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Alignment of multi-layered muscle cells within three-dimensional hydrogel macrochannels

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ABSTRACT

This work describes the development and testing of poly(ethylene glycol) (PEG) hydrogels with independently controlled dimensions of wide and deep macrochannels for their ability to promote alignment of skeletal myoblasts and myoblast differentiation. A UV-photopatterned thiol-ene mold was employed to produce long channels, which ranged from ~40 to 200 μm in width and from ~100 to 200 μm in depth, within a PEG–RGD hydrogel. Skeletal myoblasts (C2C12) were successfully cultured multiple cell layers deep within the channels. Decreasing channel width, increasing channel depth and, interestingly, increasing cell layer away from the channel base all contributed to a decreased interquartile range of cell angle relative to the long axis of the channel wall, indicating improved cell alignment. Differentiation of skeletal myoblasts into myotubes was confirmed by gene expression for myoD, myogenin and MCH IIb, and myotube formation for all channel geometries, but was not dependent on channel size. Qualitatively, myotubes were characteristically different, as myotubes were larger and had more nuclei in larger channels. Overall, our findings demonstrate that relatively large features, which do not readily facilitate cell alignment in two dimensions, promote cell alignment when presented in three dimensions, suggesting an important role for three-dimensional spatial cues.

1. Introduction

Skeletal myopathies, such as muscular dystrophy, and traumatic injuries, can lead to muscle loss [1]. Unfortunately, no therapies exist that can regenerate or restore damaged muscle to a fully functioning muscle. One potential solution offering hope is tissue engineering and the use of cell-seeded biomaterial scaffolds to regenerate muscle in vitro. An important design requirement for engineering functional muscle is myoblast alignment. Cell alignment prior to fusion of myoblasts into multinucleated myotubes is a prerequisite to achieving efficient contraction in an engineered skeletal muscle [2]. While significant research has focused on strategies to guide cell alignment in two dimensions, a three-dimensional (3-D) approach is clearly needed to engineer and re-create the dense and highly organized muscle tissue where cells are aligned in a parallel orientation.

For cells to orient in a particular direction in culture, some form of external cue must be presented to the cells. Towards this end, spatial cues like material-induced contact guidance have been used to facilitate cell alignment along a common axis [3]. Several approaches have proven effective and include patterned structures made by soft lithography [4,5], hot embossing [6], photolithography and solvent casting [7] or alternatively, electrospun networks [4,8,9]. These approaches often use small-scale topography on the order of tens of nanometers to a few microns [4–12] and have achieved excellent alignment and elongation of cells in two dimensions. This range of feature sizes represents the dimensions of native grooves in which myoblasts align in vivo during development, just prior to their fusion into myotubes [13]. Aligning of myoblasts in two dimensions in vitro has been shown to lead to improved differentiation evidenced by the formation of long multinucleated cells [5,7,9], orientation of nuclei along a common axis [5], presence of sarcomeric myosin, a key molecule of the contractile unit [6,7,9], and the development of aligned sarcomeres [9].

Contact guidance cues, however, are typically limited to features that are less than ~50 μm, where sizes greater than this often fail to promote cell alignment [6]. Alternatively, cyclic strain [15–19], static strain [20] and/or electrical stimulation [14,21,22] have all been used to achieve cell alignment, most notably in 3-D cultures. While these stimuli have resulted in improved cell alignment, the contractile forces generated by the engineered muscle

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tissues remain inferior to those of native muscle [8]. Therefore, a 3-D approach that uses spatial cues to align cells prior to the application of electrical and/or mechanical stimulation may be important to achieving functional muscle.

The goal of the present study was to develop a platform that would support high density 3-D culture of muscle cells and present cells with 3-D contact guidance cues. Using this platform, we hypothesized that relatively large feature sizes, when presented in three dimensions in the form of long channels that are macroscopically deep and wide, would support the culture of multi-layers of skeletal myoblasts and promote their alignment in three dimensions. We employed the CliPP technique recently developed by Ashley and Bowman [23] based on thiol-ene photopolymerization reactions, which offers the ability to create features with high fidelity and high aspect ratios, making this technique superior to that of traditional soft lithography. Using CliPP, channeled features ranging from ~60 to 260 μm in width and ~100 to 240 μm in depth were formed into soft hydrogels. C2C12 skeletal myoblasts served as a model cell to probe for the role of 3-D contact guidance cues, as a function of feature size, on cell alignment. Additionally, the differentiation of myoblasts into myotubes, or multinucleated muscle fibers, was examined by real time reverse transcription polymerase chain reaction (RT-PCR) and through qualitative analysis of MHCIIb for the different scaffold geometries. Overall, this study demonstrated important roles for 3-D spatial cues and culturing cells in multiple layers in promoting cell alignment in relatively large features.

2. Materials and methods

2.1. Fabrication of molds

Glass slides (VWR 48300-025) were cleaned with piranha solution (3:1 sulfuric acid to 30% hydrogen peroxide) for 4 h, rinsed three times in deionized water, rinsed once in acetone and dried. Clean slides were functionalized with methacrylate groups by reacting with 0.5 ml methacryloxypropyl-trimethoxysilane (Gelest, Inc.) at 80 °C for 4 h under argon in a cylindrical Teflon® reaction vessel. Slides were stored at room temperature under argon until use.

Mold fabrication is shown in a schematic in Fig. 1. In brief, a solution of 50 wt.% pentaerythritol tetra(3-mercaptopropionate) (PETMP, Evans Chemetics), 40 wt.% 1,3,5-triallyl-1,3,5-triazine-2,4,6-trione (TATATO, Sigma), 0.5 wt.% 2,2-dimethoxy-1,2-diphenylethan-1-one (Irgacure 651, Ciba Specialty Chemicals) photoinitiator and 0.5 wt.% aluminum N-nitrosophenyl hydroxylamine (Q1301, Wako Pure Chemicals) inhibitor was placed on a treated glass slide. The solution was covered with a second glass plate and a photomask and placed on a mask alignment system (Optical Associates Inc., San Jose, CA). The photomask was prepared from transparency film with a printed black background, containing transparent lines that were 40, 100 or 200 μm wide and 3–6 mm long. The thickness was controlled by the mask alignment instrument and was set to be either 100 or 200 μm. The monomer solution was exposed to collimated light (365 nm, 15 mW cm^{-2}) for 4.2 s. The glass slide with the patterned polymer was washed briefly with methanol to remove the unreacted monomer, then dried overnight.

2.2. Characterization of molds and their dimensions

Dried molds were cut in cross-section, then imaged using low vacuum scanning electron microscopy (LV-SEM; JSM-6480LV) at an acceleration of 1 kV. A minimum of five individual inverse channel structures was imaged on a minimum of two different molds for each set. NIH Image J was used to determine width and height of the inverse channel structures on the molds. Profilometry (Veeco Dektak 6 M Stylus Profiler) was also used to verify these dimensions by analyzing the heights of five inverse channel structures on each of two molds for each set of molds. Measurements based on SEM analysis are presented, but were statistically similar to profilometry measurements, when adjusted for sample dehydration.

2.3. Fabrication of PEG hydrogel scaffolds

Poly(ethylene glycol)diacrylate (PEGDA) (MW 3000) was synthesized as previously reported [24]. Characterization of the chemical structure through proton nuclear magnetic resonance (^{1}H-NMR) indicated that 91% of PEG ends were functionalized with acrylate groups. Additionally, acryl-PEG–RGD was synthesized from acryl-PEG-NHS (MW 3400) (Laysan Bio) and the peptide sequence NH_3-Tyr-Arg-Gly-Asp-Ser-COOH (YRGDS, Genscript), and characterized as previously reported [25]. A macromer solution was prepared with a composition of 20 wt.% PEGDA, 1 mM Acryl-PEG–RGD and 0.1 wt.% 2-hydroxy-1-[4-(2-hydroxyethoxy)phenyl] -2-methyl-1-propanone (Irgacure 2959, Ciba Specialty Chemicals) photoinitiator.

The solution was poured into a template constrained on the top by the thiol-ene mold coated with a thin layer of Teflon spray to prevent sticking, on the bottom by an untreated glass slide, and on the sides by 0.8 mm thick Teflon® spacers. The macromer solution was polymerized for 3 min on each side for a total of 6 min under a 365 nm UV lamp (UVP, model XX-20BLB) at an intensity of 4 mW cm^{-2}. The mold was then removed from the polymerized

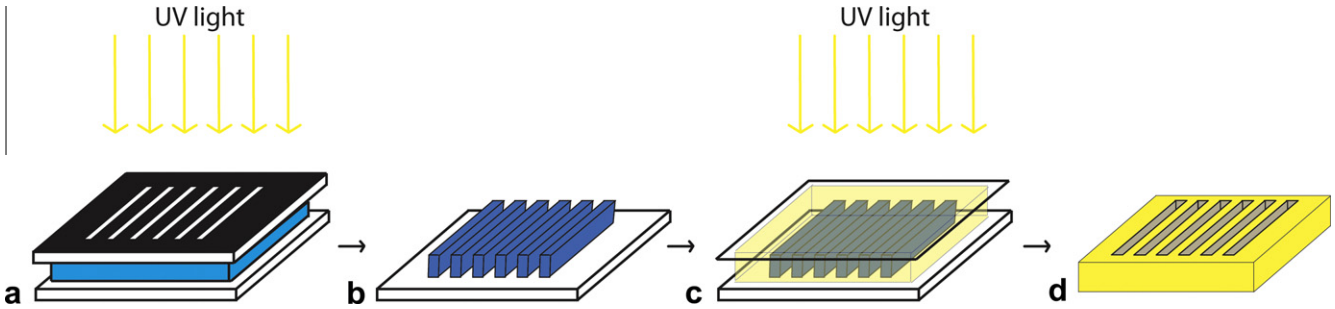

Fig. 1. Illustration of channel formation within PEG hydrogels using a photopatterned thiol-ene mold. The soft-lithography method known as contact liquid photolithographic polymerization (CLiPP) was used to generate the inverse master mold from a thiol-ene precursor solution (a). Unpolymerized solution was washed away, leaving behind the thiol-ene mold (b). A PEGDA macromer solution was placed around the thiol-ene mold secured against a glass slide and irradiated with UV to cure the PEG hydrogel around the master mold (c). Upon removal of the mold, open channels were formed in the surface of the hydrogel with dimensions that were faithful to the master mold dimensions (d).

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