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Poly(HDDA)-Based Polymers for Microfabrication and Mechanobiology

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ABSTRACT

Materials processing and additive manufacturing afford exciting opportunities in biomedical research, including the study of cell-material interactions. However, some of the most efficient materials for microfabrication are not wholly suitable for biological applications, require extensive post-processing or exhibit high mechanical stiffness that limits the range of applications. Conversely, materials exhibiting high cytocompatibility and low stiffness require long processing times with typically decreased spatial resolution of features. Here, we investigated the use of hexanediol diacrylate (HDDA), a classic and efficient polymer for stereolithography, for oligodendrocyte progenitor cell (OPC) culture. We developed composite HDDA-polyethylene glycol acrylate hydrogels that exhibited high biocompatibility, mechanical stiffness in the range of muscle tissue, and high printing efficiency at ~5 μm resolution.

INTRODUCTION

Three-dimensional microfabrication is increasingly adopted for biomedical applications, from organs-on-chips to model disease *in vitro,* to cellularized scaffolds for cell therapy and tissue regeneration [1]. Projection microstereolithography (PuSL) is an additive manufacturing technique in which complex structures are built layer-by-layer from digital masks as photopolymers are crosslinked *in situ* with ultraviolet light (Figure 1) [2,3]. PuSL enables free-form microfabrication, with enhanced accuracy, resolution (~ 0.5 μm) and reproducibility over traditional methods including solvent casting, freeze drying, soft lithography, and more recent technologies such as direct inkjet printing.

Hexanediol diacrylate (HDDA) is a short, non-viscous polymer material that has been widely used in stereolithography because it allows for short printing times and high resolution [2,4,5]. However, only few applications have been reported in biological systems, mainly in drug and gene delivery, contrary to other biocompatible hydrogel precursors such as polyethylene glycol and polyacrylamide [6-8]. In this study we explored HDDA as a biocompatible material for culture of oligodendrocytes, which are increasingly relevant in the field of neuroscience and neurodegenerative disorders [9], and developed a biocompatible, mechanically compliant and printable polymer system for biomedical research.

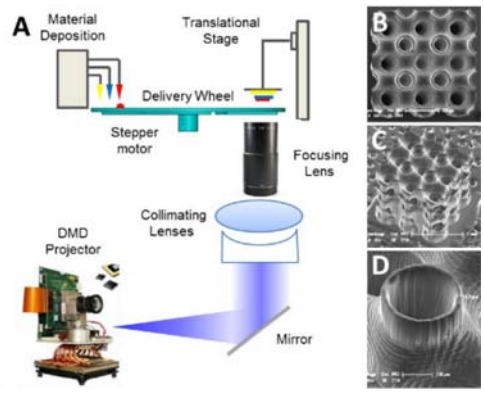

Figure 1. (a) Projection microstereolithography (PuSL) enables layer-by-layer microfabrication of three-dimensional structures with ~0.5 µm resolution. (b-d) SEM images of polyethylene glycol-based microstructures fabricated with PuSL.

MATERIALS AND METHODS

Cell culture

Oligodendrocyte progenitor cells (OPCs) were isolated from Sprague Dawley rodent mixed glial cultures, as described previously [10], and maintained in a progenitor state for 24 h in proliferation medium (DMEM, Invitrogen; Sato's modification: 5 mg/mL insulin, 50 mg/mL holo-Transferrin, 5 ng/mL sodium selenate, 16.1 mg/mL putrescine, 6.2 ng/mL progesterone, and 0.1 mg/mL bovine serum albumin; 10 ng/mL platelet-derived growth factor homodimer AA, PDGF-AA, and 10 ng/mL basic fibroblast growth factor-2, FGF-2, Peprotech). Differentiation was induced after 24 h or 48 h in differentiation medium (Sato's medium with 0.5% v/v fetal bovine serum, without PDGF-AA or FGF- 2).

Immunocytochemistry

Cells were stained with 5 µg/mL propidium iodide to identify dead cells in proliferation or differentiation media for 15 min at 37 ºC, and rinsed three times with 150 nM NaCl phosphate buffered saline (PBS). Cells were fixed with 4% paraformaldehyde, washed with PBS, permeabilized with 0.1% Triton X-100 for 5 min, and blocked with 3% normal goat serum in PBS and 0.1% Triton-100 (blocking solution) for 1 h. Primary antibodies (rat anti-MBP, 1:150 dilution, Serotec) were diluted in blocking solution and incubated at room temperature for 1 h. Samples were washed 3 times with PBS and incubated with secondary antibodies (rabbit anti-rat IgG Alexa Fluor 488, 1:150 dilutions, Invitrogen) in PBS for 1 h, followed by washing and staining of nuclei with Hoechst 33342 at a 1:1000 dilution for 5 min. Cells were imaged via epifluorescence microscopy (IX-81, Olympus), analyzed and counted with ImageJ software.

Chemical functionalization of glass substrates

Glass bottom dishes (30 mm glass diameter, Invitro Scientific) and 12 mm coverslips were rinsed with ethanol, blown dry with air, and exposed to oxygen plasma for 5 minutes. Activated dishes were functionalized with 2% v/v 3-(Trimethoxysilyl)propyl methacrylate (Sigma-Aldrich) and 1% v/v acetic acid in ethanol at room temperature for 2 h, to introduce acrylate groups on the surface and immobilize the samples to the glass via photopolymerization. Modified dishes were rinsed twice with ethanol, blown dry, and stored in a desiccator for up to 6 months.

Preparation and functionalization of poly(HDDA) and poly(HDDA)-starPEG

Polymer discs of 200 µm thickness were prepared on functionalized glass bottom dishes for atomic force microscopy (AFM)-enabled nanoindentation and biocompatibility experiments. A drop of 50 uL HDDA with 2% w/w Irgacure 819 photoinitiator was cast unto the glass, covered with an 18 mm glass coverslip and exposed to UV (8 mm light guide, OmniCure Series 2000) for 1 s at 30 mW/cm^2 (measured at the sample height). Coverslips were removed and discs were immersed in ethanol for 24 h, followed by two 24 h periods in PBS. All cell experiments were thereafter maintained in a sterile biosafety cabinet. The discs were washed 3 times with sterile PBS before coating with 50 ug/mL poly-D-lysine (PDL) in PBS overnight at 37 ºC. Poly(HDDA)-starPEG resin was prepared by mixing 10% w/w 4-arm PEG acrylate (starPEG, 20kDa arms, Creative PEGWorks), 10% w/w HDDA and 2% w/w Irgacure 819 in DMSO, and sonicating at 37ºC for 10 min. Discs were prepared as described previously, with 15 s UV exposure at 30 mW/cm^2. Bioactive samples were washed twice with sterile PBS and stored at 4 ºC for up to a week. One hour before seeding, discs were incubated in proliferation medium. Tissue culture polystyrene (TCPS) dishes were coated with 50 ug/mL PDL in water for 1 h, rinsed twice and air dried. To test non-specific protein adsorption of cytophilic molecules, polymer samples were functionalized with a mixture of PDL and poly-L-lysine-FITC in a 10:1 ratio. Fluorescence intensity was normalized to the respective materials coated with PDL.

AFM-enabled nanoindentation

Atomic force microscope (AFM)-enabled nanoindentation measurements of 200 µm thick discs were conducted (MFP-3D Bio, Asylum Research), using cantilevers of nominal spring constant k =2.8 N/m terminating in a single crystal silicone tetrahedral probe (AdvanceTEC-FM) with an average half cone angle of 10º. The actual spring constant was calibrated via the thermal noise method [11]. Samples were equilibrated overnight and measured in PBS. Fifteen force-depth responses were collected across at least two replicate samples per material. The cantilever base velocity was 4.5 µm/s and probe retraction was triggered after reaching a maximum deflection of 264 nm. Young's elastic modulus E was calculated by fitting the Oliver-Pharr model [12] to the upper 20% of the unloading response; the elastic modulus was reported as the arithmetic mean ± standard error of measurement (n = 15) for each material.

Projection microstereolithography

The principles behind PµSL have been previously described [2]. This particular system is equipped with a light source with peak output at 365 nm and a reduction lens for high-resolution UV light transmission (Zeiss) to achieve a minimum horizontal and vertical feature size of 1-2 µm and 0.5 µm, respectively. Basic bitmap masks were generated with Paint software with feature sizes ranging from 5-30 µm. Constructs were built on functionalized 12 mm glass coverslips. Each construct consisted of 3, 10 µm thick layers, and exposed for 0.7 s and 3 s per layer for poly(HDDA) and poly(HDDA)-starPEG, respectively. The light-absorbing dye Sudan I was incorporated into the pre-polymer resins at 0.5 % w/w to adjust the light penetration depth.

RESULTS AND DISCUSSION

Poly(HDDA) hinders survival and development of OPCs

Oligodendrocytes are adherent cells that require attachment to other materials for survival, proliferation and differentiation. OPCs adopt a bipolar or tripolar morphology on typical cell

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culture substrata and become morphologically complex throughout differentiation [13]. These morphologies can be identified by visual inspection (Figure 2a). Poly(HDDA) and TCPS substrates were functionalized with PDL. OPCs seeded on poly(HDDA) adhered and remained attached to the plates through several medium exchanges, but did not spread or differentiate to exhibit characteristic processes (Figure 2b).

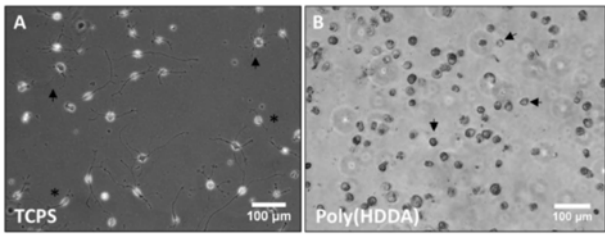

Figure 2. (a) OPCs display bipolar or tripolar morphology (asterisks) on conventional polystyrene substrata, and become progressively branched during differentiation (arrows). (b) OPCs initially adhere to poly(HDDA) but do not extend processes or develop (arrows).

Poly(HDDA) is hydrophobic and may not be amenable to conventional treatment with poly-D lysine (PDL), commonly used as a cell-adhesion promoter. Oxygen plasma is typically used to activate hydrophobic materials such as polystyrene and polydimethylsiloxane to enable ionic binding of PDL. However, plasma treatment (up to 1 h) did not promote OPC survival. Unreacted HDDA monomers and initiator can also contribute to the cytotoxicity of poly(HDDA). More rigorous post-processing of the crosslinked material with a less polar solvent such as acetone may be necessary. Future studies will combine this approach with non-contacting cytotoxicity tests to validate this hypothesis.

OPCs survive and differentiate on poly(HDDA)-starPEG gels

We furthered explored HDDA biocompatibility by engineering a composite hydrogel system of HDDA and starPEG. HDDA acts as a short crosslinker, allowing us to harness the polymer properties (high reactivity, low viscosity) that are targeted for efficient additive manufacturing with PμSL. PEG hydrogels are common components of biocompatible materials, and the multi-arm PEG acrylate variants have been exploited in tissue engineering for biofunctional matrices *in vivo* [14,15]. The poly(HDDA)- starPEG polymer exhibited improved biocompatibility compared to poly(HDDA), evident by increased hydrophilicity (Figure 3a) and non-specific adsorption of small cytophilic molecules (Figure 3b).

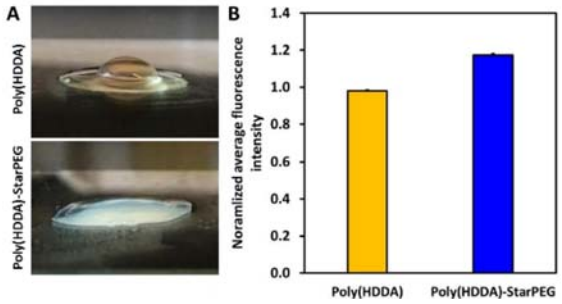

Figure 3. (a) The lower water contact angle on poly(HDDA)-starPEG shows improved hydrophilicity over poly(HDDA). (b) Enhancement of non-specific adsorption of cytophilic molecules further suggests an improvement in biocompatibility.

OPCs adhered to poly(HDDA)-starPEG and survived, with 80 ± 8.7% viability within 48 h in proliferation media, normalized to control TCPS (Figure 4g). This is comparable to reported viability of PEG diacrylate hydrogels at comparable monomer

concentration [16], and suggests compatibility with at least 10% HDDA and 2% Irgacure 819. OPCs adopted a characteristic bipolar and simple multipolar morphology (Figure 4d); similar morphologies were observed in at least three replicate experiments. Differentiation was induced by withdrawal of PDGF-AA and FGF-2 after 24 h. Cell morphology was then multipolar (Figure 4e), and on day 6 many cells expressed myelin basic protein (MBP), a marker of mature OLs (Figure 4f).

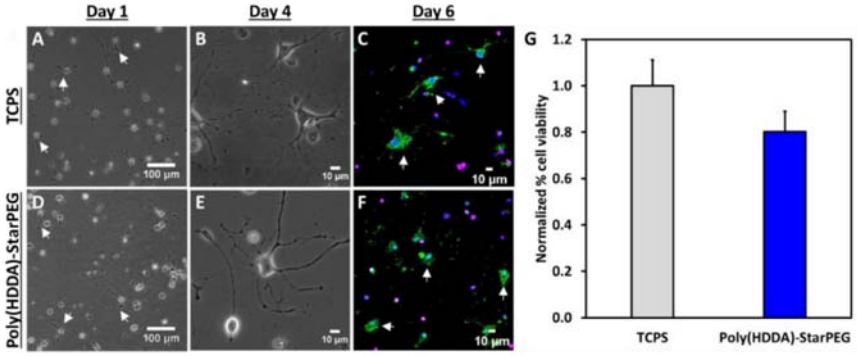

Figure 4. OPCs adopt bipolar and multipolar morphologies on poly(HDDA)-starPEG, characteristic of progenitors (d) and oligodendrocytes (e), respectively, similar to control polystyrene (a, b). (c, f) Oligodendrocytes also express MBP (green) on day 6. (g) Cell viability was 80% ± 8.7% normalized to TCPS, quantified from PI/Hoechst stains. Reported values are mean cell counts combined from 10 frames, from 1 experiment; error bars are ± SEM.

Bulk mechanics of poly(HDDA)-starPEG are relevant in the context of mechanobiology

Mechanical cues are increasingly recognized as important modulators of cell response in biology and medicine, including the effects of material stiffness on morphology and phenotype of adherent cells [17,18]. These composite polymers fabricated with HDDA as a crosslinker, and starPEG, a precursor to compliant hydrogels [19,20], are two orders of magnitude more compliant than poly(HDDA) (78.15 ± 1.5 kPa and 2.59 ± 0.11 MPa, respectively) as measured by AFM-enabled nanoindentation (Figure 5a).

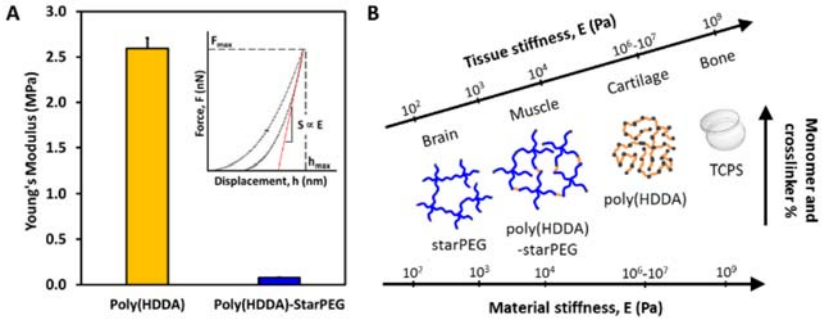

Figure 5. Young's elastic modulus *E* of bulk poly(HDDA) and poly(HDDA)-starPEG gels as measured by atomic force microscope-enabled nanoindentation (a). Gels prepared with the HDDA-starPEG resin are two orders of magnitude lower in stiffness than poly(HDDA), and

within the mechanical stiffness of muscle tissue (b). Reported values are from the mean of 15 measurements combined from at least 2 samples; error bars are ± SEM.

Previously reported *E* of poly(HDDA) are two orders of magnitude higher, likely due to differences in fitting models (e.g. Hertz versus Oliver-Pharr) and curing conditions [4]. The mechanics of the crosslinked polymers may be further tuned by varying monomer and crosslinker content, enabling the possibility to engineer materials that explore the range of physiological stiffness of biological tissues *in vitro* (Figure 5b).

Poly(HDDA)-starPEG resins are amenable to PuSL fabrication

We investigated if the new polymer system retained capabilities for microfabrication. Three-dimensional structures of poly(HDDA) and poly(HDDA)-starPEG were fabricated with PμSL (Figure 6a-b). Constructs were approximately 30 μm tall, with feature width of 5, 20 and 30 μm. Images were obtained after 24 h of immersion in PBS. Both materials achieved comparable XY resolution, with only a 4-fold increase in exposure time for poly(HDDA)-starPEG (3 s/layer), which also exhibited slightly thicker features, likely due to the higher swelling capacity of the polymer. Swelling capacity will have to be quantified and considered in the design of more complex and overhanging hydrogel structures. OPCs survived and engaged with the constructs coated with PDL, as described previously, and displayed signs of differentiation, as assessed by expression of multipolar morphology (Figure 6c). A 10% starPEG resin was tested but failed to cure at up to 20 s exposure per layer, which highlights the advantage of HDDA as an efficient crosslinker in materials with poor manufacturability at the microscale.

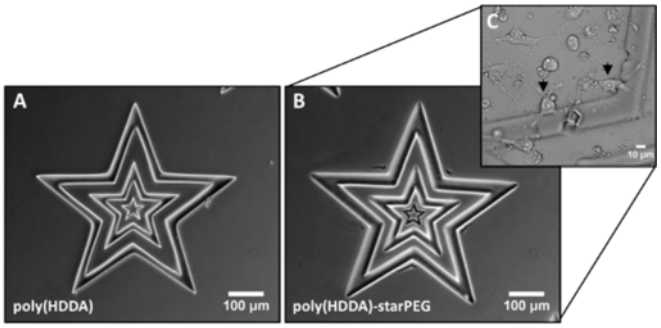

Figure 6. Three-dimensional microstructures generated with projection stereolithography. The poly(HDDA)-starPEG precursor resin is printable (b) at comparable resolution to the HDDA resin (a), with only a 4-fold (3 s/layer) increase in printing time. (c) OPCs survive and start differentiating on the printed structures displaying multipolar morphology.

CONCLUSIONS

Polymers that are readily amenable to additive manufacturing methods such as projection micro-stereolithography may not also be compatible with cell culture and with the range of material mechanical properties that reflect biological tissues, scaffolds, and cells. These results show that one such polymer based on poly(HDDA) can be modified as a composite hydrogel to attain cytocompatibility and reduce mechanical stiffness from that of thermoplastics to that of biological tissues such as muscle. Together these findings show that the materials design and

demonstration of new polymers for biocompatible additive manufacturing will increase the range of questions that can be addressed via *in vitro* mechanobiology studies and, potentially, *in vivo* translation.

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