Fabrication of 3-Dimensional Cellular Constructs via Microstereolithography Using a Simple, Three-Component, Poly(Ethylene Glycol) Acrylate-Based System

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ABSTRACT: A novel method for the production of inhibitor- and solvent-free resins suitable for three-dimensional (3D) microstereolithography is reported. Using an exemplar poly(ethylene glycol)-based resin, the control of features in the X, Y, and Z planes is demonstrated such that complex structures can be manufactured. Human mesenchymal stem cells cultured on the manufactured scaffolds remained viable during the 7 day assessment period, with proliferation rates comparable to those observed on tissue culture polystyrene. These data suggest that this novel, yet simple, method is suitable for the production of 3D scaffolds for tissue engineering and regenerative medicine applications.

INTRODUCTION

Increases in average life expectancy brought about by advances in both medicine and life style education have resulted in aging populations that suffer from a greater prevalence of a range of musculoskeletal disorders (MSDs).1 The scale of the problem is so large that MSDs such as rheumatoid arthritis, osteoarthritis, and lower back pain are now considered to be among the most important causes of disability-adjusted-life years in the world.2 Among the most promising treatment strategies for MSDs are tissue engineering and regenerative medicine, in particular, the use of adult human mesenchymal stem cells (MSCs). Studies in which MSCs have been selectively differentiated into various cell types, including osteoblasts, chondrocytes, adipocytes, ligament cells, intervertebral disc cells, myocytes, and neurons, demonstrate their multipotentiality and the highly versatile nature of these cells for tissue regeneration.3−11 As such, it is believed that MSCs may offer permanent therapies for patients suffering from degenerative and other musculoskeletal tissue defects.

Of increasing importance to tissue engineering is the ability to seed cells onto preformed, custom-built scaffolds that, in turn, will direct tissue formation. The ideal scaffold should typically be three-dimensional (3D), which can be manufactured in all possible form factors to fulfill an implant requirement anywhere in the body, and highly porous, with an interconnected pore network for cell growth and fluid transport of nutrients and metabolic waste.12 Few techniques can readily satisfy these criteria; common approaches such as salt leaching and supercritical solvent technologies typically lead to porosities between 10 and 50%, are highly process/user-dependent and generally result in salt contamination or high levels of noninterconnected micropores.13,14 Thermally induced phase separation techniques can overcome both of these problems, but the process is still particularly sensitive to material selection and quality, the methodology employed, and user experience.12,15 One alternative could be additive manufacturing in which a 3D object is constructed from multiple 2D cross sections built sequentially, where one cross-section is constructed on top of the previous layer. This technique is used extensively in rapid prototyping and allows the manufacture of 3D objects directly from a computer-aided-design (CAD) model. Such techniques are highly promising for tissue-engineered structures due to the ease of use of the machines, build areas that can be made large enough for any implant and the rapid nature of the manufacturing process. While several related methodologies, such as fused deposition modeling, 3D plotting, and selective laser sintering exist,16 of particular application to the production of cellular/tissue constructs is microstereolithography (μSL). Here ultraviolet or visible light is used to photopolymerize a cross-linkable liquid resin one 2D cross-section layer at a time using either a laser rastering or dynamic photomask approach.17−19 In dynamic masking, a single layer is exposed in one step (i.e., the whole 2D image is projected into the liquid resin) instead of being

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rastered. Such a method offers far shorter build times, the equipment is lower cost both in terms of machine purchase and running costs (dynamic masking techniques employ projector technology, instead of a laser system, making consumables and initial costs lower), and the build envelope can be changed easily.20–22

Acrylate-based resins were among the first to be used in μSL and are still undoubtedly the most prolific and versatile systems utilized to date.23–25 Furthermore, acrylates are highly susceptible to radical addition and as such, in combination with a photoradical generating chemical cause rapid propagation of carbon–carbon bond formation throughout the illuminated region. Cross-linking is brought about by employing chemical species possessing multiple acrylate bonds, resulting in the selected area being permanently solidified into the desired shape. Successful application of μSL is reliant on striking a balance between light intensity, exposure time, and photoinitiator concentration. The inclusion of a photoinhibitor is often a critical aspect of these systems. The cation of suitable species for this purpose can be problematic as a consequence of the miscibility in the resin, suitable absorption maxima, and most critically for regenerative medicine applications, biotoxicity, and lack of regulatory approval. A wide variety of property-diverse resin formulations based on acrylate chemistry have been developed for μSL ranging from biodegradable resins based on acrylate end-capped poly(alkylacylate)/poly(trimethylene carbonate)26–29 to nondegradable acrylate end-capped poly(ethyleneglycol) (PEG) hydrogels.30–32

Herein, we present a novel method to generate simple inhibitor- and solvent-free resin formulations, exemplified using commercially available acrylate end-capped low molecular weight linear PEG in combination with dipentaerythritol penta-/hexa-acrylate, with layer control to enable 3D structure formation being achieved using increased concentrations of photoinitiator. Furthermore, we show that these scaffolds are able to support human bone marrow-derived MSCs, with cells exhibiting a typical fibroblastic morphology and remaining viable for the duration of testing. Moreover, culture of MSCs on these PEG-based scaffolds led to proliferation rates comparable with that observed with tissue culture polystyrene (TCP), further supporting, as a proof of principle, the use of this material in the formation of 3D scaffolds for complex shape tissue engineering.

**EXPERIMENTAL SECTION**

**Materials.** Photoinhibitor was removed from poly(ethylene glycol) diacrylate (average $M_n = 575$ g mol$^{-1}$) and dipentaerythritol penta-/hexa-acrylate (Sigma-Aldrich) in the absence of ambient light by first dissolving the reagents in a minimum of dichloromethane before passing the solutions through basic alumina and removing the dichloromethane under reduced pressure at room temperature. Irgacure 784 (BASF) and technical grade solvents (isopropanol, dichloromethane, and ethanol, all purchased from Fisher Scientific U.K.) were used as received.

**Stereolithography.** The photolithographic resin was blended by the addition of 1.8 wt % Irgacure 784 photoinitiator into a 4:1 ratio by volume of PEG-diacrylate/dipentaerythritol penta-/hexa-acrylate with stirring. After stirring for 30 min, the resin formulation was stored in the absence of ambient light. The viscosity of the formulated resin was measured using a falling ball viscometer (Gilmont Number 3, 21 °C). Microcysteolithography (μSL) was carried out on a custom-built system described previously.23 A blue LED light engine (Enfini Uno Air) with a peak output at 465 nm was used as the projector light source. The resin tray was a 5 mm glass plate coated with a 2 mm thick layer of silicone elastomer.

**Disc Production for Cell Seeding.** Cell seeding discs were produced monolithically by varying the exposure time in a pool of the resin placed on the resin tray. The central, high optical transmission regions of the discs were exposed for 5 s, while the outer raised walls of the discs were exposed for 90 s. After production, excess free resin was removed by rinsing the constructs with isopropanol. The constructs were then subjected to post curing in a Metalight QX1 UV light box for 1 h before being washed by Soxhlet extraction for 24 h in ethanol. Discs were then stored in ethanol.

**3D (10, 3)-a Network Production.** The standard tessellation language (STL) file of the structure produced by stereolithography was based on the (10, 3)-a network described in previous literature.34 The structure was produced on a 400 μm base plate to ensure sufficient bonding to the glass build surface and using 50 μm layers and 10 s exposure per layer throughout. The structure was nominally 11.5 mm wide.

**Tensile Strength Measurements.** Dog bone type samples measuring 25.0 × 1.0 × 2.6 mm were produced via 3D fabrication and subsequently post cured under UV irradiation for 1 h in a Metalight QX1 UV chamber. Half of the samples were subjected to 24 h Soxhlet extraction with ethanol, stored under fresh ethanol after extraction, and subjected to tensile strength testing immediately. At ambient temperature and humidity, samples were axially loaded in a tensile testing machine (Deben MICROTEST) with a load cell capacity of 2 kN and crosshead speed of 0.1 mm min$^{-1}$ with a premeasured grip-to-grip separation. Data were recorded from the instrument using Deben Microtest v5.5.14 and all values reported herein were obtained from an average of five repeat specimens.

**Microstructural Analysis.** Structural analyses were performed on the fabricated articles by microcomputed tomography (μ-CT) using a SkyScan 1174 scanner (manufactured by Bruker MicroCT). Scans were carried out at 40 kV and a current of 1 mA with a sample pixel resolution of 18 μm over 180° with a rotation step size of 0.5° with 2400 ms total exposure time at each step. Reconstruction to cross-sectional slices were made using the SkyScan NRecon program which uses a modified Feldkamp algorithm.

**Acquisition and Expansion of Human Mesenchymal Stem Cells.** Bone marrow aspirates were collected from four patients (41 and 65 year old males; 42 and 75 year old females) following total hip replacement surgery with full patient consent and approval from the North West Research Ethics Committee (06/Q403/238). Human MSCs were isolated from bone marrow, as previously detailed.35 Briefly, bone marrow aspirates were centrifuged at 500 × g for 10 min and the supernatant was discarded. The cell pellet was resuspended in complete isolation medium (α-minimum essential medium (Gibco) supplemented with 50 μg mL$^{-1}$ ascorbate (Sigma), 250 ng mL$^{-1}$ of amphotericin, 100 units mL$^{-1}$ of penicillin, 100 μg mL$^{-1}$ of streptomycin (Invitrogen) with 20% fetal calf serum (FCS) (Invitrogen)). RosetteSep (Stem Cell Technologies; 250 μL) was added to each 5 mL of cell suspension and incubated at room temperature for 20 min to deplete unwanted blood cells. Samples were diluted in Hank’s balanced salt solution (HBSS, Sigma; 5 mL) containing 2% FCS and 1 mL ethylenediaminetetraacetic acid (EDTA, Sigma) and mixed gently. Mononuclear cells were isolated using a Histopaque-1077 (Sigma) gradient centrifugation and cultured on tissue culture polystyrene in complete isolation medium at 37 °C, 5% CO$_2$. After 5 days, the medium containing nonadherent cells was removed and replaced with fresh complete medium (complete isolation medium containing 10% FCS). Adherent MSCs were expanded in culture in complete medium and seeded onto scaffolds. All experiments were conducted with cells at passage 4.

**Cell Seeding.** Prior to cell seeding, scaffolds were washed overnight in Dulbecco’s phosphate buffered saline (PBS; Sigma) and washed (2 × 30 min) in complete medium the following day.

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Expanded human MSC monolayer cultures were trypaned (PAA Laboratories) and viable cells were counted using trypan blue (0.4%; Sigma), as previously detailed. As preliminary experiments showed that unwashed scaffolds exhibited high levels of cytotoxicity (data not shown), only Soxhlet extracted scaffolds were used for cell seeding experiments. Scaffolds were placed in sterile 24-well tissue culture plates and cells seeded onto the scaffolds at a density of 5000 cells cm\(^{-2}\) in 20 \(\mu\)L of complete medium. After 1 h of culture at 37 °C, 5% CO\(_2\), a further 20 \(\mu\)L of complete medium was added to prevent the scaffolds from drying out. Following a further hour of culture, 1 mL of complete medium was added to each tissue culture plate well containing cell-seeded scaffolds, taking care not to dislodge adherent cells. MSC-seeded scaffolds were then cultured for 1, 3, and 7 days in triplicate. Where appropriate, media was changed every 48 h. MSCs cultured on tissue culture polystyrene (TCP) and no-cell scaffolds served as controls.

**Assessment of Cell Proliferation.** MSC-seeded scaffolds and no-cell scaffold controls were removed from tissue culture plates following 1, 3, and 7 days culture, washed twice in PBS, placed in bijoux’s containing 0.5 mL of PBS and frozen at −80 °C. For MSCs cultured on TCP, medium was removed from the wells of 48-well plates containing monolayer cells and washed with PBS. PBS (0.5 mL) was then placed into each well containing monolayer MSCs, and the plates were frozen at −80 °C. For preparation of the MSC standard curve for the proliferation assay, cells were placed into microcentrifuge tubes using a range of cell densities between 1 \(\times\) 10\(^3\) and 2 \(\times\) 10\(^3\) cells, pelleted by centrifugation at 500 \(\times\) g for 5 min, and then pellets washed, resuspended in 0.5 mL of PBS, and frozen at −80 °C. All MSCs were lysed using 3X freeze–thaw cycles and cell lysates cleared by centrifugation at 10000 \(\times\) g for 10 min. A total of 100 \(\mu\)L of each cell lysate was added in triplicate to a 96-well microplate, then 100 \(\mu\)L of Picogreen (Invitrogen) working reagent (prepared as detailed by manufacturer) added to each well and incubated at room temperature, in the dark, for 5 min. The fluorescence emission was measured using a fluorescence microplate reader (BioTek) with filters at 485 nm excitation and 528 nm emission wavelengths. A standard curve was included for each plate to enable the fluorescence emission to be converted to cell number. No-cell scaffold controls were used for preparation of the standard curve and at each time point to normalize for baseline fluorescence. A \(\lambda\)-DNA standard curve was also included on each microplate to confirm the linear efficiency of the assay according to manufacturer’s recommendations. Fluorescence values were normalized against the no-cell scaffold controls and adherent MSC number calculated using the MSC standard curve.

**Assessment of Cell Viability.** Medium was removed from the MSC-seeded scaffolds following 1, 3, and 7 days culture and the MSCs/scaffolds washed with PBS. PBS containing 2 \(\mu\)M calcein AM and 4 \(\mu\)M ethidium homodimer-1 (Invitrogen) was added to each MSC-seeded scaffold and incubated at 37 °C for 20 min in the dark. MSCs/scaffolds were visualized using a multiphoton confocal fluorescence microscope (Leica SP5). MSCs treated with 70% methanol for 30 min were used as a positive control for nonviable cell staining. Viable and nonviable cells stained green and red, respectively.

### RESULTS AND DISCUSSION

**Resin Formulation and Photocuring.** Typical stereo-lithographic resins are comprised of a blend of reactive species, tuned to provide the desired properties in the resultant photocured initiator, a photoinitiator, and a photoinhibitor, the balance between which enables the achievement of precise 3D control by stereolithography. Given the potential difficulty in identifying a suitable photoinhibitor for the process, we theorized that the presence of excess photoinitiator would lead to photoinhibition properties as a consequence of its inherent reactivity to radical species, thus, reducing its overall activity. To assess the viability of a resin in the absence of a defined photoinhibitor, a commercially available poly(ethylene glycol) diacrylate (average \(M_n = 575\) g mol\(^{-1}\)) and dipentaerythritol penta-/hexa-acrylate were blended in a 4:1 ratio by volume; the viscosity of the blended resin was measured to be 675 cP, demonstrating that this simple binary mixture was sufficiently nonviscous to be suitable for \(\mu\)SL applications. Irgacure 784 photoinitiator was added in increasing concentrations between 0.85 and 3.4 wt % and its effects upon the feature fidelity and cure thickness evaluated. Single layers of each formulation were cured for 10 s and the size and thickness of the layer were measured using a micrometer. The optimum feature fidelity in the \(XY\) plane (Figure 1a), determined by measurement of feature broadening using microcallipers, was observed between 1.7 and 2.0 wt % photoinitiator. Measurement of the cure depth with increasing photoinitiator concentration across the same range revealed that, indeed, the light penetration depth and, hence, layer thickness could be readily controlled in this manner (Figure 1b). Layers cured after 10 s of light exposure were sufficient for production of 3D structures using 50 \(\mu\)m layers to ensure sufficient curing to previous layers to prevent cohesive failure. In this manner, a simple, commercially available, three component, inhibitor- and solvent-free system could be formulated with sufficient control over the curing pattern and depth to produce well-defined 3D structures by the micro-stereolithography process.

**Mechanical Properties.** To quantify the mechanical properties of the resultant photocured materials from the
optimized resin formulation, tensile strength testing of dog bone type specimens was undertaken. The materials demonstrated elastic deformation until failure (Figure 2a) with a Young's modulus \( E \) of 137.9 ± 12.3 MPa, ultimate tensile strength (UTS) of 10.5 ± 0.9 MPa, and elongation at break of 7.7 ± 0.9\% from 10 specimens. In each case, no yield point was observed which indicates that failure occurs at microstrain points within the structure.

The application of acrylate-based components for the \( \mu \)SL resin dictated that the samples be thoroughly washed prior to cell testing to remove traces of the potentially toxic precursor materials and ensure high cell viability. This process was typically performed by Soxhlet extraction of the postcured constructs with ethanol. Given the possibility of the ethanol to transesterify the ester linkages within the structures, tensile testing was also performed on dog bone samples that had been treated in this manner. A marked reduction in the UTS (2.7 ± 0.6 MPa) and elongation at break (1.7 ± 0.4\%) of the specimens was observed after Soxhlet extraction; however, such dramatic differences in \( E \) were not apparent (153.5 ± 7.8 MPa).

These data are consistent with mechanical failure occurring at the site of microstrains/cracks, defects that were exaggerated by swelling and deswelling during Soxhlet extraction and the subsequent evaporation of solvent during testing and are not a result of the cleavage of the ester linkages contained within the material, which would have also resulted in a lowering of the Young's Modulus. Both before and after extraction, the materials demonstrated biologically relevant strength profiles with values in the range of those observed in human musculoskeletal tissues.\(^{37}\)

**Construction of Cell-Testing Discs and 3D (10, 3)-a Network.** Constructs suitable for the facile performance of assessment of cell viability and proliferation were created with a cylindrical profile with a raised rim, surrounding a cell seeding surface of 8 mm diameter, to aid in cell containment and a maximum diameter of 10 mm such that they sit in multiwell plates. Cell seeding discs were produced by varying the exposure time in two layers in a pool of resin on the resin tray to create the pseudo-3D structure (Figure 3). Using a micrometer, the outer wall of the disc was measured as 600 \( \mu \)m thick and the center membrane was measured to be 260 \( \mu \)m.

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**Figure 2.** Typical stress vs strain curves for dog bone type specimens subjected to tensile strength testing. (A) Non-Soxhlet extracted samples and (B) Soxhlet extracted samples.

**Figure 3.** Digital microscopy image of a cross-section through a cell seeding disc showing pseudo-3D structure.

**Figure 4.** (A) CAD representation and (B) and digital macro photograph of the 3D (10, 3)-a structure produced by \( \mu \)SL. The overall dimensions of the structure produced by \( \mu \)SL as shown above were 11.50 × 11.50 × 11.00 mm (scale bar = 225 \( \mu \)m). The overall dimensions of the 3D CAD model as shown were 11.75 × 11.75 × 11.20 mm.

**Figure 5.** Number of adhered MSCs on scaffolds and TCP following 1, 3, and 7 days of culture. Dotted line indicates number of seeded cells (2500) and * denotes significance \( p \leq 0.05.\)
While these constructs demonstrate to an extent the effect of curing times on the prepolymer resin, the fabrication of a (10, 3)-a network was undertaken to demonstrate the utility of this photoinhibitor-free approach to 3D μSL. Comparison of the 3D CAD structure of the (10, 3)-a network with the overall dimensions of $11.75 \times 11.75 \times 11.20$ mm to the actual fabrication of the structure produced by μSL resulted in an accurate model devoid of unwanted inclusions with overall dimensions of $11.50 \times 11.50 \times 11.00$ mm (Figure 4). This is consistent with an overall isotropic shrinkage of approximately 6% and, notably, feature fidelity and total channel interconnectivity were preserved. Such shrinkage is commonly caused by factors such as leeching of solvating fluids from the structure, errors in the focus of the μSL instrument, and inherent stresses/curing shrinkage in the final article. However, given the absence of solvating fluids in this resin, we postulate that this minimal shrinkage is a combination of both cure shrinkage and focal errors. Further analysis of the structure by μ CT was employed to obtain visualizations of the article and confirm that no inclusions/defects were present in the structure that were not readily observable by eye (i.e., in the center). In this manner it was demonstrated that all elements of the STL design file were successfully constructed during the build process and that the designed interconnectivity of all channels throughout the structure was maintained (Supporting Information). While the resultant scaffolds had a slight yellow coloration immediately after the curing process, attributed to the photoinitiator, postcuring led to the material ultimately bleaching to produce a translucent material.

**Cell Proliferation and Viability.** MSCs were seeded onto scaffolds at a density of 5000 cells cm$^{-2}$, equaling a total of 2500 cells per disc (Figure 5). The number of MSCs adhered to scaffolds and TCP following 1 day of culture was 4743 ± 1708 and 6090 ± 1554 cells, respectively. By day 3, the number of adhered MSCs increased on both scaffolds and TCP surfaces (18710 ± 7805 and 17420 ± 1464 cells, respectively), with these increases in cell number reaching significance when compared to day 1 ($p \leq 0.05$; Figure 5). Increasing the time in culture to 7 days resulted in a further increase in adherent cell number, with the number of MSCs adhered to scaffolds and TCP reaching 25715 ± 15806 and 65159 ± 15333, respectively. The increased number of adherent cells observed at day 7 reached significance on TCP compared to days 1 and 3 ($p \leq 0.05$) and, compared to day 1, only ($p \leq 0.05$) on scaffolds. There was no significant difference in adherent cell number between MSCs seeded on scaffolds or TCP at any of the three time points analyzed ($p > 0.05$). Importantly, these results suggest that MSCs are able to adhere and proliferate on the PEG scaffolds in a manner similar to that observed with TCP. Our findings are in agreement with those previously reported by Lu et al., where PEG-coated TCP was shown to support attachment and proliferation of human MSCs. Although μSL manufactured PEG scaffolds have recently been proposed for tissue engineering applications by Zhang and co-workers, cell adhesion was only realized through the addition of gelatin methacrylate or by the functionalization of PEG with RGD motifs. Such modifications result in elevated manufacturing costs, complexity and build duration, highlighting the benefits of an “off-the-shelf” PEG-based resin for rapid fabrication of cell-supporting scaffolds, as reported here.

The viability of the MSCs was indicated by cytoplasmic green staining and a lack of red nuclear staining at all three time points studied, with cells displaying fibroblastic morphology (Figure 6A–C,E–G). The density of cells adhered to the surface of the scaffolds appeared to increase with increasing culture duration, supporting the cell proliferation data (Figure 6D,H), indicating nonviable cells and confirming the validity of the assay. To our knowledge, this is the first report of cultured cells on solid 2D PEG scaffolds formed from a precursor resin; however, PEG-based 2D and 3D hydrogels have previously

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**Figure 6.** MSCs from four different patients were seeded onto scaffolds at a density of 5000 cells/cm$^2$ (2500 cells per scaffold) in triplicate and allowed to adhere. MSCs were cultured in complete medium for (A, E) 1 day, (B, F) 3 days, and (C, G) 7 days before being washed in PBS and incubated with 2 μM calcein AM and 4 μM ethidium homodimer-1 (Invitrogen) for 30 min at 37 °C in darkness. (D, H) MSCs treated with 70% methanol for 30 min were used as nonviable cell controls. MSCs were visualized using a multiphoton confocal microscope (Leica SP5) with (A–D) 20× objective and (E–H) 63× objective. Green cytoplasmic and red nuclear stain indicates viable and nonviable cells, respectively. Scale bars are representative of 50 μm.
been shown to support cells and even lead to specific lineage (e.g., osteogenic and chondrogenic) differentiation of MSCs. The high levels of MSC viability and proliferation reported for the PEG scaffold provide a "proof of principle" for development of a cell-supporting, complex scaffold fabrication system.

**CONCLUSIONS**

In conclusion, we have demonstrated the preparation of a photolithographic resin from commercially available materials that is suitable for building highly precise 3D structures by microstereolithography. Furthermore, the simplicity of this approach is demonstrated by the absence of a requirement for a photoinhibitor or solvent that can lead to unpredictable effects on the resultant materials. The scaffolds were demonstrated to be suitable for the culture of human mesenchymal stem cells, with proliferation on the material comparable to that observed with tissue culture plastic. We therefore propose the use of microstereolithography-produced, PEG-based scaffolds for complex structure tissue engineering. Extended cell culture studies will be needed to evaluate the potential for the material to control differentiation of MSCs down specific tissue lineages.

**ASSOCIATED CONTENT**

μCT analysis video demonstration. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

The authors declare no competing financial interest.

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