PREFACE

Skeletal muscle is one of the abundant tissues of the human body, constitutes 45% of the body mass. Skeletal muscle tissue has a great significance in a wide range of motor activities. Any damage to skeletal muscles leads to partial or complete loss of motor functions, which adversely affect the patient`s health and quality of life. Skeletal muscle loss may occur due to inheritable diseases, acquired disorders, accidental injuries, and cancer. In normal conditions, skeletal muscle tissue has excellent regenerative potential owing to the presence of muscle specific stem cells or satellite cells. Under the conditions of significant muscle loss, their regenerative capabilities prove to be insufficient. Application of approaches, including autologous muscle flap transfer and myogenic cell transplantation, has met with limited success. Tissue engineering approach has emerged as a possible broad area, which may lead to the development of artificial skeletal muscle tissues. There are various challenges to the approach including (1) choice of a myogenic cell, (2) suitable biomaterial as a scaffold, which provides an environment promoting enhanced cell growth and differentiation, (3) mimicking the intricate, sophisticated architecture of the tissue with uniformly aligned myofibers leading to muscle tissues with maximized force generation. (4) recreation of a vascularized and innervated environment, which ensures the survival of the tissue by maintaining the high metabolic demand of the tissue along with the supply of electrochemical signals.

With the advent of enabling technologies in tissue engineering, various strategies have emerged to establish appropriate conditions for cells-embedded tissue engineered scaffolds; that leads to the formation of functional tissues in vitro. Among these strategies, microfabrication, optogenetics and bioprinting based strategies show the potential to meet the various challenges in skeletal muscle tissue engineering. The current thesis presents the development of multiple strategies towards fabrication of skeletal

muscle tissues in vitro using the enabling technologies such as three-dimensional (3D) bioprinting, microfluidics, micropatterning, and optogenetics.

With the aim of applying 3D bioprinting strategy, an inexpensive customized bioprinting system was utilized in combination with a printable bioink blend comprising of gelatin, sodium alginate, and hydrolyzed collagen type-I to fabricate C2C12 cells laden skeletal muscle like constructs. The sequence of processing steps utilizes the thermoreversible property of gelatin, rapid crosslinking capabilities of alginate and cell adhesive nature of collagen; that altogether makes the printability of viable cell laden constructs possible. The bioink is optimized for its rheological properties along with the swelling and porosity-based properties. Live dead staining of the printed constructs shows a gradual increase in percentage of live cells from 80% post printing stage to above 90% after 14 days of culture. Fluorescence staining of the actin cytoskeleton with nuclei confirms the cellular networking and communication. In total, a simple and cost-effective strategy was developed by combining inexpensive bioprinting system and novel bioink composition for the potential development of skeletal muscle like tissue models.

The thesis also elucidates the strategy of creating optically sensitive cells by utilizing optogenetics based technology. The optogenetic tool employed is a plasmid DNA (AAV-CAG-ChR2-GFP responsible for co-expression of a non-selective cationic channel channelrhodopsin-2 (ChR2) along with green fluorescent protein. A liposomal-based transfection method is employed to transfect the isolated plasmid DNA in three secondary cell lines, namely C2C12, HepG2, and NIH-3T3 cells. A significant difference in fluorescence intensity (GFP) is observed between transfected cells and control cells for each of the cell lines. The transfection of C2C12, HepG2, and NIH-3T3 cells was found significantly noticeable after a period of nine, six, and three days, respectively. The percentage of transfected cells is found to be 58.16%, 41.26%, and 46.04% for C2C12,

HepG2, and NIH 3T3 cells, respectively. The study delivers methodology to produce optically controllable C2C12, HepG2, and NIH-3T3 cells.

The thesis also explored the strategy of micropatterning in order to mimic skeletal muscle`s intricate, and sophisticated architecture. For this purpose, a plasma based micropatterning technique known as microchannel flowed plasma process (μCFP) was developed. The micropatterning procedure involves the application of organosilanes octadecyltrichlorosilane (OTS) and 3-aminopropyltriethoxysilane (APTES) to create alternative hydrophobic and hydrophilic domains. Applying the μCFP, micropatterns of different hydrophilic widths including 20 μm, 200 μm, and 1000 μm were created. Incubation of the micropatterned surfaces with 5-fluorescein isothiocyanate (FITC) solution followed by fluorescence microscopy reveals the development of micropatterned substrates. Further, culturing of secondary myoblasts C2C12 myoblasts leads to their gradual alignment in all the micropatterns. The alignment of myoblasts follows a trend where the cells align first in 20 μm followed by 200 μm and finally 1000 μm when observed until 8 days of culture.

Owing to the greater physiological relevance of primary skeletal muscle cells, the thesis explores the isolation, culture, and characterisation of primary myoblasts from rat hind limb muscles. Isolation of cells from rat hind limbs involves dissection of muscles, enzymatic digestion, mechanical trituration followed by plating of cells. Characterization of isolated cells reveal the satellite cell origin of the cells. Nearly 65% of the cells stained positive for Pax7, which is a biomarker for satellite cells. The isolated cells during culture show polygonal, bipolar, and elongated morphologies. The dynamics of these morphologies reveals that the isolated cells first start growing with polygonal morphology, differentiate to bipolar morphology (myoblasts), and then subsequently fuse to form elongated morphology (myotube). Microscopic techniques like bright field

microscopy, live cell imaging and fluorescence microscopic based observations along with morphological characterization of the cells. Calcium dynamics studies show an increase in the peak with the differentiated status of the cells. Functional behaviour of formed myotubes is characterized by using calcium dynamics and kymograph-based approach for spontaneous twitching in myotubes. All these observations and morphometric analysis show that the isolated cells form rat hind limb muscles are of satellite cell origin, and they show highly dynamic behaviour as the myogenesis proceeds from the stages of adhesion and proliferation to the appearance of bipolar myoblasts and finally differentiate into myotubes through alignment and fusion of the myoblasts.

Moving one-step further, the thesis assessed the influence of micropatterning based contact guidance cues over primary myoblast alignment, differentiation, and myotube formation. For this purpose, primary myoblast cells (i.e., satellite cells) isolated from rat hind limb muscle are characterized and cultured for 14 days on micropatterned surfaces having varying widths (20, 200 and 1000 μm) of hydrophilic regimes developed by microchannnel flowed plasma process (μCFP). Several characteristic parameters of muscle differentiation, including the fusion index, maturation index, and average width of the myotubes are quantified. Also, analysis of nuclear parameters such as area and variation in the number of nuclei concerning a change in myotube width is done. The functional behavior of cultured myotubes exhibiting spontaneous contractions is assessed through kymograph to determine the twitch frequency. Also, we evaluate the degree of alignment of myotubes on micropatterned substrates through examining the orientation order parameter and two dimensional fast Fourier transform analysis. Altogether, the studies reveal that the μCFP based micropatterned surfaces not only support differentiation and formation of myotubes but, also lead to contact guidance cues which act as an important regulator for controlling the size and degree of alignment of myotubes

during the myogenesis process. Overall, it provide a set of morphological and image processing based methods and identification of specific parameters to characterize the in vitro satellite cell myogenesis.

To summarize the thesis, multiple strategies like bioprinting, optogenetics and micropatterning were applied in order to mimic the structural, functional and dynamic micrenvironment features necessary for the fabrication of skeletal muscle tissue.

Keywords:

3D bioprinting, Optically sensitive cells, Channelrhodopsin, Microchannel flowed plasma process, Micropatterning, Myotube alignment, differentiation, Myogenesis, 2D FFT analysis, Orientation order parameter.