis and maintaining homeostasis; chondrocytes interact with HAc in the pericellular environment via CD44 receptors located on the cell surface.^[37,38] For this reason, HAc is often incorporated into scaffolds for articular cartilage tissue engineering to enhance regeneration.^[39,40]

Here we report on the design, fabrication, and evaluation of an integrated bi-layered scaffold for OTE based on a degradable poly(2-hydroxyethyl methacrylate) (polyHEMA) hydrogel. Each layer of the bi-layered scaffold was designed with defined pore sizes and bioactive molecules, such as HAc and HAp, selected specifically to support regeneration of articular cartilage and subchondral bone. The sphere-template fabrication technique was applied to obtain an integrated bi-layered scaffold with controlled, monodisperse pore sizes within each layer and highly interconnected networks. Degradable polyHEMA, which amalgamates cartilage and bone layers of the scaffold, was synthesized by ATRP of HEMA. Mechanical properties of the construct as well as the cytotoxicity of the scaffold and its degradation products were determined. Then an in vitro study was carried out where the cartilage and bone layers of the integrated bi-layered scaffold were co-cultured with differentiated to chondrocytes human mesenchymal stem cells (hMSCs) and primary hMSCs, respectively. The ability of the bi-layered scaffold to support simultaneous matrix deposition and adequate cell growth and differentiation in each layer during four weeks of in vitro co-culture in the absence of supplied soluble growth/differentiation factors was examined.

2. Experimental Section

2.1. Materials

The following chemicals were purchased from Sigma and were used without further purification: HEMA (ophthalmologic grade, 99%), polycaprolactone (PCL) diol (Mn ~530 g/mol), methacryloyl chloride (97%), triethylamine (TEA) (99.5%), dimethylsulfoxide (DMSO) anhydrous (99.9%), copper(I) chloride (CuCl) (99%), 2-chloropropionyl chloride, 2,2'-bipyridyl, hexadecyltrimethylammonium bromide (CTAB) (99%), methacrylic anhydride (94%), diammonium hydrogen phosphate ((NH₄)₂HPO₄), calcium nitrate (Ca(NO₃)₂·4H₂O), phenol red-free Dulbecco's modified eagle's medium (DMEM), chondroitin sulfate, 1,9-dimethylmethylene blue (DMMB), magnesium sulfate (MgSO₄). Uncrosslinked poly(methyl methacrylate) (PMMA) microspheres of two size ranges (sphere diameter 200 μm and 38 μm) were purchased from Kupa Inc. HPLC grade

dichloromethane, acetonitrile and acetone were purchased from EMD Chemicals Inc. Vybrant MTT cell proliferation assay kit, MSC fetal bovine serum (FBS), 1% antimycotic/antibiotic and alpha minimum essential medium (α -MEM) were purchased from Invitrogen. Medical grade hyaluronan (HAc) (Mw 60 kDa) was purchased from Lifecore Biomedical.

2.2. Synthesis of Methacrylated HAc (HAcMA)

An aqueous solution of HAc (600 mg, 1% w/v) was mixed with an aqueous hexadecyltrimethylammonium bromide (CTAB) solution (550 mg, 1% w/v) [*molar ratio 1 CTAB molecule to 1 COOH moiety*] at room temperature to form the precipitate HAc-CTA.^[41] Then pure HAc-CTA was filtered, washed with distilled water and lyophilized. HAc-CTA was dissolved in DMSO to form a 0.5% w/v solution. Next, methacrylic anhydride (MA) was added dropwise to the solution under N₂ atmosphere [*molar ratio 1 MA to 1 OH moiety*]. The reaction mixture was stirred at room temperature for 24 h. For the purpose of chemical analysis, CTA salts were removed in situ via salt exchange with an excess volume of 0.2 M NaCl. HAcMA was purified via dialysis against distilled water for three days and lyophilized. The degree of methacrylation (51%) was determined with ¹H-NMR by spectroscopy (Bruker DRX 500 MHz; D₂O as the solvent) by examining the ratio between integral area under the peak at δ 6.0 and carbohydrate protons.^[42] For hydrogel scaffold fabrication, HAc-CTA-MA was purified by precipitation with distilled water and ethanol, and lyophilized.

2.3. Synthesis of HAp Nanoparticles

HAp nanoparticles were prepared using a modified wet chemical procedure reported previously.^[43] Briefly, 400 mL of 5% w/v (NH₄)₂HPO₄ solution (pH 10.4) was added slowly (1.5 mL/min) into 300 mL of 19% w/v Ca(NO₃)₂·4H₂O solution (pH 10.4). Precipitates of HAp nanoparticles, obtained after mixing the two solutions, were aged for 24 h and washed with deionized water until pH decreased to 7. The precipitates were dried at 80 °C and calcined at 600 °C to remove the any organic compounds.

2.4. Sphere-Templating Scaffold Fabrication

2.4.1. Template Preparation—PMMA microspheres were fractionated with an ATM model L3P Sonic Sifter into two groups with different diameters: 188 ± 26 and 35 ± 3 μ m. The microspheres with nominal diameters of 188 ± 26 μ m were transferred to a mold (composed of two microscope slides separated by a 1 mm thick Teflon spacer) and sonicated for 10 minutes for optimal packing. The packed microspheres were then sintered for 20 h at 140 °C to obtain PMMA templates with neck sizes (interconnects between the beads) approximately 30% of the microsphere diameter. The 35 ± 3 μ m diameter microspheres were coated with HAp nanoparticles (1% w/w) by intensive grinding together of the beads with HAp using a mortar and a pestle. The coated microspheres were transferred to a mold, sonicated and sintered for 20 h at 190 °C as described above.

2.4.2. Scaffold Fabrication—The integrated bi-layered scaffold is comprised of a degradable polyHEMA hydrogel with theoretical Mw of 20 kg/mol that was synthesized by ATRP based on the procedure previously reported by Atzet et al.^[36] First, the 35 and 188 μ m PMMA microsphere templates (section 2.4.1) were stacked one on top of the other in a glass mold (composed of two microscope slides separated by a 2-mm thick Teflon spacer). Next, under an inert atmosphere, the mold was infiltrated with a nitrogen-purged reaction mixture consisting of HEMA (2 g, 0.0154 mol), bi-functional PCL-based initiator (0.08 g, 1×10^{-4} mol), PCL dimethacrylate (PCLDMA) (0.32 g, 4.6×10^{-4} mol, 3% mol/mol_{HEMA}), HAc-CTA-MA (40 mg, 2% w/w_{HEMA}), 2,2'-bipyridyl (0.0198 g, 2×10^{-4} mol), and CuCl (0.039 g, 2.5×10^{-4} mol) dissolved in 1 mL DMSO. Polymerization of HEMA by ATRP

was performed for 24 h at room temperature. Next, the PMMA bead template surrounded by the polymerized polyHEMA hydrogel was removed from the mold and placed in dichloromethane to dissolve the PMMA microspheres. The resulting porous scaffold, composed of a degradable polyHEMA gel, was washed in acetone and then hydrated in distilled water. Finally, CTA salts were removed in situ via salt exchange with an excess volume of 0.2 M NaCl, returning the grafted HAc to its hydrophilic state.

2.5. Mechanical Testing

Mono-layered scaffolds of the individual layers of the bi-layered scaffold were also fabricated and analyzed along with the integrated bi-layered scaffold. Cylindrical hydrated and dry acellular samples (5 mm × 10 mm) were tested in unconfined compression between impermeable platens using a Universal Testing Machine (Instron 5543) with load cells of 10N or 500N, respectively, using ASTM D695-10 as a guide. The compression tests were performed at room temperature with a constant strain rate of 1.3 mm/min up to 50% reduction in specimen height. The compressive modulus was calculated as the ratio of stress to strain of the initial linear region of the stress-strain diagram. At least seven replicates from each sample were tested.

2.6. Scanning Electron Microscopy

Acellular scaffolds and cell morphologies within the cultivated scaffolds were characterized by scanning electron microscopy (SEM) (FEI SEM XL Siron). To observe scaffolds' morphology, lyophilized samples were sputter coated with 7 nm of Au/Pd (SPI Supplies). To observe cell morphology and cell infiltration into a scaffold, histological sample cross-sections of approximately 1 mm thick previously embedded for histological sectioning were deparaffinized, rehydrated, lyophilized and then Au/Pd sputter coated as described above.

2.7. In Vitro Cell Culture Study

2.7.1. hMSC Culture—Primary human bone marrow derived MSCs were received frozen at passage 1 and stored in liquid nitrogen until use.^[44] Cells were thawed and placed in T75 tissue culture polystyrene flasks with 13 mL of base medium (α -MEM, 10% MSC FBS, 1% antimycotic/antibiotic). Cells were cultured at a density of 500 000 cells per flask at 37 °C and 5% CO₂. After 24 h the medium was replaced with fresh medium. The culture medium was changed every 2–3 days. At 90% confluency, medium was removed and 4 mL of 0.25% trypsin-EDTA was added and cells were incubated at 37 °C, 5% CO₂ for 10 minutes. Cell solution was diluted with 8 mL of base medium and centrifuged at 600 g for 5 minutes. Cell pellets were re-suspended in base medium, diluted in trypan blue, and counted using a hemocytometer. Cells were then seeded in T75 flasks as described above. Cells obtained at passages 2–3 were used in this study.

2.7.2. Cytotoxicity

2.7.2.1. Bi-layered Scaffold Cytotoxicity: MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole), is reduced to purple formazan in living cells; MTT assays are essentially cell viability assays. Scaffolds were disinfected with 70% ethanol, washed with sterile PBS and placed in base medium for 24 h. Simultaneously, 24-well culture plates were seeded with hMSCs (1×10^4 cells/well) and cultured at 37 °C, 5% CO₂ for 24 h, then the medium was removed and replaced with 0.5 mL of scaffold eluent. The cells were incubated with medium eluted from the scaffold ($n = 6$) for an additional 24 h; cells washed with phenol red-free Dulbecco's modified eagle's medium (DMEM) then exposed to 0.5 mL of phenol red-free DMEM medium containing 10% of MTT solution (5 mg/mL MTT reagent in PBS) for 4 h. DMSO (1 mL) was then added to extract a blue product after vigorous pipetting. Tissue culture polystyrene (TCPS) ($n = 6$) and latex ($n = 6$)

were used as the negative and positive controls, respectively. A 200 μ L aliquot from each well was transferred to a 96-well plate and the absorbance was measured at 550 nm with a microplate reader (tunable VERSAmax microplate reader, Molecular Devices).

2.7.2.2. Degradation Product Cytotoxicity: Scaffolds were hydrolyzed in 0.1 N NaOH, the solution was neutralized and degradation products were extracted with chloroform. The organic phase was dried over magnesium sulfate, filtered, and chloroform was evaporated to obtain a white powder. The degradation products were dissolved in base medium at various concentrations (0.1–15 mg/mL) and the effects on cell viability assessed as above.

2.7.3. Chondrogenic Differentiation of hMSCs—Recombinant transforming growth factor beta 1 (TGF- β 1, PeproTech) was used to induce chondrogenic differentiation of hMSCs. TGF- β 1 was reconstituted to a concentration of 10 ng/ μ L in 4 mM HCl supplemented with 1 mg/mL bovine serum albumin (BSA), sterile filtered, and frozen at -20°C until use. hMSCs from passage 3 were transferred to 15 mL poly-propylene tubes (250 000 cells per tube) and centrifuged at 600 g for 5 minutes. Base medium was removed and 500 μ L of chondrogenic medium (base medium supplemented with 10 ng/mL of TGF- β 1) was added and the cells were maintained in a cell culture incubator at 37°C , 5% CO_2 for a period of 14 days and chondrogenic medium was replaced every 2–3 days. After 2 weeks the cell pellet was partially digested in sterile collagenase (125 CDU/mg, Sigma) solution (1 mg/mL) in α -MEM for 40 minutes. Cells were isolated, collected and counted using methods outlined above.

2.7.4. Seeding of the Bi-Layered Scaffold—The polyHEMA-based bi-layered scaffolds were punched into 5 mm diameter disks, disinfected with 70% ethanol, washed with sterile PBS and soaked in base medium (α -MEM, 10% MSC FBS, 1% antimicrobial/antibiotic) at 4°C overnight. Scaffold samples were placed into individual wells of 48-well non-treated polystyrene plates. First, the 38 μ m pore layer of each scaffold was seeded with 420 000 hMSCs in 900 μ L of base medium. The scaffolds were incubated at 37°C , 5% CO_2 ; the base medium was changed on day 2. On day 3, the scaffolds were inverted and the 200 μ m pore layer of each scaffold was seeded with 2.5×10^6 differentiated to chondrocytes hMSCs and 900 μ L of base medium was added. All scaffolds were cultured for an additional four weeks and the medium was changed every 2–3 days.

2.7.5. Histological and Immunohistochemical Analysis—After the in vitro culture studies, the scaffolds were fixed with a methanol/glacial acetic acid solution (9:1 v/v) for 18 h at 4°C . Next, the sample scaffolds were dehydrated through a graded ethanol/xylene series and embedded in paraffin. Paraffin blocks were sectioned (5 μ m thick) and placed onto charged glass slides, deparaffinized and rehydrated. For histological analysis, sections were stained with (1) Mayer's hematoxylin and eosin (H&E) to visualize cell infiltration and distribution, (2) Alizarin Red to determine calcium deposition, (3) Masson's trichrome to explore collagen production and alignment, and (4) Alcian blue (pH 1) to identify sulfated glycosaminoglycans (GAG). Immunohistochemical (IHC) analysis for type II collagen was performed using standard avidin-biotin complex peroxidase-based antibody staining (Vectastain, Vector Laboratories). Rehydrated paraffin sections were heat-pretreated for antigen retrieval in Tris/EDTA buffer (pH 9.0). Primary mouse anti-human monoclonal antibody for type II collagen (1:50 dilution) was obtained from Santa Cruz Biotechnology. Secondary horse anti-mouse antibody (1:400 dilution) was obtained from Vector Laboratories. Sections with omitted primary antibody served as controls. Images were obtained with a Nikon Eclipse E800 upright microscope equipped with MetaMorph® software (version 6.0, Molecular Devices).

2.7.6. Biochemical Analysis

2.7.6.1. Alkaline Phosphatase Assay: Alkaline phosphatase (ALP) activity was quantified at two and four weeks of cell culture to evaluate osteogenic differentiation. For this purpose, medium was aspirated off of each well and bi-layered scaffolds ($n = 3$) were rinsed with sterile PBS. Samples were then transferred to new wells and 200 μL of lysing solution (Cell Lytic™, Sigma) was added. Samples were incubated in the lysing solution for 20 minutes at room temperature and then frozen at $-20\text{ }^{\circ}\text{C}$ until use. Lysate from each sample was assayed in triplicate ($n = 3$) using a clear bottom 96-well plate. The ALP activity of the samples (IU/L) was measured using a commercially available assay kit (QuantiChrom™ Alkaline Phosphatase Assay Kit). Absorbance values were recorded with a microplate reader. The final reaction volume of 200 μL in each well was composed of 150 μL of working solution and 50 μL of cell lysate or standard solution ($n = 3$).

2.7.6.2. Glycosaminoglycan (GAG) Binding Assay: GAG total concentration was measured using cell lysate (see above) collected after two and four weeks of cell culture. A standard curve was created using chondroitin sulfate solutions with known concentrations ranging from 0 to 30 $\mu\text{g/mL}$. Using a 96-well plate, 50 μL of cell lysate or standard solution ($n = 3$) was added to 150 μL of 1,9-dimethylmethylene blue (DMMB) solution, consisting of: 16 mg DMMB, 3.04 g glycine, 2.37 g sodium chloride, 95 mL 0.1 M HCl, 1 L distilled water. The absorbance was measured at 525 nm using a spectrophotometer (FLUOstar Omega, BMG LABTECH).

2.7.6.3. Protein Assay: Cellular protein concentration was measured using cell lysate (see above) collected after two and four weeks of cell culture. The total protein content of the lysate was measured using a commercially available assay kit (Pierce® BCA Protein Assay Kit). The final reaction volume of 210 μL in each sample well was composed of 200 μL of working solution and 10 μL of the cell lysate or standard solution ($n = 3$). Absorbance of the solution was measured using a spectrophotometer (FLUOstar Omega; BMG Labtech) at a wavelength of 570 nm, then converted to protein content using an albumin standard curve to determine the amount of intracellular protein.

3. Results and Discussion

3.1. Fabrication of the Integrated Bi-Layered Scaffold

The sphere-templating fabrication technique was applied to obtain an integrated bi-layered scaffold with controlled, monodisperse pores within each layer and highly interconnected networks. This specific approach to sphere-templating involves forming a crosslinked polymer scaffold around a template of sintered spherical porogens of uniform size, and then removing the template to produce a porous 3D hydrogel.^[18] In this study, PMMA and HAp-coated PMMA (1% $w_{\text{HAp}}/w_{\text{PMMA}}$) microspheres having diameters of $188 \pm 26\text{ }\mu\text{m}$ and $35 \pm 1\text{ }\mu\text{m}$, respectively, were placed into their respective molds and sintered to lightly fuse the microspheres (Figure 1A, steps 1–3). Next, the PMMA and HAp-coated PMMA templates were placed side-by-side in a single mold and infiltrated with a reaction mixture of HEMA containing HAcMA (2% w/w_{HEMA}). ATRP of HEMA that will be discussed below resulted in a composite of degradable polyHEMA hydrogel grafted with HAc and PMMA microspheres (Figure 1A, steps 4–5). Dissolution of PMMA microspheres (Figure 1A, step 6) resulted in the integrated bi-layered scaffold (Figure 1B–D). A SEM image of the scaffold cross-section (Figure 1B) demonstrates its bi-layered structure. The designated cartilage layer of the developed scaffold was composed of degradable polyHEMA with covalently incorporated HAc and was designed to have pore diameters of 200 μm (polyHEMA(200)/HAc). Lien et al. reported that scaffolds with pore diameters of 200–500 μm support efficient proliferation and distribution of chondrocytes that maintained their chondrogenic

phenotype.^[45] The objective of incorporating HAc into the polyHEMA scaffold was to encourage chondrogenesis.^[46–49] The designated bone layer was decorated with HAp nanoparticles and engineered with pore diameters of 38 μm (polyHEMA(38)/HAp). Although the most popular pore size range for bone tissue engineering scaffolds is 300–500 μm ,^[50] we choose a pore diameter of 38 μm because our previous studies have shown that scaffolds with pore diameters approximately of 30–40 μm are optimal for achieving vascularized, relatively non-fibrotic integration of the material within host tissue.^[23–29] Note that bone tissue should be highly vascularized (hence the 30–40 μm pores) while the cartilage layer should have low vascularity—large pores have been found in our studies to lead to low vascularity.^[23] The presence of HAp nano-particles from the PMMA beads during fabrication should encourage stem cell infiltration and differentiation due to its osteoconductive and osteoinductive properties.^[51–55] It is important to note that, due to our fabrication methods, HAc was also present in the bone layer of the bi-layered scaffold. The presence of HAc in the area designated for bone regeneration may be advantageous: HAc has been shown to play an active role in the osteogenic differentiation of MSCs.^[56,57]

SEM images of the bi-layered scaffold, polyHEMA(200)/HAc/polyHEMA(38)/HAp, also demonstrate that the sphere-templating fabrication results in a porous, highly interconnected network with precise control over pore size in each layer: approximately 200 and 38 μm for the designated cartilage and bone layers, respectively (Figure 1B–D). At high magnifications, it appears that the HAp was homogeneously distributed throughout the designated bone layer (Figures 1C–D). As shown on Figure 1E, a thin, *non-porous* degradable polyHEMA layer approximately 10–20 μm thick formed between the bone and cartilage layers of the scaffold. The layer is formed as a result of diffusion of the monomer mixture between two PMMA sphere templates that were sintered separately and then assembled together prior to the infiltration step during fabrication process. The presence of such a layer is desirable since it has the potential to prevent osseous tissue from growing into the designated cartilage layer^[7] while allowing inter-diffusion of soluble bioactive agents during the regeneration process that ultimately may permit independent chondrogenesis and osteogenesis in each scaffold compartment.

As was mentioned before, the cartilage and the bone layers of the integrated bi-layered scaffold have been amalgamated together via a degradable polyHEMA hydrogel. The hydrogel was synthesized by ATRP of HEMA, as shown in Figure 2A, using a slight modification to the procedure previously reported by Atzet et al.^[36] The reaction was simplified by replacing the multi-solvent system with DMSO during the infiltration step of the fabrication process—DMSO was the only solvent that we found that forms a homogeneous solution of all reagents, but in which PMMA is not soluble.^[19,22] HEMA was polymerized in the presence of a bi-functional degradable PCL-based macroinitiator and PCLDMA to obtain a polyHEMA hydrogel with degradable sites within the backbone and crosslinked regions (Figure 2A). For our study, the hydrogel was designed to have a backbone with theoretical M_w before and after degradation of 20 kg/mol and 10 kg/mol, respectively, by setting the monomer/initiator molar ratio to 154.^[36] The presence of the methacrylated bio-active molecule, HAc-CTA-MA, in the reaction mixture during ATRP of HEMA covalently incorporated HAc into the poly-HEMA hydrogel structure. To maximize the integration of HAc with the polyHEMA matrix, it was desired to maximize the degree of methacrylation.^[58] To do so, the native HAc molecule was complexed with an ammonium salt (CTAB) to improve its solubility in non-aqueous solvents, thus enabling more control over the methacrylation reaction (Figure 2B). In addition, the CTA-complexed HAc-CTA-MA was soluble in DMSO, which allowed the HAc-CTA-MA to be incorporated into the ATRP of the HEMA. After polymerization of the hydrogel, the CTA salt was removed via an aqueous salt exchange to return the HAc to its natural hydrophilic state.

To examine the moduli of the materials that we synthesized, unconfined compression tests of designated bone and cartilage mono-layered scaffolds were carried out separately to determine the compressive Young's moduli then compared to values for the integrated bi-layered scaffold. Although the mechanical properties reported for bi-layered scaffolds have been often measured in the dry state,^[14,16] we tested our materials in both the hydrated and dry states (Figure 3).

The compressive Young's moduli of dry bone mono-scaffold, cartilage mono-scaffold, and bi-layered scaffold were 62 ± 6 , 30 ± 6 , and 39 ± 7 MPa, respectively (Figure 3A). The compressive Young's moduli of hydrated bone and cartilage mono-scaffolds and of bi-layered scaffolds were 0.110 ± 0.001 , 0.044 ± 0.003 , and 0.09 ± 0.01 MPa, respectively (Figure 3B). Dry materials were significantly stiffer compared to hydrated materials, as expected; however, the compressive modulus of the hydrated scaffold is more relevant to study since the scaffold will presumably be implanted in its swollen form. Both hydrated and dry materials showed similar trends in comparing the different architectures: due to a smaller pore diameter and presence of HAp the bone mono-scaffold exhibited a higher modulus in comparison to the cartilage mono-scaffold, while the compressive modulus of the bi-layered scaffold was in between the compressive moduli of the bone and cartilage mono-scaffolds. The Young's moduli of both the hydrated bone and cartilage mono-scaffolds were significantly lower than values reported for native bone and cartilage tissues. The Young's modulus of human cancellous bone can vary from 0.1 to 4.5 GPa depending on the bone density and trabecular orientation,^[59,60] and the Young's modulus of human articular cartilage, which is inhomogeneous and anisotropic tissue, has been reported in the range 0.41–0.85 MPa.^[61] However, it is expected that surrounding tissue will provide protective support for the bi-layered implant immediately after implantation thus providing external mechanical support prior to cellular infiltration. Also, titanium plates can provide early-stage support. ECM production and accumulation in each layer will lead to increased mechanical properties with time.

3.2. In Vitro Studies

3.2.1. Cytotoxicity—The cytotoxicity of the bi-layered scaffold and its degradation products toward hMSCs were evaluated. Results show (Figure S1) that neither the scaffold itself nor its degradation products in the concentration range 0.1–15 mg/mL were cytotoxic.

3.2.2. In Vitro Scaffold Cell Culture Studies—Figure 4A depicts our scaffold seeding protocol where the designated bone layer of the bi-layered scaffold was seeded with primary hMSCs and the designated cartilage layer was seeded with chondrocytes that were differentiated from primary hMSCs prior to seeding. Once seeded, both types of cells were then cultured within the bi-layered scaffold for four weeks and the development of bone and cartilage matrices were quantified. Hypothesizing that biophysical conditions and the microstructure of each layer would direct cell behavior, cells were cultured only in standard base medium, without the presence of any soluble growth/differentiation factors.

Figure 4B shows a representative histological image of the bi-layered scaffold seeded with hMSCs in the bone layer and chondrocytes in the cartilage layer after two weeks of culture. The image visualizes two different types of cells growing simultaneously in each sub-layer of the integrated bi-layered scaffold.

We now present in vitro data that characterizes poly-HEMA(38)/HAp and polyHEMA(200)/HAc layers separately during four weeks in culture, documenting the potential of the bi-layered scaffold to support osteogenesis and chondrogenesis. Although we present results for the two layers of the scaffold separately, it is important to realize that primary hMSCs and

chondrocytes were co-cultured simultaneously within the two layers of the scaffold, without the addition of endogenous growth/differentiation factors.

3.2.3. Cell Development in the Bone Layer, polyHEMA(38)/HAp—Development of a mineralized matrix within the designated bone layer, polyHEMA(38)/HAp, was analyzed via histological staining, SEM, and a biochemical assay. The polyHEMA(38)/HAp layer of the bi-layered scaffold was seeded with primary hMSCs, which proliferated and migrated throughout the entire thickness of the designated bone layer during four weeks of culture (Figure 5A–B). Comparing the H&E stained histology image at four weeks (Figure 5B) to the image after one week of culture (Figure 5A), it is apparent that the cell density increased both within the polyHEMA(38)/HAp layer and on its surface. hMSCs seeded on the 38- μ m bone layer of control scaffolds that did not present HAp-coated pores, polyHEMA(38), did not infiltrate the scaffold after four weeks of culture (Figure 5C). These results were not surprising since HAp is reported to exhibit osteoconductive properties.^[51–53] Similarly, SEM images of the polyHEMA(38)/HAp layer after four weeks of culture show that the cells formed a continuous network throughout the thickness of the designated bone layer (Figure 5G) and increased their density (Figures 5H–I) compared to scaffolds after one week of culture (Figure 5D–F). In addition, at one and four weeks of culture, the cells appear to be attached to the scaffold. After closer examination, the walls of the pores appear to be coated with a crystalline substance, assumed to be the mineralized ECM deposited by differentiated hMSCs. We hypothesized that the presence of HAp induced osteogenic differentiation of the primary hMSCs. To confirm this hypothesis, the bi-layered scaffold was stained with Alizarin red after two and four weeks of culture with hMSCs as shown in Figure 6A and 6B, respectively.

Figure 6A and 6B demonstrate positive staining for Alizarin red, which indicates that calcium was present and the deposition of calcium increased with time. These results are supportive of the idea that HAp, due to its osteoinductive nature,^[54,55] triggered hMSCs differentiation along an osteogenic pathway, even in the absence of soluble growth factors. To exclude the possibility that the positive staining was due to calcium from HAp within the polyHEMA(38)/HAp layer, an acellular polyHEMA(38)/HAp layer was stained with Alizarin red and showed no staining (Figure 6E). We assumed that HAp nanoparticles were embedded within the polyHEMA hydrogel and that the dye could not reach the nanoparticles to chelate the calcium. Although the nanoparticles were embedded within the hydrogel, HAp released inductive ions into the medium, which could differentiate uncommitted cells into the osteolineage. Additional negative controls, such as seeded scaffolds without HAp (Figure 6F) and seeded poly-HEMA(38)/HAp scaffolds that were decalcified prior to Alizarin red staining (Figure 6C–D), all demonstrated negative staining supporting the suggestion that calcium deposition was due to the osteogenic differentiation of the hMSCs.

An increase in ALP activity, which is an early sign of osteoblastic differentiation in culture, was detected at levels 13.7 ± 1.2 to 20.9 ± 0.7 [(IU/L)/mg protein] at weeks two and four, respectively. These assay results support our assertions that hMSCs differentiated to osteoblasts or osteoblast-like cells that deposited a mineralized ECM as they migrated and proliferated through the designated bone layer of the bi-layered scaffold. It is important to note that our cell seeding carried out in a co-culture system, and the effect of the seeding conditions on stem cell differentiation should not be ignored.^[49] Although presence of HAp was concluded to be a primary factor in directing hMSCs behavior, HAC, which is also present in the hydrogel, may have played a role in encouraging osteogenic differentiation.^[56]

3.2.4. Cell Development in the Cartilage Layer, polyHEMA(200)/HAc—The polyHEMA(200)/HAc layer of the bi-layered scaffold was seeded with chondrocytes that were obtained by culturing primary hMSCs in pellet culture in chondrogenic medium (10 ng/mL TGF- β 1) for 14 days.

H&E stained histological images of the cross-sections of the polyHEMA(200)/HAc layer seeded with chondrocytes after four weeks of culture are shown in Figure 7A–B. Viable cells are visible within a dense ECM (Figure 7A–B), which had been deposited throughout the scaffold layer. The morphology of the chondrocytes appears to be dependent on the location of the cells. Cells appear elongated at the outer surfaces and mostly round in pores closer to the core of the construct. The alignment of collagen fibers with the cell deposited ECM (verified by Masson's tri-chrome staining, Figure S2) correlated with changes in cell morphology: when randomly oriented, the morphology generally exhibited inside of the core of the construct, chondrocytes retained their spherical morphology; when collagen fibers exhibited defined alignment, primarily on the surface of the scaffold or within the thinner interconnects between pores, chondrocytes adopted the alignment orientation of the collagen fibers and exhibited a flattened morphology. Similar zonal morphology in chondrocytes behavior was previously reported by Li et al.^[62] ECM formation and cell morphology after four weeks of culture were also characterized by SEM, as shown in Figure 7C–D. Dense ECM infiltrated the entire cartilage layer, extending through interconnecting channels to adjacent pores (Figure 7C–D). Higher magnification revealed chondrocytes having a spherical morphology within the scaffold, which were embedded in abundant ECM (Figure 7E–F); cells were seen as separate cells (Figure 7E) or as cell clusters (Figure 7F).

The sulfated GAGs within aggrecan are an essential component of articular cartilage ECM. Alcian blue-stained histological images of the polyHEMA(200)/HAc layer verify that the ECM produced by the cells was rich in sulfated GAGs and was distributed evenly throughout the thickness of the cartilage layer after four weeks in culture (Figure 8A–B); the positive staining indicates that the cells (pink) are surrounded by sulfated GAGs (blue). The biosynthesis of GAGs was also evaluated using a DMMB assay. Accumulation of GAGs within the poly-HEMA(200)/HAc layer was quantified as 3.5 ± 0.3 and 5.0 ± 0.4 (μ g/mg protein) after two and four weeks of culture, respectively, indicating an increase in GAG production over time. Type II collagen is a specific component of articular cartilage ECM, which we desire to regenerate. Figure 8C–D shows microscopy images of IHC analysis, indicating positive staining for type II collagen.

We propose that these encouraging results described above were guided by the biocomposition of the cartilage layer of the scaffold. HAc was incorporated for the purpose of enhancing chondrogenesis;^[46–49] maintaining the chondrocyte phenotype is critical for achieving chondrogenesis, which requires that the cells obtain and preserve a rounded shape.^[63] This was achieved first through pellet culture of primary hMSCs, then by in vitro culture within HAc containing of polyHEMA hydrogel supplemented with HAc. Through its interaction with other matrix proteins and binding to chondrocyte transmembrane receptors (i.e., CD44), HAc plays a role in maintaining the chondrocyte phenotype, which is exemplified by clusters of round cells surrounded by type II collagen and GAG matrix.^[64] Additionally, previous work has shown that the co-culture of MSCs and chondrocytes in close proximity increases collagen and GAG content, and decreases hypertrophy of differentiated cells.^[49] Therefore, we may assume that in addition to the presence of HAc, chondrogenesis might be also attributed to the co-culture of hMSCs and chondrocytes within the scaffold. We plan additional experiments to explore cell behavior in each of the two scaffold faces.

4. Conclusions

Herein we describe the first attempt of the simultaneous cultivation of two different cell types, hMSCs and chondrocytes, within separate dedicated layers of an integrated bi-layered scaffold for OTE, using standard cell culture medium devoid of growth/differentiation factors. In this study, we have demonstrated the ability to reproducibly manufacture integrated bi-layered scaffolds with pre-designed structural architecture, pore morphology and bio-molecular composition using a sphere-templating technique. The scaffold supported the simultaneous growth of chondrocytes and hMSCs by providing a suitable environment for cell attachment, infiltration, proliferation, and differentiation of hMSCs to osteoblasts (for the designated bone layer) and retention of chondrocyte phenotype (for the designated cartilage layer). Concurrent deposition of ECM typical to each cell type during culture was promoted in standard base medium without the addition of soluble/differentiation growth factors. Based on the scaffold design and on the preliminary in vitro results we hypothesize that upon implantation (cellular and/or acellular) the integrated bi-layered scaffold, poly-HEMA(200)/HAc//polyHEMA(38)/Hap, would trigger similar simultaneous zone regeneration of bone and articular cartilage. We plan to quantify the regenerative capacity of these scaffolds in vivo in the near future. Due to its promising biological behavior, the integrated bi-layered scaffold described here is a promising candidate for healing of an osteochondral defect.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Steps of the sphere-templating technique to fabricate an integrated bi-layered scaffold; (B) SEM image of the cross-section of the integrated bi-layered scaffold polyHEMA(200)/HAc/polyHEMA(38)/HAp (scale bar 500 μm); (C,D) magnified images of the designated bone layer, polyHEMA(38)/HAp (scale bars 50 and 10 μm , respectively) and (E) a magnified image of the interface area between the designated cartilage and bone layers (scale bar 50 μm).

Figure 2. (A) ATRP of HEMA to obtain degradable polyHEMA hydrogel. (B) Synthesis of HA-CMA.

Figure 3. Compressive moduli of (A) dry and (B) hydrated designated bone and cartilage mono-scaffolds compared to the integrated bi-layered scaffold.

Figure 4.

(A) Schematic design of the in vitro cell study and (B) representative image of H&E-stained histological cross-section of the polyHEMA(200)/HAc//polyHEMA(38)/HAp scaffold seeded with hMSCs and chondrocytes in the bone and cartilage layers, respectively, after two weeks of culture.

Figure 5.

H&E stained histological cross-sections of polyHEMA(38)/HAp layer seeded with hMSCs after (A) one and (B) four weeks of culture; (C) histological image (H&E) of the cross-section of the control polyHEMA(38) layer without HAp seeded with hMSCs, after four weeks of culture (scale bar 100 μm); (D) SEM images of the cross-section of polyHEMA(38)/HAp layer seeded with hMSCs after one (scale bar 200 μm); (E) (scale bar 20 μm); (F) (scale bar 5 μm) and four (G) (scale bar 200 μm); (H) (scale bar 50 μm); (I) (scale bar 10 μm) weeks of culture at different magnifications.

Figure 6. (A, B) Alizarin red stained histological cross-sections of polyHEMA(38)/HAp seeded with hMSCs; (C, D) decalcified polyHEMA(38)/HAp controls seeded with hMSCs; (E) poly-HEMA(38)/HAp not seeded with cells; (F) polyHEMA(38) (w/o HAp) seeded with hMSCs. Scaffolds that were incubated for two (A, C) and four weeks (B, D, E, F) are represented.

Figure 7. (A,B) H&E stained histological cross-sections of polyHEMA(200)/HAc layer seeded with chondrocytes after four weeks of culture; (C,D) SEM images of the scaffold cross-section at different magnifications (C scale bar 200 μm; D scale bar 50 μm); (E,F) representative SEM images of chondrocytes within the polyHEMA(200)/HAc layer after four weeks of culture (scale bar = 2 μm).

Figure 8.

Alcian blue-stained histological images at various magnifications of the cross-section of the polyHEMA(200)/HAc layer seeded with chondrocytes after four weeks of culture (A,B; blue-turquoise = proteoglycans; violet = nuclei/cytoplasm; * = scaffold). Histological images of IHC staining for type II collagen of the cross-section of the polyHEMA(200)/HAc layer seeded with chondrocytes after four weeks of culture: (C) the sample; (D) control (omitting primary antibody).