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Polyurethane acrylates as effective substrates for sustained in vitro culture of human myotubes

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ABSTRACT

Muscular disease has debilitating effects with severe damage leading to death. Our knowledge of muscle biology, disease and treatment is largely derived from non-human cell models, even though non-human cells are known to differ from human cells in their biochemical responses. Attempts to develop highly sought after in vitro human cell models have been plagued by early cell delamination and difficulties in achieving human myotube culture in vitro. In this work, we developed polyurethane acrylate (PUA) materials to support long-term in vitro culture of human skeletal muscle tissue. Using a constant base with modulated crosslink density we were able to vary the material modulus while keeping surface chemistry and roughness constant. While previous studies have focused on materials that mimic soft muscle tissue with stiffness ca. 12 kPa, we investigated materials with tendon-like surface moduli in the higher 150 MPa to 2.4 GPa range, which has remained unexplored. We found that PUA of an optimal modulus within this range can support human myoblast proliferation, terminal differentiation and sustenance beyond 35 days, without use of any extracellular protein coating. Results show that PUA materials can serve as effective substrates for successful development of human skeletal muscle cell models and are suitable for long-term in vitro studies.

Statement of Significance

We developed polyurethane acrylates (PUA) to modulate the human skeletal muscle cell growth and maturation in vitro by controlling surface chemistry, morphology and tuning material's stiffness. PUA was able to maintain muscle cell viability for over a month without any detectable signs of material degradation. The best performing PUA prevented premature cell detachment from the substrate which often hampered long-term muscle cell studies. It also supported muscle cell maturation up to the late stages of differentiation. The significance of these findings lies in the possibility to advance studies on muscle cell biology, disease and therapy by using human muscle cells instead of relying on the widely used animal-based in vitro models.

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1. Introduction

Biomaterial-enabled tissue engineering is an active area of research that holds potential to replace and restore damaged tissues, including skeletal muscle tissue [1]. With the regulatory and societal push to replace animal testing, there has been an increasing demand for in vitro cell models that can serve as alternative test platforms. Human cell-derived models are highly sought after as these would closely mimic in vivo human tissue in their biophysical and biochemical responses. Tissue engineering for regenerative medicine and development of in vitro cell models require biocompatible material substrates that provide the right set of chemical and physical cues to interface with cells in a manner that supports their attachment, proliferation and differentiation into the right functional phenotype [2,3]. In particular,

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differentiation of human myoblasts into myotubes and the sustained long-term culture of human myotubes have been exceedingly difficult to achieve in vitro. As highlighted by Trapecar, M. *et. al.* [4] few reports on in vitro human skeletal muscle cell culture exist in the literature. With notable differences between responses of animal and human muscle cells to biochemical and biophysical stimulations [5–8], there is a pressing need for human cell-based in vitro models to advance our understanding of human muscle degeneration, disease and treatment. To date, the limited ability of substrates to retain myotube attachment beyond 1–2 weeks [8] poses a significant barrier to the use of human skeletal myofibers for meaningful in vitro studies.

Sustained attachment and culture of human myotubes require new biomaterials to be developed. Studies have shown that skeletal muscle cells are heavily influenced by substrate stiffness [3,9–12]. Prior work had predominantly focused on biomaterials with mechanical properties that mimic healthy muscle tissue, with $E_{surface}$ = ca. 12 kPa [10]. Materials developed and studied were thus of the 8 kPa -133 MPa range [10]. A study of polyacrylamide gels with surface modulus varied from 8 to 17 kPa [13] demonstrated that the best-performing substrate for muscle cell culture was one that had intermediate mechanical properties closest to that of healthy muscle tissue (~12 kPa). This result resonated with studies done on both murine skeletal muscle cells (C2C12 cell line) [10] and murine skeletal muscle stem cells [12]. Romanazzo *et.al.* [11] studied the differentiation of C2C12 cells cultured on poly-ε-caprolactone materials with modulus ranging from 910 kPa to 133 MPa. This study concluded that increasing substrate modulus adversely affected C2C12 differentiation. Collectively, these studies seem to indicate that materials with modulus close to muscle tissue (~12 kPa) are optimal, with softer or stiffer materials leading to poor muscle cell differentiation outcomes. It has been generally assumed that biomaterials which mimic natural muscle tissue stiffness would be most well-suited as substrates for in vitro muscle cell culture. Nonetheless, since skeletal muscles also attach to bones via tendons in vivo, we hypothesized that stiffer materials with a modulus more similar to tendon or bone could hold promise as well. The moduli of tendon and bone tissues range from hundreds of MPa to several GPa [14–17], and biomaterials with moduli in this range have yet been tested as human muscle cell culture substrates.

In this study we explored the use of stiffer tendon or bone-like materials (with modulus in the 100–1000 s of MPa) for muscle cell culture. We selected to use transparent and rubbery polyurethane acrylate (PUA) materials as previously reported [18], because polyurethanes have proven to be a biocompatible class of materials used successfully in the treatment of damaged tissues [19–21] and commercial biomedical devices such as catheters, blood bags, and cardiovascular devices [2]. We believe that the previously demonstrated ability to pattern UV crosslinkable PUA with nano to micron-scaled surface topographies [18] provide further opportunities to improve cell culture outcomes with topographical patterning. Here we investigated the use of flat PUA with varied tendon-like modulus as biomaterial supports for in vitro differentiation and long term culture of human skeletal muscle cells. In particular, we highlight that the approach we used to tune PUA modulus enabled us to achieve variations in tendon to bone-like surface modulus (from 150 MPa to 2.4 GPa) with conserved surface chemistry and roughness, as characterized in our study. This is a unique feature that allows independent inquiry into the effects of substrate mechanical properties on cell behavior. Here, the effects of PUA surface modulus on human myoblast attachment, proliferation and differentiation are reported. Our findings show that PUA materials with optimal modulus enable sustained human myotube cultures in vitro.

2. Materials and methods

2.1. Materials

Ebecryl® 265 radiation curing resin was obtained from Allnex (Selangor, Malaysia). Trimethylolpropane ethoxy triacrylates (TMPEOTA) were purchased from Cytec (total number of ethoxy group repeating unit (***n***) = 3, New Jersey, USA) and DSM-AGI Corporation (***n*** = 9 and 20, Taipei, Taiwan). The photoinitiators, additol HDMAP and additol CPK, were purchased from Cytec (New Jersey, USA). 1H, 1H, 2H, 2H-perfluorodecyltrichlorosilane was purchased from Alfa Aesar (distributed by VWR Singapore Pte. Ltd., Singapore).

2.2. Preparation of PUA substrates

The PUA mixture and PUA cured samples were prepared using methods reported previously [18]. In the current study, 43 mg TMPEOTA, 1.5 µl additol HDMAP and 1.5 mg of additol CPK were added for every 100 mg Ebecryl® 265 radiation curing resin. The PUA curing time was 5 min. PUA samples were prepared by sandwiching the PUA liquid mixture in between two pre-silanized glass molds and subsequently exposing it to UV light for 5 min. The sample thickness was controlled by using glass coverslips as spacers. PUA samples used for in vitro cell study was of dimension 1.5 cm × 1.5 cm × 0.1 cm. Prior to use in cell cultures, the PUA samples were extracted in MilliQ® ultrapure water (resistivity 18.0 MΩ, Merck MilliPore Division, Merck Pte. Ltd., Singapore) with a ratio of 3.3 ml water/cm^2 sample area at 50 °C for 3 days to remove any uncured or leachable components. Water was replenished daily. The extracted substrates were subsequently oven dried at 37 °C overnight and UV sterilized before use.

2.3. Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR)

ATR-FTIR analysis was performed on a Bruker Vertex 80V FTIR spectrometer (Bruker Singapore Pte. Ltd., Singapore). Samples were pressed onto the diamond internal reflection element of the ATR accessory. Spectra were recorded from 4000 to 600 cm^{-1} at 4 cm^{-1} resolution for 32 scans. The FTIR spectra were analyzed using the integrated OPUS operation and evaluation software (Bruker Singapore Pte. Ltd., Singapore).

2.4. Scanning electron microscope (SEM)

PUA films were coated with a thin layer of gold using a sputter coater 108 (Cressington Scientific Instruments Ltd., Watford, UK) to render the surface conductive and imaged using a JEOL JSM 6360LA SEM (JEOL, Massachusetts, USA).

2.5. Nanoindentation experiment

Nanoindentation test was performed using Agilent G200 Nanoindenter (Agilent Technologies, California, USA). The tip geometry was Berkovich. The surface approach and strain rates were 10 nm/s and 0.05/s, respectively. Maximum depth was set to 3000 nm. Surface modulus was obtained from the plateau of modulus displacement curves, as shown in Fig. A.1 in Supplementary information.

2.6. Swelling study

PUA sample was immersed in a mixture of 1:10 v/v dichloromethane (DCM):isopropyl alcohol (IPA) at room temperature. The

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