Matrix Property-Controlled Stem Cell Differentiation for Cardiac and Skeletal Tissue Regeneration

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Doctor of Philosophy in the Graduate School of The Ohio State University

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2015

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Abstract

When ischemia, caused by diseases such as myocardial infarction (MI) or atherosclerotic peripheral artery disease (PAD), happens in myocardium or skeletal muscles, the depletion of oxygen and nutrients can cause the immediate death of muscle cells, the formation of stiff scar tissues, followed by the mechanical and functional properties loss of heart/skeletal muscles. In order to treat these diseases, it’s necessary to: 1). fast re-establish the blood flow of ischemic tissues; 2). fully regenerate the cardiac/skeletal muscles to restore the tissue functions.

One of the widely used approaches to reach these treatment goals is stem cell transplantation. By using novel biomaterial-based scaffolds (gels, foams or fibrous networks), stem cells may be delivered into the injured area, differentiate into cardiomyocytes/myofibers and help the regeneration of local tissues. In the first part of this work, physical induction approaches for stem cell differentiation is presented. Using an electrospinning method, fibrous scaffolds based on hydrogel and polyurethane (PU) were fabricated and cardiac differentiation of cardio-sphere derived cells (CDCs) was successfully induced through the control of scaffold mechanical and morphological properties (fiber diameter, density, alignment, single fiber modulus and scaffold macro modulus). In a hydrogel system, the matrix modulus was successfully decoupled from the chemical structure, composition and water content properties, and a matrix tensile
modulus of around 20kPa was found to better induce the myogenic differentiation of mesenchymal stem cells (MSCs) cultured under normal condition.

In the other hand, due to the harsh local environment caused by ischemia, the transplanted cells usually have low survival and differentiation rates. To solve this problem, cells were delivered in hydrogels with angiogenesis factor basic fibroblast growth factor (bFGF) or oxygen release microspheres (ORM) to conquer the local low oxygen and low nutrient conditions. The second part of this work focuses on the application of this delivery system in vivo using a mice hindlimb ischemia model. Results showed that MSC survival and myogenic differentiation rates were significantly improved both in vitro and in vivo with the delivery of bFGF or ORM under ischemic condition. In addition, a dramatic increase of muscle fiber regeneration, blood flow recovery as well as the mechanical/functional (muscle contractility, fatigue resistance and mice running ability) properties was observed. These results indicate the great potential of this cell-gel-biomolecule system in the treatment of muscle ischemia.

To better understand how the matrix modulus affects the stem cell differentiation, we developed a novel approach using digital image correlation (DIC) and finite element modeling (FEM) to calculate the cell-generated tractions. This is presented in the third part of this work, and our results demonstrated that MSCs with higher myogenic differentiation exerted larger tractions to their surrounding matrix.
Dedication

This document is dedicated to my father in heaven, my parents Guoyin Xu and Renmei Yu, without whom none of my success would be possible.

Have I not commanded you? Be strong and of good courage; do not be afraid, nor be dismayed, for the LORD your GOD is with you wherever you go. (Joshua 1:9)
Acknowledgement

I would like to thank my advisors, Dr. Jianjun Guan and Dr. Peter M. Anderson for their patient guidance and motivation during my graduate study at the Ohio State University. I would like to thank them for all the kindly help and advises not only on my academic study, but also on my learning of life. I want to appreciate the time, expertise and support from my committee members: Dr. John Lannutti and Dr. Steven Niezgoda. I would like to thank Dr. Zhenqing Li, Mr. Xiaolei Guo, Mr. Xiaofei Li, Mr. Zhaobo Fan, Mr. David Gutschick, Dr. Xiang Chen and Dr. Harshad Paranjape for their kindly help and insightful discussions of this work.

I would like to thank Dr. Minghuan Fu and Dr. Zhihong Li for their expertise on animal surgery. I would also thank many of my co-workers from whom I learned a lot on biology and medicine: Dr. Haichang Li (Ma Lab), Dr. KiHo Park (Ma Lab), Ms. Ying Liu (Liu Lab), Dr. Mahmood Khan (Angelos Lab) and Dr. Xiaoyun Xie (Liu Lab). I also want to express my appreciation to Mr. Xinyu Zhu, Mr. Matthew Joseph and Mr. Jeremy Seidt for their kindly help and discussions on some of the experiments. I also want to thank my friends Ms. Xi Wang, Ms. Jing Li, Ms. Jia shi, Ms. Yongyu Yan and Mr. Francisco Carrasquillo, who have made my graduate life so fantastic.

This work was supported by National Science Foundation (1160122 and 1006734), the
American heart association and The Ohio State University Materials Research Seed Grant Program, funded by the Center for Emergent Materials, an NSF-MRSEC, grant DMR-1420451, the Center for Exploration of Novel Complex Materials, and the Institute for Materials Research.
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   Differentiation of Mesenchymal Stem Cells Using Thermosensitive Hydrogels. Acta
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Fields of Study

Major Field: Materials Science & Engineering
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List of Abbreviations

AFM: atomic force microscope        FAK: focal adhesion kinase
bFGF: basic fibroblast growth factor FA: focal adhesion
BPO: benzoyl peroxide               FBS: fetal bovine serum
CDC: cardiosphere-derived cells     FEM: finite element modeling
CHF: congestive heart failure      H&E: hematoxylin and eosin
CLI: critical limb ischemia        IGF-1: insulin-like growth factor 1
CSC: cardiac stem cells            iPSC: induced pluripotent stem cells
DIC: digital image correlation      KLF4: Kruppel-like factor 4
DMD: Duchenne muscular dystrophy   LCST: lower critical solution
dsc: differential scanning calorimetry temperature
DPBS: dulbecco’s modified phosphate LDPI: laser Doppler perfusion imager
buffer saline                       MHC: myosin heavy chain
ECM: extracellular matrix           MI: myocardial infarction
EDL: extensor digitorm longus        MPC: muscle precursor cells
EPR: electron paramagnetic resonance MSC: mesenchymal stem cells
ERK1/2: extracellular-signal-regulated OCT: optimal cutting temperature
kinases 1/2                          ORM: oxygen release microspheres
ESC: embryonic stem cells
PAD: atherosclerotic peripheral artery disease
PDI: polydispersity index
PDGFBB: platelet-derived growth factor-BB
PDMS: poly-dimethylsiloxane
PEG: polyethylene glycol
PEUU: poly (ester urethane) urea
RGD: arginine-glycine-aspartic acid tripeptide
ROS: reactive oxygen species
RVE: representative volume element
SEM: scanning electron microscope
SGTT: sol-gel transition temperature
STAT3: signal transducer and activator of transcription 3
TA: tibialis anterior
TCP: tissue culture plate
TGF-1: transforming growth factor-beta 1
VEGF: vascular endothelial growth factor
1.1 Introduction of cardiac & skeletal muscle tissue engineering

In recent years, cardiac diseases have become the leading cause of death throughout the world. There are around 2.5 million people die annually from severe cardiovascular diseases such as myocardial infarction (MI) and congestive heart failure (CHF) in the United States alone [1]. MI, commonly known as heart attacks, is usually caused by the blood supply interruption due to a collection of lipids and/or white blood cells on the walls of the arteries. After MI, inflammation may happen followed by cardiomyocyte apoptosis, formation of the fibrous scars, increase of the stress burden for the surrounding myocardium tissues, and finally CHF [2]. The regeneration capacity of human myocardium is so insufficient for severe injuries that heart function after MI or other serious cardiac diseases cannot be fully restored by itself. In normal state, new cardiomyocytes in mammals or human beings can rarely divide. Even after an injury, the remaining cardiomyocytes have limited capacity to initiate the DNA synthesis and to re-enter the cell division cycles [3]. Thus one of the most crucial factors to cure cardiac diseases is to deliver proper types of cells to the infarcted locations and/or to induce the transplanted cells’ cardiac differentiation into fully-functional cardiomyocytes in vitro or in vivo. In order to reduce post-MI mortality, various surgical interventions such as
mechanical circulatory devices and drugs have been developed in recent decades [4]. None of these methods, however, can fully restore the patients’ cardiac functions [4].

Skeletal muscle is one of the major components of the body. It comprises around 40% of the total body weight and provides a multiple array of concise motor functions [5]. Under normal situations, skeletal muscles can repair themselves by the removal of injured myofibers and synthesis of new myofibers through the activation of satellite cells [6], which generally stay quiescent within muscle fibers. While severely injured by diseases such as tissue ischemia, muscle teratoma or genetic defects, muscle degeneration will happen due to the limited number and proliferation capability of the satellite cells, followed by impaired healing, permanent loss of muscles volumes and tissue functional deficiency [7]. The treatment goals for the skeletal muscle regeneration approaches are to 1) fast recover the blood perfusion of the damaged tissues; 2) recover the mechanical and functional properties of the injured muscles. However, current surgical procedures and pharmacological treatment show limited efficacy [8, 9].

Stem-cell therapy and cardiac/skeletal muscle tissue engineering have been researched with the goal of better maintaining the myocardium/skeletal muscle function. The stem-cell therapy directly delivers cells into the injured tissues. Cells that have been already utilized in stem-cell therapy include induced pluripotent stem cells (iPSCs) [10, 11], embryonic stem cells (ESCs) [12, 13], mesenchymal stem cells (MSCs) [14, 15], cardiosphere-derived cells (CDCs) [16], skeletal myoblasts [17, 18] and cardiac stem cells (CSCs) [19]. Studies found that after the injection or mobilization of certain cell
types such as bone-marrow-derived cells, cardiomyocytes/skeletal muscle cells were found in the damaged area of the tissues and the muscle function was partly improved [13], indicating that these cells may be capable of migrating to the injured local locations and differentiating into desired cell types (cardiomyocytes for cardiac regeneration and skeletal muscle cells for myogenic regeneration) or inducing the survived cardiomyocytes/satellite cells from other positions to the failure area [20]. Nevertheless, this method is still limited by the insufficient survival, engraftment and differentiation rate of the injected cells. The leaking of cells during the delivery and apoptosis of the injected cells in the local harsh ischemic environments has been suggested as possible reasons [21]. To better address these problems, tissue engineering could be an alternative strategy.

In cardiac & skeletal muscle tissue engineering, biomaterials and regulatory factors can be combined together to closely mimic the extracellular environment in natural tissues to improve the transplanted cell survival, proliferation, differentiation and maturation. Natural biomaterials including collagen [22], matrigel [23], self-assembling peptide [24], decellularized extracellular matrix [25], and synthetic polymers such as poly(lactide-co-glycolide) (PLGA) [26], polycaprolactone (PCL) [27], poly(glycerol-sebacate) (PGS) [28] and polyurethane (PU) [29, 30] are now widely used in cardiac & skeletal muscle tissue engineering. Ideally, the biomaterials should be biocompatible and biodegradable. They should possess naturally occurring cardiac/skeletal muscle-like nanofibrous structures and anisotropic mechanical properties, providing an instructive microenvironment for the cells to attach, grow, migrate and differentiate. Some of these biomaterials can also be
used to deliver protein, gene or RNAs together with the cells. Studies have discovered that by controlling the properties of the biomaterials such as the matrix stiffness [31, 32], topography [33] or chemical structures [34], cardiac/myogenic differentiation of the stem cells can be significantly improved. In this chapter, cardiac & skeletal muscle tissue engineering – especially the in vitro approach – will be introduced and discussed. Approaches using biochemical and biophysical properties of the biomaterial-based scaffolds to induce stem cell cardiac/myogenic differentiation as well as their advantages and limitations will also be presented and discussed here.

1.2 Cardiac & skeletal muscle regeneration approaches

Tissue engineering is an interdisciplinary field in which the principles of the life sciences and material engineering are applied to develop proper tissue substitutes that can fully restore, maintain and improve the tissue/organ functions. Cardiac/skeletal muscle regeneration approaches can be divided into three categories: scaffold-free, in which cell-based sheets or aggregates are implanted for cardiac/skeletal muscles regeneration; in vivo, where cells are mixed with or seeded into biomaterials and then injected or implanted into the injured cardiac/skeletal muscles; and in vitro, in which cells are seeded into a 3-D scaffold to develop into a comparatively mature and functional tissue construct in vitro before implantation into the injured areas for regeneration.

1.2.1. Scaffold-free cardiac/skeletal muscle regeneration

Okano’s group first developed the scaffold-free method in 1999 by using a temperature-sensitive surface for the cultivation of cardiac cells [35]. This method aimed to produce
the most native-similar tissue by forcing the seeded cells to create their own cellular environment without biomaterials. Usually the culture dishes were coated by poly(N-isopropylacrylamide) (PNIAAm), a thermo-sensitive polymer that is hydrophobic at 37 °C, allowing the cells to attach to and grow on, and hydrophilic at 32°C causing cell sheets to detach [36]. In utilizing this approach, the collected cardiomyocytes cell sheets were layered one by one to form 3-D tissue constructs. These constructs were found to have electrical communication and macroscopically spontaneous beating capacity, and even became vascularized in vivo within four weeks [36]. Besides cardiomyocytes, stem cells including adipose tissue derived mesenchymal stem cells (ADMSCs) have also been applied in scaffold-less cardiac tissue engineering [37]. Cell sheets were cultured and obtained similarly to the cardiomyocyte sheets before transplantation into a sub-acute rat MI model [37]. A thick stratum consisting of cardiomyocytes, newly formed vessels and undifferentiated ADMSCs has been observed in vivo. The scaffold-free cell sheets also acted through paracrine pathways and triggered angiogenesis, improving cardiac function in rats with MI. As for the skeletal muscle regeneration, satellite cells were usually co-cultured with fibroblast to self-assemble into cylindrical muscle constructs [38]. Fibroblasts were used to produce and assemble the in vitro-like extra cellular matrix (ECM) to support the proliferation and myotubes formation/myogenic differentiation of satellite cells. Carosio et al, for example, fabricated 3D vascularized skeletal muscle tissues based on myogenic cells, fibroblast and endothelial cells [39]. Their results indicated that these produced scaffold-less constructs demonstrated histological continuity with endogenous muscle fiber in vivo and were capable of generating
significantly more contraction forces in a mice whole muscle graft model where extensor digitorm longus (EDL) muscle was excised.

This detachment method allows the cells to maintain the necessary connection to the extracellular matrix (ECM) they have already produced, making it easier for them to attach to the host tissues without any mediator. Also the absence of other additives reduces the problems of immune rejections. However, the application of this scaffold-less method is limited to three or four layered cell sheet number due to the oxygen and nutrient diffusion distance as well as the constructs’ extremely low mechanical properties especially low elastic modulus and strength.

In order to increase the cell sheet number, an approach stacking cells sheets onto pre-vascularized tissue constructs using multiple surgeries was carried out by Shimizu et al. [40]. On the other hand, Murry et al. produced a scaffoldless cardiac patch by using a rotating orbital shaker to aggregate the cardiomyocytes [41]. Pre-vascularized patches were also prepared by the co-culture of cardiomyocytes, fibroblasts and endothelial cells. Active contraction and electrical pacing have been investigated. What’s more, these pre-vascularized patches exhibited mechanical properties more similar to natural myocardium and better integration with the host tissues than those made only from cardiomyocytes [41]. In order to increase the modulus and strength of the constructs, collagen gel-reinforced cell sheets were developed [42]. However, collagen gel was not an ideal material because of its low elastic modulus, strength and flexibility. Thus, combination of cells with biomaterials that have higher mechanical properties, especially flexibility and
modulus, to the naturally occurring ECM in myocardium/skeletal muscles tend to be a better choice for cardiac/skeletal muscle regeneration.

1.2.2. In vivo cardiac/skeletal muscle regeneration

In vivo cardiac/skeletal muscle tissue engineering involves the combination of biomaterials with cells and the injection of the mixture. This approach can improve cell retention during the injection due to the higher viscosity of the biomaterial-cell mixture. At the same time, these biomaterials can act as a framework for the cells to attach on as well as a mechanical support for the damaged muscle tissues. The biomaterials widely used for in vivo cardiac/skeletal muscle tissue engineering include naturally occurring gels such as collagen [22], fibrin [43], alginate [7] and self-assembling peptides [44], as well as synthetic hydrogels [45-47].

Christman et al. used fibrin gel as an injectable cell-carrier and mechanical support for the ischemic myocardium in their study [21]. Results showed that the injection of fibrin glue together with myoblasts significantly preserved the infarcted myocardium wall thickness and improved cardiac functions after MI in rats. In another study, Page R.L. et al. efficiently delivered the human muscle cells using fibrin microthreads for the treatment of large defect in skeletal muscles [48]. By using this delivery system, scar tissue formation was reduced and new skeletal muscle regeneration was improved. In addition, transplanted cell migrated as early as 2 days after implantation, and integrated with host cells to form mature muscle fibers and connective tissues. The functional recovery of muscles, as assessed by the intermittent tetanic force of tibialis anterior (TA)
muscles, was also significantly improved in animals receiving implants 4 months after surgery.

Nevertheless, in vivo cardiac/skeletal muscle tissue engineering has some limitations. First, even though the biomaterials can provide a matrix for the cells to attach on and at the same time physically protect the cells from immune rejections, the cell engraftment with the host tissues as well as their growth and differentiation are still limited without an ideal biochemical and biomechanical microenvironment. Second, since the injectable biomaterials are mostly biodegradable, their protection and functional aids can only last for a finite time. Last but not the least, these gels usually have slow gelation rate, which may lead to a blood flow block. In order to solve these problems, synthetic hydrogels with controllable degradation rate, tunable gelation rate and chemically modifiable functionalities such as PEG-based hydrogel [47], poly(vinyl alcohol) (PVA)-based hydrogel [49] and poly(N-isopropylacrylamide) (PNIPAAm)-based thermo-sensitive hydrogels [50] were developed. Growth factors and other biomolecules such as Arg-Gly-Asp (RGD) peptide [44] were also bonded to the hydrogels to improve the growth, graft and differentiation of the transplanted cells.

This in vivo cardiac/skeletal muscle tissue engineering method relies mainly on the delivered cells to improve the cardiac/myogenic functions, but fails to take full use of the biomaterials. In native myocardium/skeletal muscles, however, the decrease of tissue functions is the result of both cell (cardiomyocyte/skeletal muscle cells) death and extracellular microenvironment change. Thus, employing biomaterials that can closely
mimic the natural muscle tissues structurally, mechanically and chemically is necessary to better maintain and fully improve the tissue regenerations.

1.2.3. In vitro cardiac/skeletal muscle regeneration

In vitro cardiac/skeletal muscle tissue engineering focuses on seeding cells on pre-formed 3-D scaffolds and controlling the cell survival, proliferation and differentiation processes by precise adjustment of the in vitro culture conditions before implanting the constructs in vivo. The basic requirement for the produced cardiac/skeletal muscle constructs is to possess a similar structure and mechanical properties – especially stiffness and flexibility – to natural muscle tissues, contain high densities of cells, and have enough thickness for clinical application. Ideally, the implanted constructs should be able to integrate with the host tissues, deliver the electrical and mechanical signals within the matrix to the transplanted cells and promote angiogenesis for long-term improvement of the muscle functions. The scaffold properties and the in vitro cultivation process both play important roles in the properties of the produced tissue constructs. Figure 1.1 presents an overview of the in vitro cardiac/skeletal muscle tissue engineering process.
Figure 1.1. An overview of in vitro cardiac/skeletal muscle regeneration process: Cells are seeded into scaffolds made of natural or synthetic polymers, and the tissue constructs are then cultured in vitro under specific conditions to develop into mature tissues before implantation in vivo.

1.2.3.1. Scaffold selection

Scaffolds that have been applied in the field of in vitro cardiac/skeletal muscle tissue engineering can be divided into gel, foam and fibrous forms. Because of their injectability, gels can be delivered by catheters, thus avoiding large surgeries. The pre-formed foam and 3-D fibrous network scaffolds, on the other hand, can well mimic the structure of the natural cardiac/skeletal muscles better by precise control of the pore size, pore shape, fiber diameters and orientations.

Collagen is one of the main components in ECM of the cardiac/skeletal muscles. It is of great importance as a structural support as well as a mechanical property provider of the
muscles. As a result, significant attention has been paid on using collagen as a matrix for cell delivery. While employing the collagen gels, cardiomyocytes/myogenic cells or stem cells were seeded into the collagen solutions and tissue construct was obtained by a simple gelation process [51, 52]. Studies found that by delivering the mixtures of collagen gel and rat neonatal cardiomyocytes into the local injured areas, the contractility of the infarcted heart had been gradually improved, and the maximum beating force was investigated on 18\textsuperscript{th} day after implantation [53]. The collagen gel was later further modified by the matrigel gel, a basement membrane protein matrix, mixed with neonatal cardiomyocytes and cultured in vitro for 12 days before being implanted into the infarcted hearts. Vascularization had been observed within the tissue constructs 14 days after implantation. By four weeks, the heart wall thickness was dramatically increased and the myocardial dilation was significantly attenuated [53].

Sponge is another widely applied scaffold form for in vitro cardiac/skeletal muscle tissue engineering. Large surface area and high porosity make them excellent structures for the cells to attach, migrate and communicate with each other. Furthermore, the interconnected pores also offer spaces for capillary growth. Preformed foam can be prepared by biomaterial solution lyophilization [54], salt leaching [55] or microfabrication techniques to create complex geometries [56]. In order to induce the cell alignment, the foam scaffolds are usually designed with ordered channels or accordion-like honeycombs [56]. Interestingly, the accordion-like scaffolds based on poly (glycerol sebacate) (PGS) have been found to possess anisotropic mechanical properties closely-matched to the native cardiac/skeletal muscles with controllable elastic modulus by
changing the polymer curing time. Better cell alignment and contractility inducible by electric field have been observed using this scaffold. One major limitation of the foam scaffolds is the difficulty to seed cells homogeneously within them. Despite the interconnected pores, cells usually have limited ability to migrate into the inner parts of the scaffolds. Additionally, the limited diffusion of the nutrients and oxygen also constrain the cell migration depth into the scaffolds. Finally, a risk of residual chemicals may exist if harsh chemical conditions are used to produce porous structures.

Fibrous networks have also been utilized for in vitro cardiac/skeletal muscle tissue engineering because of their capacity of closely mimicking the structure (cardiac muscle/myofibers in native healthy tissues are in the form of aligned fibers, and collagen fibers within the cardiac/skeletal muscles are aligned nanofibers) and anisotropic mechanical properties of natural tissues. These scaffolds are usually fabricated by electrospinning, a drawing method in which micro or nano-fibers are produced from polymer solutions with an electric field charging between an output needle and a collector. By changing the processing parameters such as polymer solution concentration, output speed, electric voltage or mandrel (collection part) rotating velocity, one can control the many properties of the scaffolds including fiber diameter, density, alignment, single fiber modulus and the scaffold macroscopic mechanical characteristics. The fibrous networks have a large surface to volume ratio which can contribute to higher oxygen and nutrient permeability, better cell migration and communication. However, it is always limited by the inhomogeneous distribution and limited migration depth of the seeded cells. Recently, Guan et al. carried out a method to produce electrospun 3D
fibrous elastase-sensitive polyurethane scaffolds seeded with mesenchymal stem cells (MSCs) via electrospraying [14]. Cells were successfully and homogeneously distributed throughout the full thickness of the scaffolds, and no significant difference in cell growth kinetics, cell morphology or MSC multipotency was found when comparing the electrosprayed MSCs with the non-electrosprayed ones. The fabricated scaffolds possessed a similar fibrous and anisotropic structure as well as stress-strain response compared to native porcine myocardium. What’s more, 3-D alignment of the seeded cells within the scaffolds was obtained by strengthening the constructs during the in vitro cultivation. Cardiac differentiation of MSCs was also observed, indicating the tissue constructs’ potential of further application in vivo for cardiac regeneration.

1.2.3.2. In vitro cultivation

Except for the scaffold forms, the in vitro cultivation condition including the dynamic environment, electrical stimulation and mass transfer is also an important aspect of tissue construct production. Bioreactors have been widely used to enhance the mass transfer between the tissue construct and its surrounding environment as well as to provide a controllable basic condition such as PH, temperature, oxygen and nutrient contents for the tissue constructs.

Because of the complexity of the in vivo environment, sophisticated bioreactors have been designed and applied for cardiac/skeletal muscle tissue cultivation in vitro. In static state, oxygen and nutrients can only diffuse to a thickness of 100um within the constructs [55], which limits the size/thickness of non-vascularized tissue constructs. The use of
rotating, perfusion or spinner flasks increases the mass transport, which helps the oxygen and nutrients transfer. The spinner flasks may not fit the cardiac/skeletal tissue constructs cultivation very well because the eddies generated by the turbulent flow may be destructive for the seeded cells [57]. Rotating flasks offer a dynamic environment with low shear stress and comparatively high mass transfer [58], but they cannot produce the necessary normal stresses that would occur in vivo [59]. As for perfusion flasks, they can provide excellent oxygen and nutrients transfer to the cells. However, shear stresses are present during the perfusion process, which may lead to a final cell function difference [60]. As a result, methods such as oxygen carriers within the constructs [61, 62], biomolecules modification [7, 43] or pre-vascularization of the system [39, 41] have been carried out recently to better supply the oxygen and nutrients for many cellular processes.

In native myocardium or skeletal muscles, the orderly pacing of electrical signals and macroscopic contraction are of great importance in the tissue organization and functionalization process [63-65]. Radisic et al. found that by applying a 1Hz, 5 V/cm, 2ms monophasic square electrical wave, the cell alignment within the constructs was improved and the synchronic contraction was significantly increased by 7 times after 8 days’ cultivation in vitro [66]. This study also suggested that the conductive and contractile properties of the tissue constructs might depend on the application time of the electrical initiation. If the electric stimulation is applied too early, the electric signals may inhibit cardiac protein accumulation and yield poor contractility. If applied too late, they may no longer have contribution to the functional assembly of the cells [67]. Researches have already found that the electric pulses can cause the hyperpolarization and
depolarization of the cell ends, inducing cells to align according to the electrical field and to promote the formation of gap junction [68]. The increased gap junctions, at the same time, allow the cells to communicate with each other to transfer signals, which lead to a better synchronized contraction.

Considering the fact that the natural heart/skeletal muscles undergo mechanical stretch and contraction while functioning, mechanical strains are also applied during the in vitro cultivation. Zimmerman et al. produced a neonatal rat cardiomyocyte-embedded tissue construct based on collagen/matrigel matrix and cultured the tissue constructs in vitro under a 110% uniaxial stretching at a frequency of 2Hz [69]. Inter-connected and oriented cardiac muscle bundles were observed and the implanted tissue constructions showed contractile properties similar to natural myocardium. What’s more, the engineered cardiac tissues showed non-delayed electrical coupling to the host tissues and also significantly prevented the chambers’ further dilation, improving the cardiac functions after MI.

1.3 Biomaterials properties-guided cardiac/myogenic differentiation of stem cells
While using biomaterials to build up a native-like microenvironment for the seeded cells in cardiac/skeletal muscle tissue engineering, both the chemical and physical properties of the biomaterials are crucial for cell proliferation, differentiation and integration with the host tissues. The features of physical microenvironment that have been seen to moderate stem cells fate in tissue constructs include the matrix stiffness [31], fiber alignment [71, 72], fiber diameter [72, 73] and so on. Additionally, the chemical properties of the applied biomaterials such as the ligand composition [74], special ligand concentration [75] and biodegradability [76] can also affect the cellular processes of the stem cells.
1.3.1. Biophysical properties-guided cardiac/myogenic differentiation of stem cells

1.3.1.1. Matrix stiffness

Decades ago, ECM was thought to be a passive component of various tissues and only act as a support for the cells. However, studies have investigated that the ECM patterns are changing in a specific manner during the embryonic development, and its component such as laminin, collagen or fibronectin are expressed differently in different stages [77].

Figure 1.3. Matrix elasticity (A) and the differentiation of Naive MSCs (B) [31].
Many studies have shown that the extracellular matrix stiffness of the tissue constructs can significantly affect the stem cells’ fates. Hematopoietic stem cells, for example, when cultured on tropoelastin, an elastic biomaterial, showed a two-fold or three-fold expansion capacity compared with those cultured on non-elastic biomaterials [78]. Engler et al. found that mesenchymal stem cells (MSCs) committed to the lineage specified by matrix elasticity instead of other factors after several weeks’ culture in vitro. Soft matrices made of collagen-coated polyacrylamide that mimic brains’ elasticity are neurogenic, stiffer gels with similar modulus as muscles are myogenic and relatively rigid matrices that mimic collagenous bones are osteogenic [31]. In addition, attempts have also been made to produce 3-D matrix that could change its stiffness with time. For instance, Young et al. synthesized a hydrogel that stiffened from around 1kpa to 8kpa over a period of 300 hours by crosslinking thiolated-hyaluronic acid (HA) hydrogel with poly (ethylene glycol) diacrylate (PEGDA) via a slow Michael-type addition [79]. This method was aimed to mimic the modulus change process of the native heart as the cells developed into mature adult myocardiocytes. A 3-fold upregulation of expressions in mature cardiac specific markers as well as around 60% more functional cardiac muscle fibers were observed compared with those cultured in static condition on polyacrylamide hydrogels, indicating the possibility of improving the cardiac regeneration by a precise control over the matrix stiffness.

Beningo et al. have found that when the matrix had a higher modulus/stiffness, the fibroblast cell integrins binding to the ECM developed more mature focal adhesions and mediated signaling pathways that could influence cellular processes [80]. Because
cardiac/myogenic cells can spread and elongate on surfaces, it seemed like this integrin-mediated signaling might also happen in them. On the other hand, a softer matrix allows cells to transfer signal through a larger contractile strain for the same contraction force [81]. As a result, for the purpose of cardiac/skeletal muscle regeneration, an optimal stiffness should be determined and biomaterials should be designed accordingly by varying the physical structure or chemical properties of the polymers.

1.3.1.2. Scaffold topography

Besides the influence of scaffold/matrix mechanical properties, the micro and nano topography of the scaffolds also play a significant role in the remodeling of the myocardium/skeletal muscles. In natural cardiac/skeletal muscles, cell may encounter various levels of topography from macro level such as the shape of blood vessels to micro level includes the projections and morphology of other surrounding cells, and to nano level such as the collagen banding and protein conformation. All of these topographies have potential to influence the cell behavior and functionalities. As mentioned above, pre-formed scaffolds such as porous foams or nanofibrous networks are widely used for in vitro cardiac/skeletal muscle tissue engineering. For porous sponge-like scaffolds, the interconnected pores provide cells with channels to migrate and communicate as well as a supply of oxygen and nutrients, thus having a significant influence on the seeded cell distribution, density, migration and adhesion. For example, Yeong et al. fabricated a porous polycaprolactone (PCL) scaffold with a porosity of 85% and interconnected micropores of 40-100 um [82]. After seeding C2C12 myoblasts into the scaffolds and cultured the tissue constructs in vitro, a high cell density of 1.2*10^6
cells/mL was observed after 4 days. Furthermore, fusion and differentiation of the myoblasts were investigated on Day 6 and further confirmed by myosin heavy chain immunostaining after 11 days’ cultivation [82].

As for the fibrous scaffolds, topography such as the fiber alignment and fiber diameter can also make a difference on stem cells fates. Ordered topographical features such as parallel linear fibrous structure have been seen to be able to induce the cell actin cytoskeleton and the cell-matrix focal adhesion direction via the contact guidance phenomenon. A good example is that bone marrow stromal cells cultured on electrospun poly(ester urethane) urea (PEUU) elastomer substrates assumed a more spindle-shaped morphology with the increasing degree of fiber alignment. Concurrently, the transcription factor scleraxis expression decreased as the alignment increased [33]. Fiber diameters, on the other hand, may be capable of regulating cell spreading, orientation, proliferation and differentiations, resulting from the fact that cells grown on fibers exhibit a higher cells aspect ratio and smaller projection than those grown on smooth substrates [83]. MC3T3-E1 osteoprogenitor cells, for instance, have shown an increasing cell density as the fiber diameter increases from 0.14 um to 2.1um in the absence of osteogenic factors when cultured on electrospun scaffolds made of copolymers poly(ethylene glycol)-poly(lactide acid) (PEG-PLA) and poly (lactide acid) (PLA).
Figure 1.4. A. Diagrammatic representation of two stream electrospinning method. B. Confocal images of MSCs in PLGA-fibrin scaffold after 14 days culture. a. cardiac troponin (green), nuclei (orange) and fibers (blue). b. α-sarcomeric actinin (red), nuclei (blue) and fibers (green). c. Tropomyosin (red), nuclei (blue) and fibers (green) [84].

For other types of nanotopography within the scaffolds, Evelyn et al. found that human mesenchymal stem cells (hMSCs) could be controlled to differentiate into neuronal lineage when seeded onto scaffolds with nanogratings of 350 nm. Their nuclei and cytoskeleton were aligned and elongated along the nanogratings, which suggested the possibility that cells may explore the topographical cues and relay the information to the nucleus to initiate a certain cellular process [85]. One explanation for the effect of topography on cells fate is that the various topography of the scaffolds could influence
the available surface for protein adsorption, which restricts the ECM deposition and finally the number of initial cell focal adhesion sites [85], and the change of cell focal adhesion sites is critical for many cellular processes including cell attachment, migration, growth and differentiation.

1.3.2. **Biochemical properties-guided cardiac/myogenic differentiation of stem cells**

Natural polymers usually perform specific biochemical, structural and functional roles in the body due to the presence of multifunctional groups and high bioactivity. Synthetic materials, however, lack the many biological activities that are commonly owned by natural polymers to influence the growth and differentiation of cells. Their advantages lie in the fact that the polymer composition and ligands concentration can be easily tailored to obtain specific mechanical properties and degradation rate for different tissue engineering. Furthermore, the synthetic polymers can also be used to carry biomolecules such as RGD peptide [44, 86] or growth factors [43] for the stimulation of specific cellular processes so as to recreate full functional tissues. The strategies used to carry biomolecules include non-covalent immobilization (by electrostatic or hydrogen bonding), physical encapsulation and covalent bonding to the functional groups of the biomaterials. Researches have already found that both the bioactivity including ligand composition [74] and concentration [75] as well as the biodegradability [76] of the biomaterials can have a significant effect on the transplanted cells’ fate in cardiac/skeletal muscle tissue engineering.

1.3.2.1. **Ligand composition**
Cells receive much of the information in the form of biochemical cues from their local extracellular microenvironment. Protein signals, for instance, can bind to integrins, a transmembrane receptor that locates on the cell surface, and the associations between them can dictate the cell attachment, spread, proliferation and differentiation rates [87]. In cardiac/skeletal muscle tissue engineering, on top of the structural support function, the binding sites in the biomaterials make it possible for the scaffolds to serve as a delivery system of these regeneration cues to the injured cardiac/skeletal muscles. Growth factors such as hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF) [88, 89], insulin-like growth factor 1 (IGF-1) [90, 91], basic fibroblast growth factor (bFGF) [91, 92], transforming growth factor-beta 1 (TGF-1) [93], hormone EPO and granulocyte-colony stimulating factor (G-CSF) have already been found to have cardio/myogenic-protective effects or help with the tissue fast angiogenesis [94]. On the other hand, mechanical linkages such as the focal adhesions between cells and the extracellular matrix can be formed by bonding the arginine-glycine-aspartic acid (RGD) tripeptide onto the biomaterials to promote cell-matrix interactions.

Davis et al. bonded biotinylated IGF-1, a cardiomyocyte division and differentiation factor, to the self-assembling peptide nanofibers and injected the mixture into infarcted rat myocardium [90]. The bonded IGF-1 was found to be able to induce Akt phosphorylation, decrease the activation of caspase-3, a marker of cell apoptosis, and increase the mature cardiac marker troponin I (CTnI) expression within the cardiomyocytes, indicating that IGF-1 modified self-assembling peptide nanofibers could promote long-term activation of survival pathways and induce the maturation and
functional development of transplanted cells. After injection into the infarcted rat myocardium, a steady and sustained IGF-1 delivery was provided by the system for twenty-eight days. In addition, myocyte cross-sectional area was increased by 25% and caspase-3 cleavage was decreased by 28% compared with the control groups (self-assembling nanofibers only or untethered IGF-1) when combining the IGF-1 modified peptide with cardiomyocytes. A good example of applying the RGD-bonded biomaterials was carried out by Schussler’s group [64]. RGD was coupled with clinically approved collagen matrix in order to improve transplanted cardiomyocyte viability, contractile performance and differentiation. The results showed that the RGD modified collagen scaffolds increased the transplanted cardiomyocytes viability up to one month in vitro. At the same time, the seeded cells were better aligned, elongated and contained centrally positioned elongated nuclei together with cross striation and a three-fold increased contractile performance over the non-modified scaffolds. All of these results indicated a comparatively successful sarcomeric differentiation of cardiomyocytes.

These works showed a great potential of the biomaterials, especially synthetic biomaterials used in cardiac/skeletal muscle tissue engineering, by taking use of their functional groups to bond biomolecules with specific properties to control the cellular processes especially the differentiation of transplanted cells within the scaffolds and finally develop a native-like and functional tissue constructs for clinical use. One of the big challenges with this method lies in the fact that the biochemical cues in vivo are so complex that we are still quite far away from fully understanding it. Furthermore, delivery of proteins to a receptor that is already activated by the endogenous proteins may
be detrimental [95]. Finally, the factors should be targeted to specific cell types to avoid their possible toxic influence on other cells.

1.3.2.2. Ligand concentration

In addition to the ligand composition, the overall adhesion ligand concentration is of great importance in inducing the cellular processes. Cell morphology, for example, has been found to change with RGD ligand concentration. The cells tended to spread isotropically in the matrix with a higher surface ligand density and extend randomly on surfaces with lower adhesion sites [96]. Another good example is cell migration. Due to the limited number of adhesion sites, cells grown on low RGD concentration surfaces are usually unable to migrate. On the other hand, when seeded on surfaces with very high RGD concentrations, the cells obtain such strong interactions that it’s very difficult for them to disrupt the molecular bonds to migrate. The ligand bulk or spatial distribution is especially critical for protein delivery with chemotactic properties: a certain chemotactic gradient had to be developed to deliver these chemokines [97]. Fugetaxis, in which cells are repelled instead of attracted, may happen with a steep chemotactic gradient [98].

In addition to the ligand bulk concentration, the ligand line concentration, which can also be treated as local spacing, is also critical in regulating cell functions. Mooney et al. cultured MC3T3-E1 cells into RGD-modified alginate-based hydrogel with different RGD ligand spacing [75]. The proliferation rate of seeded cells was upregulated from 0.59±0.08 to 0.73±0.03 per day and the cell differentiation was dramatically promoted, as demonstrated by a 4-fold increase of the secretion rate of the osteocalcin, a typical
osteoblast differentiation marker in the late stage, with a ligand spacing from 78nm to 36nm.

1.3.2.3. Biodegradability

Optimally, the biomaterials used for cardiac/skeletal muscle tissue engineering should degrade in a controllable rate without causing toxicity. They should last long enough to guide the integration of transplanted cells with the host tissues, but not too long to inhibit the eventual cell-cell physiological coupling in the myocardium/skeletal muscles [99]. Traditional methods that have been applied to control the polymer degradation rate include varying cross-linking densities, molecular weight and combination of polymers with different degradation rates.

The degradation rate of the scaffolds affects the cellular processes by influencing the biomolecule release rate. In cardiac/skeletal muscle tissue engineering, the temporal delivery of the biomolecules has to be taken into consideration since the expression of a specific factor at the correct time and place is particularly important for normal cardiogenesis/myogenesis [100]. A given signal that is able to induce differentiation in one stage might fail to induce or even inhibit differentiation in another stage. As a result, delivering key proteins and releasing them at the right time with a proper speed is crucial. Recently, different strategies have been carried out for controllable local release of biomolecules based on the biomaterials’ biodegradability. For example, PH-sensitive biomaterials have been developed for protein delivery based on the fact that the cardiac/skeletal tissues suffer a PH change after the ischemia due to the lactic acid
production during anaerobic metabolism [101]. By incorporating PH-responsive groups such as sulfonic acids or carboxylic [76], polyelectrolyte hydrogels were designed to be stable in the physiologic PH while swell in low PH to release their loadings. Recently, biomaterials that are capable of degrading according to the oxidative stress were also developed [102], showing a potential as delivery systems for biomolecules in cardiac/skeletal muscle tissue engineering given that the reactive oxygen species (ROS) are upregulated during the early stage after ischemia (by heart attack or skeletal muscles injuries/diseases).

1.4 Discussion

Cardiac/skeletal muscle tissue engineering has made huge strides in the past few decades to improve the potential methods available for patients with severe cardiac/skeletal muscle failures compared with traditional strategies. Table 1.1 lists some of the experimental results in cardiac/skeletal muscle tissue engineering during the past few years. Despite the fact that in vitro cardiac/skeletal muscle tissue engineering has been successfully demonstrated to be able to improve the cardiac/skeletal muscle regeneration, and the various biophysical and biochemical cues mentioned above have been intensively studied these years to obtain a better control over the many cellular processes including the cell attachment, migration, division and differentiation, there are still some significant problems associated with this strategy. First, the tissue constructs cannot provide a native-like microenvironment for the complete development of functional cardiac/skeletal muscle tissues. Accordingly, biomaterials with matched mechanical properties
(cardiac/skeletal muscles are highly flexible and soft with elastic modulus around 1-140 kpa and fracture strain around 100% [103]) and similar fibrous structure with proper spatial and local micro and nano-topography distribution are in need.

In addition, the transplanted cells within the scaffolds usually have limited engraft, growth and differentiation rates in the long term due to the ischemic environment in the infarcted myocardium/ischemic limbs, asking for the fast angiogenesis within the scaffolds in vitro and/or in vivo. In order to stimulate angiogenesis, endothelial cells, which are capable of forming blood vessels to provide oxygen and nutrients as well as exerting tropic and inotropic effects on cardiomyocytes/myofibers [104, 105], and various angiogenesis-promoting biomolecules such as stromal cell derived factor 1(SDF-1) and vascular endothelial growth factor (VEGF) can be delivered by biomaterials. For instance, Segers et al. locally delivered protease-resistant SDF-1 by tethering it to self-assembling peptides and injected the matrix into infarcted rat myocardium [97]. Capillary density was increased from 169 ± 42 to 283 ± 27 per mm², and cardiac function, especially the ejection fraction, was increased significantly compared with control group in which native SDF-1 alone was delivered.

Finally, considering the large number of biomolecules that participate in the cardiac/skeletal muscle regeneration process, it is a big challenge to deliver all the signals at once. As a result, delivering key proteins at the right time and position is of great importance. Recently, various drugs or proteins delivery or release systems including the PH-responsive release and oxidative stress-sensitive release were carried out based on the
bioactivity and biodegradability of the applied biomaterials. A crucial aspect for the success of these systems will be the temporal orchestration as well as the direct correlations between the disease stages, release kinetics and therapeutic effects. With this in mind, a deep exploration of the stimuli change at different stages and precise design of biomaterials capable of detecting these changes with the corresponding sensing groups are essential.

The successful reestablishment of a favorable extracellular microenvironment for cardiac/skeletal muscle tissue regeneration asks for the synergistic actions between seeded cells, biomaterials-based scaffolds and multiple biomolecules such as growth factor and RGD peptide [86, 106]. Despite the problems listed above, significant progress on cell-seeded biomolecule-modified 3-D scaffold application in cardiac/skeletal muscle tissue engineering has been witnessed during the past few decades. Additionally, researchers are gaining a better and deeper understanding of the effects of various physical and chemical cues such as scaffold mechanical properties, micro or nano topography, biomaterial composition, ligand concentration and biodegradability on the cardiac/myogenic regeneration process. Such an increasing understanding of the cells’ response to their microenvironment provides an improved and encouraging insight for future studies into a more rational and proper design of the scaffolds to better regenerate the damaged cardiac/skeletal muscles.
Table 1.1. Experimental results of some cardiac/skeletal muscle regeneration studies.

<table>
<thead>
<tr>
<th>Scaffold type</th>
<th>Cell type</th>
<th>Culture Condition</th>
<th>Animal model</th>
<th>Improvement in cardiac function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matrigel hydrogel</td>
<td>Mouse ESCs</td>
<td>In vivo</td>
<td>Lewis rats</td>
<td>W.S. ↑, F.S.↑, no LV dilation</td>
<td>[23]</td>
</tr>
<tr>
<td>Self-assembling peptide nanofibers modified by IGF-1</td>
<td>Mouse cardiomyocytes</td>
<td>In vivo</td>
<td>Sprague-Dawley rats</td>
<td>Activate Akt, C. D↑, increase myocyte cross-sectional area, decrease caspase-3 cleavage</td>
<td>[90]</td>
</tr>
<tr>
<td>Accordion-like honeycomb sponge based on PGS</td>
<td>Neonatal rat heart cells</td>
<td>Static in vitro culture with electric pulse</td>
<td>--</td>
<td>Native-like stiffness, electric field induced cell contractility, C.A. ↑</td>
<td>[56]</td>
</tr>
<tr>
<td>Electrospun PU nanofibers</td>
<td>Mouse MSCs</td>
<td>In vitro culture with constant stretch</td>
<td>--</td>
<td>Native-like structure and stress-strain response, C.A. ↑, C.D. ↑</td>
<td>[14]</td>
</tr>
<tr>
<td>Collagen gel</td>
<td>Neonatal rat heart cells</td>
<td>In vitro culture with dynamic stretch before implantation</td>
<td>Wistar rats</td>
<td>Undelayed electrical coupling, prevent LV further dilution, W.S. ↑, F.S. ↑</td>
<td>[69]</td>
</tr>
</tbody>
</table>

Continued
Table 1.1 continued

<table>
<thead>
<tr>
<th>RGD modified collagen scaffold</th>
<th>Mouse cardiomyocytes</th>
<th>Static in vitro culture</th>
<th>--</th>
<th>High coupling yields, C.V.↑, C.D↑, High cell contractility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrin Microthreads</td>
<td>Human adult skeletal cells</td>
<td>In vivo</td>
<td>Female nude SCID mice</td>
<td>Reduced scar formation, transplanted cells participate in healing process, M. R. ↑, M.C.↑</td>
</tr>
<tr>
<td>--</td>
<td>Myogenic cells, endothelial cells and fibroblasts</td>
<td>Static in vitro culture</td>
<td>C57BL/6 wide type mice (Muscle graft model 1)</td>
<td>Developed 3D vascularized skeletal muscles, histological continuity with host muscles, M.C.↑</td>
</tr>
<tr>
<td>PDMS/pHEMA membrane with parallel microgrooves/collagen</td>
<td>C2C12 myoblasts</td>
<td>Static in vitro culture</td>
<td>--</td>
<td>Improved cell alignment and fusion in microgroove direction, M.D. ↑</td>
</tr>
<tr>
<td>Microporous alumina membrane modified by atelocollagen and PDMS</td>
<td>C2C12 myoblasts</td>
<td>Static in vitro culture with electric pulse</td>
<td>--</td>
<td>Increased number of stimuli-responding myotubes, M.C.↑, M.D. ↑</td>
</tr>
</tbody>
</table>

S.V.=stroke volume; W.S. =infarct wall thickness; E.F.= left ventricle ejection fraction; F.S.=fractional shortening; LV= left ventricle; C.D. = cardiac differentiation of transplanted cells; C.A.= cell alignment; C.V.=cell viability; M.R.= muscle regeneration; M.C.= muscle contractility; M.D.= myogenic differentiation.
1.5 References


[46] Li Z, Guo X, Guan J. A thermosensitive hydrogel capable of releasing bFGF for enhanced differentiation of mesenchymal stem cell into cardiomyocyte-like cells under ischemic conditions. Biomacromolecules. 2012;13:1956-64.


Chapter 2: Cardiac Differentiation of Cardiosphere-Derived Cells (CDCs) in 3D Electrospun Scaffolds

2.1 Introduction

Myocardial infarction (MI) affects more than 8 million Americans [1]. MI causes massive heart cell death and heart function decreases. Various therapeutic strategies have been used to treat MI as described in Chapter 1 [1-8]. However, normal heart function cannot be restored after MI since heart cells are less proliferative and endogenous cells are unable to produce sufficient heart cells for effective heart muscle regeneration. Stem cell therapy is a potential approach to regenerate heart tissue [2-10]. It delivers stem cells to the damaged areas (scar tissue) where they can differentiate into heart cells. However, various animal studies and clinical trials show that the success rate for delivered stem cell differentiation is low and it therefore remains impractical for widespread clinical application [2-11]. Identifying and addressing the causes for the low success rate is needed for heart tissue regeneration.

The causes of low differentiation are not completely clear. The harsh biochemical environment initiated by MI compromises both stem cell survival and differentiation [2,3]. This includes low nutrient and oxygen conditions, high concentrations of reactive oxygen species (ROS) and inflammatory molecules in the infarcted hearts [2,3].
Collagen, a major extracellular matrix (ECM) in the scar tissue, may also contribute to low cell differentiation, although this has not yet been explored in current literature. Following MI, the composition, biomechanics and structure of collagen fibers change continuously [12-23]. Their stiffness gradually increases to 3 to 4 times compared with collagen in healthy heart tissue [24]. A hypothesis is that such increases may affect stem cell differentiation, consistent with recent in vitro studies showing that matrix stiffness regulates stem cell differentiation as discussed in Chapter 1[25-33]. This hypothesis can be examined by studying how collagen at different stages of MI affects stem cell differentiation.

Recent studies in stem cell biology show that stem cells can differentiate into different lineages in vitro when exposed to intrinsic properties of the matrix, such as composition, biomechanics and structure as mentioned in Chapter 1 [34]. These parameters modulate the forces exerted between the cells and matrix. Mechano-sensitive pathways subsequently convert these forces into biochemical signals that commit the cell to a specific lineage [34]. Each of these attributes is significant. Matrix composition can regulate cell fate by differential integrin binding to the matrix [34]. For example, ECM proteins can interact with a specific subset of integrins on the mesenchymal stem cell (MSC) surface, directing their differentiation into heart cells [35]. Specific domains from ECM proteins can dramatically affect cell differentiation by conformational changes that enhance force transmission as integrins bind to these ligands [36-39]. Matrix structure can also affect stem cell differentiation, by affecting integrin binding and the distribution of focal contacts between the matrix and cells [34]. For example, embryonic stem cells
(ESCs) show different differentiation behaviors in a microporous foam with solid walls compared to fibrous ones [34,40,41]. Finally, matrix mechanical properties can induce stem cell differentiation. Specifically, stem cells show lineage-specific differentiation when cultured on matrices mimicking the stiffness of native tissue [25-33]. MSCs become neurogenic, myogenic and osteogenic on matrices mimicking neural, skeletal muscle, and bone stiffness environments, respectively [25]. We have demonstrated that MSCs differentiate into cardiomyocytes in a hydrogel with 45-65 kPa modulus [32], while cardiosphere-derived cells (CDCs) differentiate into cardiomyocytes at 35 kPa modulus [31]. More recently, we demonstrated that fibrous scaffolds mimicking the global mechanical properties of healthy heart tissue can induce MSCs differentiation into cardiomyocytes [30].

Although many studies have investigated the relationship among collagen global biomechanics, collagen organization and heart function [12-23], the current literature lacks systematic studies of the effect of collagen fibers in scar tissue on stem cell differentiation. Specifically, how do collagen composition, single fiber modulus, fiber density and alignment evolve at different stages of MI and how do these changes affect cell differentiation? After being delivered into the scar tissue, stem cells adhere to collagen fibers. Fiber properties such as composition, single fiber modulus, fiber density and alignment determine the forces that a stem cell can exert on collagen fibers. Cells contact single fibers and thus sense the single fiber rather than global modulus. Fiber density and alignment determine the distribution of focal contacts between fibers and cells. The evolution in collagen composition, biomechanics and structure due to MI
introduces the possibility that differentiation might be stimulated or inhibited during certain stages. This may impact both the optimal timing for stem cell delivery into infarcted hearts and the design of matrices to promote cardiac differentiation. However, these studies cannot be conducted in vivo, as other parameters like cytokines and glycosaminoglycans (GAGs) may concurrently affect cell differentiation, making it difficult to isolate the effects of collagen fibers alone [2,3].

Towards the goal of elucidating the relationship between stem cell differentiation and changes in collagen fiber properties after MI, we created a novel in vitro model based on fibrous scaffolds that mimic structure of the collagen matrix. By modulating fiber composition and fabrication parameters, scaffolds with different single fiber moduli, global moduli, alignment, and fiber density were obtained. CDC cardiac differentiation in the scaffolds was then investigated and correlated to these properties in this chapter.

2.2 Materials and methods

2.2.1. Materials

N-isopropylacrylamide (NIPAAM, TCI) was recrystallized with hexane for three times before use. 2-Hydroxyethyl methacrylate (HEMA, Alfa Aesar) and acrylic acid (AAc, Acros) were purified by passing through an inhibitor-remover column. Polycaprolactone (PCL) diol (Mn = 2000, Acros) was dried under vacuum at 55°C overnight before use. β-butyrolactone (VWR), gelatin type A (Acros), hexafluoroisopropanol (HFIP, Oakwood), tin(II) trifluoromethanesulfonate (Sn(OTf)2, VWR), 1,6-diisocyanatohexane (HMDI,
Acros), 1,4-diaminobutane (DAB, Acros), stannous octoate (Sn(Oct)$_2$, Pfaltz & Bauer), and dimethyl sulfoxide (DMSO, Fisher) were used as received.

2.2.2. Poly(ester urethane) urea synthesis

Poly(ester urethane) urea (PEUU) was synthesized from PCL, HMDI and DAB according to our previously established method [42]. In brief, 12.37 g of PCL was dissolved in 150 mL DMSO in a 3-neck flask with nitrogen protection. Following addition of 2 mL of HMDI and 6 drops of Sn(Oct)$_2$, the mixture was heated up to 70°C to start the reaction. After 3 h, the solution was cooled to room temperature. A solution of DAB (0.544 g) in 100 mL DMSO was then added dropwisely to the flask. The reaction was conducted overnight under stirring. The resulting PEUU solution was precipitated in excess cool DI water and dried under vacuum. The synthesized PEUU has a glass transition temperature ($T_g$) of -46°C, tensile strength of 4.7±0.6 MPa, breaking strain of 946±78%, and Young’s modulus of 2.2±0.3 MPa.

2.2.3. Hydrogel synthesis

The hydrogel was based on NIPAAM, AAc, and a macromer based on HEMA and oligo ($\beta$-butyrolactone). The macromer was synthesized by reacting HEMA with $\beta$-butyrolactone (molar ratio 1:6) under 110°C with nitrogen protection for 1 h. Sn(OTf)$_2$ was used as a catalyst. After the reaction, the mixture was cooled to room temperature, dissolved in THF, and precipitated in cool DI water. The precipitate was then dissolved in ethyl acetate, dried over anhydrous magnesium sulfate, and filtered. The solvent was
removed under reduced pressure. The resulting macromer was abbreviated as HEMA-oHB6.

The hydrogel was synthesized by free radical polymerization using benzoyl peroxide (BPO) as the initiator\cite{43,44}. The molar ratio of NIPAM/AAc/HEMA-oHB6 was 86/4/10. Stoichiometric amounts of NIPAM, AAc and HEMA-oHB6 were dissolved in dioxane in a three-necked flask. The monomer concentration was controlled at ~5 wt%. The solution was bubbled with nitrogen for 15 min before BPO solution (in dioxane) was injected into the flask. After 24 h of reaction at 70°C, the solution was cooled to room temperature and precipitated with hexane. The polymer was purified twice using THF/diethyl ether. The resulting polymer was finally dried under vacuum overnight.

The synthesized hydrogel had a sol-gel temperature of ~14 °C as determined by DSC. This allows the hydrogel to maintain a solid state at body temperature. The degradation product poly (NIPAM-co-AAc-co-HEMA) has 4 % of AAc, which has been demonstrated to have a LCST above 37 °C\cite{45}. Therefore, the degraded polymer can be dissolved in body fluid and removed from the body. The hydrogel was highly flexible with a breaking strain >300% and Young’s modulus of 11.1±3.5 kPa (after gelation at 37°C, gel solution was prepared at 10% w/v in DPBS).

2.2.4. *Mouse cardiosphere-derived cells (CDCs) culture*

Mouse CDCs were isolated from the explant of exocardium biopsies as described previously\cite{46}. The CDCs were cultured in Iscove’s Modified Eagle Medium (IMDM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals) and
1% penicillin-streptomycin (Invitrogen) under normal culture conditions (21% O₂, 5% CO₂, 37°C). Cells at passage 13-17 were used. Previous work has demonstrated that CDCs at these passages preserved phenotype and multipotency [47].

2.2.5. Effect of electrospraying on CDC survival and growth

To investigate the effect of electrospraying voltage on CDC survival and growth, CDCs were electrosprayed under 10, 15 and 20 kV. Before electrospraying, cells were suspended in the culture medium containing 2% gelatin A. Some of the electrosprayed CDCs were then stained with Trypan Blue to determine percentage of cells that survived during electrospraying. The remaining cells were seeded in a 96-well culture plate to determine if electrospraying affected cell growth. CDCs without electrical treatment were used as the control. After 1, 3 and 5 days of culture, an MTT assay was used to determine cell viability [48].

2.2.6. Fabrication of tissue constructs by electrospinning & electrospraying

A two-stream electrospinning and electrospraying setup was employed to generate fibrous tissue constructs as described previously [30]. CDCs were suspended in IMDM with 2% gelatin, then loaded into a syringe and charged at +10 kV for electrospraying. Gelatin not only increases the viscosity of the cell suspension ease for electrospraying, but it also help preserve the viability of CDCs during electrospraying [30]. The total number of CDCs used for each tissue construct was 100 million. The cell suspension was injected at a fixed feeding rate of 15 mL/h. The electrospinning stream included solution of hydrogel and polyurethane blend (15%, w/v in HFIP). The hydrogel/polyurethane ratio
was controlled at 90/10, 70/30, and 50/50 wt%, respectively. The solution was charged at +15 kV and two different flow rates (4.5, and 7.5 mL/h) were used. The fibers and CDCs were collected on a rotating mandrel (diameter 11.5 cm, rotation speed 1000 rpm) charged at -10 kV. After ~40 min of fabrication, the tissue constructs were immersed in culture medium overnight before transferred to spinner flasks for 1 week in vitro culture. The spinner flasks were used to ensure sufficient delivery of oxygen and nutrition to the whole tissue constructs. The nomenclature for the resulting constructs is shown in Table 2.1.

Table 2.1. Fabrication parameters for tissue constructs.

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>Hydrogel content</th>
<th>PEUU content</th>
<th>Pumping speed (mL/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) HG9PU1/7.5</td>
<td>90%</td>
<td>10%</td>
<td>7.5</td>
</tr>
<tr>
<td>(B) HG9PU1/4.5</td>
<td>90%</td>
<td>10%</td>
<td>4.5</td>
</tr>
<tr>
<td>(C) HG7PU3/4.5</td>
<td>70%</td>
<td>30%</td>
<td>4.5</td>
</tr>
<tr>
<td>(D) HG5PU5/4.5</td>
<td>50%</td>
<td>50%</td>
<td>4.5</td>
</tr>
</tbody>
</table>

2.2.7. *In vitro culture of tissue constructs*

After incubating in culture medium for 24 h, part of the construct was cut for dsDNA analysis, staining, imaging, and mechanical testing. The rest was transferred into a spinner flask for further culture. Each spinner flask was supplemented with 100 ml of culture medium. The samples were harvested after 3 and 7 days of culture for dsDNA analysis, RNA extraction for gene expression test, and immunohistochemistry studies.
2.2.8. CDC survival and growth in tissue constructs

To determine cell distribution in the constructs after fabrication, the samples were stained with phalloidin rhodamine for F-actin after 24 h or 7 days of culture. Confocal images were taken using Olympus Fluoview 1000 Laser Scanning Confocal microscopes.

dsDNA assays were employed to determine the number of living cells in tissue constructs. In brief, tissue constructs collected at each chosen time point were washed with fresh PBS and digested with papain solution overnight at 60°C [44]. PicoGreen (Invitrogen) was then added to the digested solution followed by fluorescent intensity measurements using a fluorescence plate reader at an excitation wavelength of 480 nm and emission wavelength of 520 nm. Standard dsDNA with graded concentrations was used to calibrate the fluorescence intensity vs. DNA concentration. The dsDNA content for each construct was normalized by the weight of the construct.

2.2.9. Construct characterization

Tissue construct morphology was determined using scanning electron microscopy (SEM). The constructs were fixed with 4% paraformaldehyde at 37°C for 1 h. After rinsing with PBS, the constructs were dehydrated by 50%, 70%, 80%, 95% and 100% ethanol solutions in sequence. Hexamethyldisilazane was then used to chemically dry the samples. To image the interior structure of the tissues, the surface layer of the constructs was peeled off. To image construct cross-section, the constructs were frozen in liquid nitrogen and quickly cut by a blade. The samples were finally sputter-coated with gold and imaged with a FEI NOVA nanoSEM. Fiber diameters were determined from SEM
images of interior structure using Image J software, while fiber densities were calculated from SEM images of tissue construct cross-sections. Fiber orientation distributions within the tissue constructs were calculated by using a Hough transformation algorithm in Matlab, based on the SEM images of the scaffolds.

Single fiber modulus was measured by a BioScope Catalyst Atomic Force Microscope (Bruker Corporation) equipped with a water bath. The testing was conducted in PBS at 37°C. The samples were scanned with the Tap 150A probe. The tip parameters are as follows: $k = 5$ N/m, nominal tip radius = 8 nm, Poisson’s ratio = 0.5. After visualizing and identifying single polymer fibers, mapping of the mechanical properties on single fiber was performed under PeakForce Quantitative Nanomechanical (QNM) imaging (Standard Amplitude) mode. For each group, multiple polymer samples were measured (2-3 per group), and several locations (4-6) were scanned. The scan area of each location was about $50 \times 50 \mu m^2$, and the scanning rate was 0.5 Hz. For modulus measurement, a ramp size of $2 \mu m$ (Deflection Mode – Relative, Threshold – 30 nm) was taken at each data point. Minimum of 500 data points (force-displacement curves on individual fiber) were obtained for each location. Data was then analyzed using NanoScope Analysis software (Bruker Corporation).

Mechanical properties of the tissue constructs were assessed by uniaxial tensile test performed on a TestResources 1000R load frame (model 1322) equipped with a 50 lb load cell in a 37°C water bath. A cross-head speed of 10 mm/min was employed. At least eight $20 \ mm \times \ 2.5 \ mm$ size samples were tested for each construct.
The resulting uniaxial tensile mechanical response of the tissue constructs was related to the hydrogel/polyurethane fiber angular distribution following the constitutive development of Sacks et al. [49,50]. Here, it is assumed that a representative volume element (RVE) can be identified to capture the microstructure in average sense. The RVE is treated as a three-dimensional continuum and assumed to be small relative to the macroscopic scale of the constructs. The hydrogel/polyurethane fibers are modeled as a hyper-elastic solid. Within the RVE, the following assumptions are made:

1. The tissue is idealized as a planar fiber network, and hydrostatic effect from surrounding water is not modeled.

2. The undulation of the hydrogel/polyurethane fibers is assumed to gradually disappear with stretch on the scaffold. The load required to straighten the fibers is negligible compared to the load transmitted once the stretched fibers are straightened.

3. The fiber strain can be calculated from the transformation of the global strain tensor to the local fiber basis.

4. The strain energy function of the constructs is the sum of the individual fiber strain energies.

5. The mechanical contribution of cells is not explicitly modeled.

From assumption 3, the uniaxial strain $\varepsilon$ along each fiber is expressed in terms of the
global tissue strain tensor $E$ and fiber axis direction $N$,

$$
\varepsilon = N^T \cdot E \cdot N, \text{ where } N_1 = \cos \theta, N_2 = \cos \theta, N_3 = 0
$$

(2.1)

Thus, $\theta$ is the angle subtended by the fiber axis and the tensile direction (1-axis), which is parallel to the circumferential direction of the mandrel during fiber spinning. Assumption 1 imposes $N_3 = 0$.

From assumption 4, the strain energy per unit volume of scaffold is

$$
W = V_f \int_{-\pi/2}^{\pi/2} R(\theta) w(\varepsilon) d\theta
$$

(2.2)

Here, $w$ is the strain energy per unit volume of fiber, $R(\theta)$ is the distribution of fiber orientations, and $V_f$ is the volume fraction of fibers in the RVE. The 2nd Piola-Kirchoff stress $S$ along the 1 (tensile) axis and the corresponding local stress $\sigma$ along the fiber axis are

$$
S = \frac{\partial W}{\partial \varepsilon} \quad \text{and} \quad \sigma = \frac{\partial W}{\partial \varepsilon}
$$

(2.3)

Here, $E$ is the Green Lagrange strain in the RVE along the tensile axis. Using Eqns. (1-3) furnishes a tensile stress-strain relationship for the RVE [51]

$$
S = V_f \int_{-\pi/2}^{\pi/2} R(\theta) \sigma(\varepsilon) [\cos^2 \theta - \nu \sin^2 \theta] d\theta
$$

(2.4)
The bracketed term \( \frac{d\varepsilon}{dE} \) = \( \frac{d\varepsilon}{dE} \), where Poisson's ratio of the RVE is defined by \( \nu = -\frac{E_T}{E} \), the ratio of the transverse and axial strains during a tension test.

Expressions for the elastic modulus of the scaffold at small and large strain can be developed from Eq. (4). The fiber orientation distribution function \( R(\theta) \) can be determined by a fit based on modified Cauchy distribution:

\[
R(\theta) = \frac{a}{\pi} + \frac{1-a}{\pi c [1+(\theta - L)^2/c^2]}
\]  

(2.5)

where \( a \) represents the random orientation component, \( c \) is a shape parameter, and the location parameter \( L = 0 \) since the samples are aligned to scaffold axis. In the small strain regime, the elastic modulus of the fiber is described by a relatively small value \( m_0 \), valid during the initial stages of stretch before the fibers become straight. If \( \sigma = m_0 \varepsilon \) is inserted into Eq. (4), the elastic modulus \( M_0 \) of the RVE at small strain is

\[
M_0 = V_f m_0 A_0, \quad A_0 = \int_{-\pi/2}^{\pi/2} R_0(\theta)[\cos^2 \theta - \nu \sin^2 \theta]^2 d\theta
\]  

(2.6)

In the opposing limit when the applied strain is sufficiently large to fully straighten the fibers, the incremental modulus of the fibers is assumed to have a larger value \( m_L \). In this limit, the fibers are assumed to be fully aligned at \( \theta = 0 \), so that \( A = 1 \) and the RVE modulus at large strain is

\[
M_L = V_f m_L
\]  

(2.7)
2.2.10. CDC cardiac differentiation in tissue constructs

CDC cardiac differentiation in tissue constructs was determined by real-time RT-PCR and immunohistochemistry. Cells cultured on tissue culture plate (TCP) were used as a control. Tissue constructs were collected after 3 or 7 days of culture in spinner flasks. RNA was extracted from the constructs by TRIzol (Sigma). Approximately 1 µg of RNA was used to synthesize cDNA using High Capacity cDNA Reverse Transcription Kits (Applied Biosystem). Real-time RT-PCR was performed with a Mastercycler ep gradient S thermal cycler (Eppendorf) and Platinum Taq DNA Polymerase (Life Tech.) with primers listed in Table 2.2. Fold differences were calculated using the standard ΔΔCt method with β-actin as the housekeeping gene [52,53].

Table 2.2. Primers used for real time RT-PCR to check the cardiac differentiation of CDCs.

<table>
<thead>
<tr>
<th>Transcription</th>
<th>Prime sequences</th>
<th>Tm (°C) a</th>
</tr>
</thead>
<tbody>
<tr>
<td>GATA4</td>
<td>Forward TAAATCTAAGACGCCAGCAG</td>
<td>59.8</td>
</tr>
<tr>
<td></td>
<td>Reverse TGCCCCATAGTGAGATGACAG</td>
<td>61.4</td>
</tr>
<tr>
<td>CTnT</td>
<td>Forward TACATCCAGAAGACAGAGCG</td>
<td>61.4</td>
</tr>
<tr>
<td></td>
<td>Reverse CTCTCAGTTGGTTCTTCATTCAAG</td>
<td>60.7</td>
</tr>
<tr>
<td>MYH6</td>
<td>Forward GAGGAGATGCGAGATGAGAG</td>
<td>61.6</td>
</tr>
<tr>
<td></td>
<td>Reverse CGGTTTGTCTTGAAGTAGAGC</td>
<td>61.3</td>
</tr>
<tr>
<td>CACNA1c</td>
<td>Forward CAGAAACTACAGGAGAAGAGG</td>
<td>59.5</td>
</tr>
<tr>
<td></td>
<td>Reverse AAGAAGAGGATCAGGTTGGT</td>
<td>60.5</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Forward AAGATCAAGATCATTGCTCCTC</td>
<td>61.2</td>
</tr>
<tr>
<td></td>
<td>Reverse GGACTVATCGTACTCCTG</td>
<td>59.5</td>
</tr>
</tbody>
</table>

a TmS were calculated by NIH PerlPrimer.
For immunohistochemical analysis, the tissue constructs were fixed with 4 % paraformaldehyde at 37 °C for 1 h. After rinsing with fresh PBS, the constructs were frozen with OCT and sectioned into 10 µm thick slices. The slices were then blocked with 10% goat serum in 0.1 % Triton X-100 for 1 h, followed by incubation with mouse monoclonal anti-cTnI and anti-CX43 (Abcam) antibodies respectively at 37°C overnight. After rinsing with PBS, the corresponding secondary antibodies were added and incubated for another 1 h. Slices without primary antibody but with secondary antibody treatment were used as a negative control. The slices were finally stained with Hoechst 33342 for nuclei. Images were taken with an Olympus FV1000 filter confocal microscope.

2.2.11. CDCs adhesion on hydrogel/polyurethane blends
CDCs were seeded on hydrogel/polyurethane film to evaluate cell adhesion capacity. Three blends with different hydrogel/polyurethane ratios (90/10, 70/30 and 50/50, wt%) were used. The hydrogel/polyurethane blends were cut into 6 mm discs and fitted into 96-well plates. Each well was seeded with $2 \times 10^5$ cells. After 24 h of culture, the culture medium was removed and samples were washed carefully three times with DPBS. Cells cultured on non-treated tissue culture plate (TCP) were used as a control. The dsDNA content was tested as described above in section 2.2.8.

2.2.12. Statistical methods
Data are expressed as mean ± standard deviation. Statistical comparisons were performed by ANOVA using JMP. Significant difference is defined as $p < 0.05$. 
2.3 Results

2.3.1. Effect of electrospraying voltage on CDC survival and growth

CDCs were electrosprayed at different voltages to investigate the effect of electric field on cell survival and growth. Table 2.3 shows that greater than 97% of the electrosprayed cells survived under 10, 15, and 20 kV. There was no significant difference in the cell survival rate between the control and electrosprayed groups. The electrosprayed cells were able to proliferate during the culture with similar growth kinetics to cells that were not electrosprayed (Figure 2.1).

![Figure 2.1](image-url)  
Figure 2.1. Viability of CDCs 1, 3, 5 days after electrospraying at different voltages (0, 10, 15, and 20 kV).
Table 2.3. Survival rates of CDCs after electrospraying.

<table>
<thead>
<tr>
<th>Voltage (kV)</th>
<th>0</th>
<th>10</th>
<th>15</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival rate</td>
<td>97.2%</td>
<td>97.7%</td>
<td>97.6%</td>
<td>98.9%</td>
</tr>
</tbody>
</table>

2.3.2. Fabrication and characterization of tissue constructs

Tissue constructs with a high density of CDCs were successfully fabricated using a technique with simultaneous fiber electrospinning and cell electrospraying. The electrosprayed CDCs and electrospun fibers were homogenously distributed in the constructs (Figures 2.2 and 2.3). The fibers assumed diameters typically greater than 1 µm with no significant difference between different constructs (Table 2.4). The fiber density and alignment were dependent on the pumping speed of polymer solution (Table 2.4). An increase in pumping speed from 4.5 to 7.5 mL/h significantly increased fiber density (p < 0.05) while decreased fiber alignment (p < 0.05).

Table 2.4. Fiber diameter, density, and alignment of different constructs*.

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>(A)</th>
<th>(B)</th>
<th>(C)</th>
<th>(D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter $d$ (µm)</td>
<td>$1.50 \pm 0.12$</td>
<td>$1.28 \pm 0.09$</td>
<td>$1.47 \pm 0.22$</td>
<td>$1.37 \pm 0.26$</td>
</tr>
<tr>
<td>Density $n$ (#/100µm$^2$)</td>
<td>$46.1 \pm 2.0$</td>
<td>$25.5 \pm 3.2$</td>
<td>$22.7 \pm 2.8$</td>
<td>$22.2 \pm 3.7$</td>
</tr>
<tr>
<td>Alignment $A_0$</td>
<td>$0.45 \pm 0.06$</td>
<td>$0.61 \pm 0.07$</td>
<td>$0.60 \pm 0.02$</td>
<td>$0.59 \pm 0.04$</td>
</tr>
</tbody>
</table>

*Fiber diameter and density were detected by Image J based on the SEM images.

Fiber alignment was calculated by Matlab based on the SEM images using eq. (6).
Figure 2.2. Distribution of cells at different depths throughout the tissue constructs at day 1 (A) and day 7 (B).
Figure 2.3. SEM images of tissue constructs cross sections, surfaces and fiber alignment/orientation distributions calculated based on the images. a: HG9PU1/7.5; b: HG9PU1/4.5; c: HG7PU3/4.5; d: HG5PU5/4.5.
Typical stress-strain curves of the four tissue constructs are presented in Figure 2.4 and the tensile mechanical properties are summarized in Table 2.5. The constructs were highly flexible and relatively strong with tensile strains and strengths greater than 362% and 440 kPa, respectively. The constructs were also relatively soft with small strain tensile moduli $M_0$ ranging from $4.8 \cdot 10^1$ to $4.6 \cdot 10^2$ kPa. The tensile modulus and strength were dependent on the PEUU/hydrogel ratio and polymer solution pumping speed. Table 2.5 demonstrates that an increase in PEUU/hydrogel ratio significantly increased tensile strength and modulus ($p < 0.05$). At the same PEUU/hydrogel ratio, increasing the polymer solution pumping speed from 4.5 to 7.5 mL/h increased the tensile strength ($p < 0.05$) but not modulus ($p > 0.05$).

![Figure 2.4](image-url)

*Figure 2.4. Representative stress-strain curves of the tissue constructs tested at 37°C and in aqueous condition.*
Table 2.5. Mechanical properties of different tissue constructs.

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>(A)</th>
<th>(B)</th>
<th>(C)</th>
<th>(D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tensile Modulus $M_0$ (kPa)</td>
<td>48.1 ± 10.9</td>
<td>60.4 ± 5.0</td>
<td>222.7 ± 32.5</td>
<td>463.1 ± 37.7</td>
</tr>
<tr>
<td>Tensile strength (kPa)</td>
<td>629.4 ± 97.5</td>
<td>440.8 ± 73.3</td>
<td>892.0 ± 67.3</td>
<td>2019.9 ± 283.9</td>
</tr>
<tr>
<td>Elongation</td>
<td>4.54 ± 0.87</td>
<td>3.62 ± 0.89</td>
<td>5.10 ± 0.69</td>
<td>7.17 ± 1.34</td>
</tr>
</tbody>
</table>

The modulus of the single fibers in tissue constructs was measured by AFM in a 37°C water bath. These AFM-based moduli are denoted $m_A$ and ranged from 130 kPa to 1 MPa. They depended on the PEUU/hydrogel ratio but not pumping speed (Figure 2.5). In general, fibers with a higher PEUU/hydrogel ratio assumed a greater modulus ($p < 0.05$).

Figure 2.5. AFM characterization of scaffold morphology (a) and single fiber modulus of scaffolds (b).
2.3.3. *CDC adhesion on hydrogel/PEUU blends and cell survival in tissue constructs*

To investigate if fiber composition had an effect on CDC attachment, the cells were seeded on 2D films of PEUU/hydrogel blends with different ratios (90/10, 70/30 and 50/50), and cell adhesion was characterized after 24 h of culture using dsDNA test. Figure 2.6 demonstrates that there was no significant difference among all three films with different PEUU/hydrogel ratios (*p* < 0.05). This indicates that the PEUU/hydrogel ratio did not affect CDC adhesion. Interestingly, the cell adhesion on PEUU/hydrogel films was much greater than that on the tissue culture plate.

![Graph](image.png)

Figure 2.6. CDC adhesion on 2D films of hydrogel/polyurethane blends with different ratios (90:10, 70:30 and 50:50, wt%).
Figure 2.7. dsDNA content of tissue constructs after 1 and 7 days of culture in spinner flasks.

CDCs survived in the fabricated tissue constructs during the 7-day culture period in spinner flasks. Comparison of the dsDNA content in each tissue construct after 1 and 7 days of culture showed no significant difference between the two time points (p > 0.05, Figure 2.7). The thickness of the scaffolds is around 150um, more than the 100um diffusion distance of oxygen and nutrients in static state [30]. The use of spinner flask increases the mass transfer, which helps the oxygen and nutrients to diffuse further inside the tissue constructs [30].

2.3.4. Cardiac differentiation of CDCs in tissue constructs

The cardiac differentiation of CDCs in tissue constructs was evaluated at the mRNA level by real-time RT-PCR, and at the protein level by immunohistochemistry. At the mRNA level, mature cardiac markers cTnT, MYH6, and CACNA1c were significantly up-regulated in the constructs (A) and (B) (Figure 2.8).
Figure 2.8. Gene expression of CDCs in tissue constructs after 7 days of culture in spinner flasks.

a: CACNA1c; b: cTnT; and c: MYH6.
Figure 2.9. CX43 expression (red) and CTnI expression (green) of CDCs in tissue constructs after 7 days of culture in spinner flasks. Cell nuclei were stained by Hoechst (blue). a: HG9PU1/7.5; b: HG9PU1/4.5; c: HG7PU3/4.5; d: HG5PU5/4.5.

Meanwhile, almost no mature cardiac markers were expressed in the other two constructs. Overall the construct (A) exhibited the highest expressions. At the protein level, CDCs in constructs (A) and (B) were both cTnI and CX43 positive (Figures 2.9). However, the cells in constructs (C) and (D) were both cTnI and CX43 negative. These results demonstrate that construct (A) most significantly stimulated CDC cardiac differentiation.
2.4 Discussion

The purpose of this chapter was to determine optimal physical properties of fibrous tissue constructs that lead to cardiac differentiation of CDCs. This work represents the first step towards elucidating the relationship between stem cell cardiac differentiation and changes in collagen fiber properties after MI. Various studies have demonstrated that matrix properties such as composition, biomechanics and structure affect stem cell differentiation [25,54-58]. However, most of these studies were conducted either in 2D environment or in non-fibrous scaffolds. These studies therefore cannot elucidate stem cell differentiation in the 3D fibrous scaffolds that resemble the morphology and structure of the native ECM. For fibrous scaffolds, physical properties such as modulus, and fiber diameter and alignment potentially affect cell differentiation, as they affect mechano-pathways and focal adhesion of cells [25,54-58]. In this chapter, we found that for fibers with the same cell adhesive property, the macroscopic tensile modulus of the scaffold at small strain ($M_0$), fiber area fraction $A_f$, and fiber alignment $A_0$ determined cell differentiation.

2.4.1. Geometric and mechanical properties of tissue constructs

Key trends among the processing, geometric, and mechanical parameters are observed among the summary results presented in Table 2.6. Tissue constructs were fabricated through simultaneous electrospinning of polymer fibers and electrospraying of cells. Here, pumping speed $P$ and hydrogel content $H$ were varied. The use of both compliant hydrogel (~10 kPa) and relatively stiff PEUU (~2.2 MPa) enabled constructs with heart tissue-like mechanical properties. In particular, the small strain tensile moduli of the
constructs ranged from $M_0 = 48$ to 461 kPa. This is within the modulus range of the native heart tissue during a beating cycle (10-500 kPa) [59].

The trends in fiber volume fraction $V_f$ and fiber alignment $A_0$ at small strain show peculiar differences for construct (A) compared to the others. Here, $V_f = n \pi d^2/4$ is used, where $n$ and $d$ are reported in Table 2.4. The large $V_f$ for construct (A) stems from a considerably larger $n$ and modestly larger $d$ (Table 2.4). Apparently, the larger pump speed generates greater packing of fibers. The fiber alignment function $A_0$ is also smaller, indicating a broader fiber distribution about the perfectly aligned ($\theta = 0$) case. This is consistent with a larger pump speed, which increases the speed of the impinging polymer stream relative to the fixed mandrel speed. The SEM images in Figure 2.3 confirm the larger $V_f$ and smaller $A_0$ for construct (A), compared to construct (B). Comparison of cases (B-D) shows that alignment and volume fraction are controlled primarily by pump speed rather than hydrogel fraction. This is surprising since the results (to follow) show a large variation in fiber modulus, which could in principle affect $V_f$ and $A_0$.

These geometric and processing features translate into a range of macroscopic moduli at both small strain ($M_0$) and large strain ($M_L$). Constructs (A) and (B) have comparable values of $M_0$ that are ~25% or less than for cases (C) and (D). The comparable values demonstrate that $M_0$ can be held relatively constant by increasing pump speed at constant hydrogel fraction, thereby increasing $V_f$ while decreasing $A_0$. $M_L$ is larger in case (A) vs. (B), which is consistent with a larger $V_f$. But overall, the large increase in $V_f$ produces
rather modest gains in $M_L$. Comparison of cases (C-D) shows that increases both the small and large strain moduli are achieved by decreasing the hydrogel fraction.

The data also address how the local fiber moduli at small and large strain depend on pump speed and hydrogel fraction. The fiber moduli $m_A$ (Figure 2.5) from AFM can be complemented by estimates of $m_0$ and $m_L$ using eqs. (6-7). In particular, the small strain fiber moduli $m_0$ are obtained by inserting experimental values of $V_f, A_0$ and $M_0$ into eq. (6), and the large strain counterpart $m_L$ is obtained by inserting $V_f$ and $M_L$ into eq. (7). All three fiber moduli ($m_A, m_0, m_L$) increase as the PEUU fraction increases ($H$ decreases). Changing the pump speed produces no significant difference ($p>0.05$) in fiber modulus. The results indicate that $m_0$ is $\sim$80% of $m_L$, consistent with the effects of increased polymer chain alignment and overall fiber straightening at larger strain.

A relevant experimental issue is the most efficacious approach to estimate fiber modulus. AFM is desirable both because it is not inferred indirectly through formulae (eqs. 6-7) that require measurements or assumptions about fiber alignment. However, drawbacks to AFM include the inhomogeneous stress state generated under the tip, support of the fiber, and elastic anisotropy in the radial vs. axial directions of the fiber. Drawbacks to eqs. (6-7) include the effect of cells on the measurement of $M_0$ and $M_L$. These effects were neglected in eqs. (6-7) so that the resulting values of $m_0$ and $m_L$ are expected to be upper bounds. Despite the drawbacks to each method, it is encouraging that they yielded consistent trends with pump speed and hydrogel fraction.
Table 2.6. A summary of process and response parameters of the tissue constructs*.

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>Process parameters</th>
<th>Geometric and mechanical parameters</th>
<th>Functional response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$P^{(1)}$</td>
<td>$H^{(2)}$</td>
<td>$m_L^{(3)}$</td>
</tr>
<tr>
<td>(A)</td>
<td>7.5</td>
<td>90</td>
<td>2.0±0.4</td>
</tr>
<tr>
<td>(B)</td>
<td>4.5</td>
<td>90</td>
<td>3.5±0.2</td>
</tr>
<tr>
<td>(C)</td>
<td>4.5</td>
<td>70</td>
<td>9.0±0.8</td>
</tr>
<tr>
<td>(D)</td>
<td>4.5</td>
<td>50</td>
<td>26±0.3</td>
</tr>
</tbody>
</table>

$^{(1)}$P: Polymer solution pumping speed (ml/h); $^{(2)}$H: hydrogel content in fibers (wt %);

$^{(3)}m_L$: fiber modulus at large strain (eq. 7) (10^2 kPa);

$^{(4)}A_0$: fiber alignment at small strain at $v=0.3$ (eq. 6) (10^{-1})

$^{(5)}V_f$: fiber volume fraction, experimental (10^{-1}); $^{(6)}M_0$: scaffold modulus at small strain (10^2 kPa);

$^{(7)}M_L$: scaffold modulus at large strain (10^2 kPa);

$^{(8)}$GP: gene and protein expression, gene and protein expressions were normalized first based on the results of real time RT-PCR and immunohistotology respectively, and then multiplied to give the GP value listed here.

2.4.2. Cardiac differentiation

The differentiation of CDCs towards cardiac lineage was evidenced by real-time RT-PCR (Figure 2.8) and immunohistochemistry (Figures 2.9). At the mRNA level, cardiac differentiation was dependent on the tissue construct modulus, fiber diameter, and fiber density. Overall, CDCs in the construct with the lowest modulus ($M_0 = 48$ kPa) and the highest fiber volume fraction ($V_f = 8.5\times10^{-3}$) demonstrated the highest expressions of
mature cardiac markers cTnT, MYH6 and CACNA1c. Statistical analysis showed that $M_0$ had a significant effect on the expressions of MYH6 and CACNA1c. Interestingly, $V_f$ played an important role in the cTnI expression where a larger $V_f$ led to a higher extent of expression. At the protein level, CDCs in both the $M_0 = 48$ and 60 kPa constructs were positive to cTnI, but not in the 220 and 460 kPa constructs. For CX43 expression, although all constructs had the expression, cells in the 48 and 60 kPa constructs had remarkably higher expression than those in the 220 and 460 kPa cases.

Both the real-time RT-PCR and immunohistochemical results of scaffolds (B), (C) and (D), which have very similar fiber diameter $d$, density $n$ and alignment $A_0$ (p > 0.05 ) but different scaffold modulus, demonstrate that the optimal modulus for CDC cardiac differentiation in fibrous scaffolds is around 50 kPa. This value is slightly higher than that of CDC cardiac differentiation in 3D hydrogels. As reported in our previous study, the optimal modulus for CDC cardiac differentiation in PNIPAAm-based hydrogels is 31-40 kPa [31,60]. The discrepancy may result from different microenvironment in the hydrogel and fibrous scaffold.

**Figure 2.10** shows the results of a statistical analysis of the gene and protein expressions, leading to a fit of the functional performance

$$GP = (B \times V_f) \times \exp[K(M_0 - C)^2 + D]$$

(2.8)

The fitted function captures the experimental points well ($R^2=0.98$, p<0.05, p<0.0001, p<0.0001 for $V_f$, $M_0$ and $M_0^2$ respectively). Over the scaffold modulus range $M_0 = 50$-500
kPa, the lower values produced higher cardiac differentiation of the seeded CDCs. It is unclear whether even smaller $M_0$ might yield further increases in $GP$.

![Graph showing simulated relationship of gene/protein expression (GP), fiber volume fraction ($V_f$), and small strain scaffold modulus ($M_0$).]

Figure 2.10. Simulated relationship of gene/protein expression (GP), fiber volume fraction ($V_f$), and small strain scaffold modulus ($M_0$).

The results for Cases (A) and (B) in Table 2.6 show that $GP$ depends on more than the fiber and construct moduli. In particular, cases (A) and (B) have similar $M_0$ ($p>0.05$) and $m$ ($p>0.05$ for $m_0$, $m_L$ and $m_A$ from AFM tests). Yet $GP$ for (A) is ~7 times that for (B). This may due to the larger $V_f$ and smaller fiber alignment $A_0$. This is confirmed in Figure 2.3, based on the larger fiber density in the cross sectional views and reduced fiber alignment in the side views, for (A) compared to (B). A positive coefficient $B$ in eq. (8)
also indicates that a higher fiber volume fraction increases $GP$. This was achieved primarily by increasing the fiber density. In principle, $V_f$ can also be increased with a larger fiber diameter but the effect on $GP$ is not known from the present work. The high fiber number density and tortuosity in case (A) may increase the number of cell adhesion sites on the fibers—something that may not be achieved by increasing fiber diameter alone. The different scaffold morphologies could influence the available surface for protein adsorption, affecting cellular matrix deposition and the number of initial cell focal adhesion sites [57]. A change in cell focal adhesion sites could mediate signaling pathways that influence cellular processes including cell migration, growth and differentiation [58-60].

### 2.5 Conclusions

In this chapter, ECM-like fibrous tissue constructs with different global moduli, single fiber moduli, fiber density and alignment were fabricated using a simultaneous fiber electrospinning and cell electrospraying technique to investigate the effect of construct mechanical and morphological properties on CDC cardiac differentiation. The fabricated fibers had similar cell adhesion properties. The CDCs remained alive within the constructs during a one-week culture period. Constructs with a small strain global modulus of 48 kPa, a larger fiber volume fraction of $\sim 9 \cdot 10^{-3}$, and decreased alignment ($A_0 = 0.45$, eq. 6) were found to stimulate cardiac differentiation of seeded CDCs most effectively as evidenced by gene expressions of cardiac troponin T (cTnT), calcium channel (CACNA1c), and cardiac myosin heavy chain (MYH6) as well as protein
expressions of cardiac troponin I (cTnI) and connexin 43 (CX43). This study suggests that ECM properties in heart tissue will have an impact on cardiac differentiation of delivered stem cells, and the timing of stem cell delivery may affect the extent of cardiac differentiation.
2.6 References


[8] Malliaras K, Marbán E. Cardiac cell therapy: where we've been, where we are, and where we should be headed. Br Med Bull. 2011; 98:161-85.


Chapter 3: Regulating Myogenic Differentiation of Mesenchymal Stem Cells (MSCs) Using Thermo-sensitive Hydrogels

3.1 Introductions

Atherosclerotic peripheral artery disease (PAD) affects more than 27 million people in North America and Europe [1, 2]. PAD decreases blood perfusion in the tissues and causes tissue ischemia. Critical limb ischemia (CLI) represents the most severe form of PAD. It is characterized by low blood perfusion, severe tissue ischemia, and degenerated skeletal muscle. Promotion of muscle repair represents one of the optimal goals for CLI treatment [1, 3-8]. However, current surgical procedures and pharmacological treatments show limited efficacy [1, 8-10]. One of the most promising strategies to achieve the treatment goal is transplantation of stem cells. Muscle regeneration can be achieved after the transplanted stem cells differentiate into myogenic lineages.

Multiple cell types have been tested in animal models and clinical trials for CLI treatment. These include bone marrow and peripheral blood mononuclear cells [11-17], mesenchymal stem cells (MSCs) [18-22], cell lines selected based on the presence of specific markers [15, 23-25], adipose tissue-derived cells [26-29], and induced pluripotent stem cells-derived cells [30, 31]. Each of these chosen cell types has shown promise for the treatment of CLI in mechanistic and preclinical studies. Among these cell
types, MSCs have attracted significant attentions due to their robust proliferation, and capability of differentiating into myogenic lineage for muscle regeneration, and into endothelial cells (ECs) for vascularization (thus fast angiogenesis) [1, 32, 33]. Therefore, MSCs have the potential to induce ischemic limb regeneration.

Various studies have demonstrated that stem cells experience low rate of myogenic differentiation in ischemic limb [34]. One of the approaches to increase myogenic differentiation is to deliver stem cells using suitable matrix, where matrix property itself and/or loaded biomolecules may not only protect the seeded stem cells but also help induce myogenic differentiation [34, 35]. As mentioned in Chapter 1, hydrogels are one type of matrices widely used to deliver cells for muscle regeneration [36]. These include collagen [37], alginate [38], fibrin [35, 39], hyaluronic acid [40], and poly(ethylene glycol) [41]. Hydrogel stiffness may be used to induce stem cell myogenic differentiation. Engler et al. found that MSCs cultured on polyacrylamide hydrogel with stiffness of 8-17 kPa differentiated into myogenic lineage [42]. This is compelling and convenient as it is not necessary to load specific biomolecules into hydrogels to induce cell differentiation. However, stem cells delivered using hydrogels are in 3D environment instead of 2D. A different modulus may be needed for myogenic differentiation. To determine the effect of hydrogel modulus on myogenic differentiation in 3D environment, the modulus is usually tuned by varying gels crosslinking density, concentration, and/or crosslinker type [43, 44]. This approach changes not only modulus, but also other properties, such as water content, chemical structure, and composition, that may potentially affect stem cell differentiation as well [45]. Thus, it is difficult to
understand decoupled effect of hydrogel modulus on stem cell myogenic differentiation.

In the earlier works of our lab, thermo-sensitive and biodegradable hydrogels based on N-isopropylacrylamide (NIPAAm), acrylic acid (AAc), and degradable 2-hydroxyethyl methacrylate-oligoester were synthesized, and hydrogel matrix elasticity was successfully decoupled from other properties such as the gel chemical structure, composition and water content [46]. In this chapter, these hydrogels were used to study how the hydrogel matrix elasticity alone affects the fate, especially growth and differentiation, of encapsulated stem cells.

### 3.2 Experimental materials and methods

#### 3.2.1. Materials

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. 2-hydroxyethyl methylmethacrylate and acrylic acid were passed through a column packed with inhibitor remover to remove inhibitor. N-isopropylacrylamide was purified by recrystallization in hexane for 3 times. 3,6-Dimethyl-1,4-dioxane-2,5-dione and β-butyrolactone (Alfa Aesar) were used as received. Trimethylene carbonate was purchased from Boehringer Ingelheim and vacuum dried before use.

#### 3.2.2. Synthesis of hydrogel polymers

Macromer HEMA-oligoHB was synthesized by ring-opening polymerization of 2-hydroxybutyrate using stannous (II) trifluoromethanesulfonate [Sn(OTf)₂] as catalyst [46, 47]. Different amounts of HB were used in order to create HEMA-oligoHB with different
HB lengths. In brief, HEMA and β-butyrolactone were charged into a 100 mL single-necked flask. The flask was immersed in a 110°C oil bath to melt the β-butyrolactone. Sn(OTf)$_2$ in THF/toluene (1/4) was then introduced into the flask. The reaction was conducted at 110°C for 1 h under the protection of nitrogen. After reaction, the flask was cooled to room temperature. The reactant was precipitated in ice water and the oil-like precipitant was collected. The product was dissolved in ethyl ester and dried with magnesium sulfate. Excess ethyl ester was evaporated under vacuum.

The hydrogel polymers were synthesized by free radical polymerization using benzoyl peroxide (BPO) as initiator [46, 48-50]. In brief, stoichiometric amount of NIPAAm, acrylic acid and HEMA-oligoHB macromers were charged into a 250 mL 3-necked flask. BPO was then added. The polymerization was conducted under 60°C overnight under the protection of nitrogen. The polymer was precipitated in hexane first, then purified twice by dissolving in THF and precipitating by ethyl ether. The polymers were finally dried under vacuum overnight. The synthesized polymers are abbreviated as PNAH-oligoHB(n), where n represents the number of repeating unit HB. Chemical composition of macromers and copolymers were valified by 400MHz $^1$H-NMR (Bruker).

3.2.3. Hydrogel preparation

To prepare a hydrogel solution, the above synthesized copolymer was dissolved in Dulbecco's modified phosphate buffer saline (DPBS) at 4°C to form a 20% (w/v) solution. The solution was then incubated at 37°C for around 30 min to form a solid hydrogel.
All the hydrogels were thermo-sensitive, exhibiting sol-gel transition temperatures between 14.6 and 20.2 °C based on DSC testing result. The gel solutions were injectable through a 26 gauge needle at low temperature below SGTT with fast gelation time within 7s at body temperature. In addition, they were highly flexible (breaking strain above 300%) and soft. The elastic matrix modulus was successfully controlled by the HB oligomer length (11.1 kPa, 20.0 kPa and 40.1 kPa for PNAH-oligoHB(6), PNAH-oligoHB(4) and PNAH-oligoHB(2), respectively) based on a uniaxial tensile test (conducted in an aqueous environment at 37°C with a strain rate of 200% /min). Importantly, water content was kept the same (around 40%) and did not change during the 2-week test period [46].

3.2.4. Mesenchymal stem cells (MSCs) encapsulation in hydrogels

Rat bone marrow-derived mesenchymal stem cells (MSCs) (a kindly gift from Dr. Mahmood Khan) were cultured in α–minimal essential medium (αMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics. The medium was changed every other day. Cells between passages 11-14 were used in this chapter. We have demonstrated in previous work that MSCs at these passages maintained their phenotype [51]. To encapsulate MSCs into hydrogels, MSCs were trypsinized from the tissue culture plates (TCP) and re-suspended in culture medium. The cell suspension was then added into hydrogel solutions and mixed thoroughly. The final cell density was 10 million/mL. 0.2 mL of cell/hydrogel mixture was then dispensed to each Eppendorf tube. Following gelation at 37°C for 20-30 minutes, suspension was removed and fresh cell culture medium was added.
3.2.5. Characterization of MSC growth in hydrogels

After 1, 7, and 14 days of culture under normal tissue culture conditions (21% O₂ and 5% CO₂, 37 °C), the survival/proliferation of MSCs in gels was assessed by double-strand DNA (dsDNA, for live cells) test and live cell tracker (CM-Dil, Invitrogen) imaging. The dsDNA content of the samples was measured by PicoGreen assay kit (Invitrogen) following the protocol described in section 2.2.8 [52]. In brief, cell-gel samples were washed with DPBS and treated with papain solution at 60 °C overnight. The dsDNA concentration in the digested solution was then measured using PicoGreen assay by a fluorescence reader, and results were demonstrated after normalized by the dry weight of the samples.

3.2.6. Characterization of MSC myogenic differentiation in hydrogels

After 7 and 14 days of culture, MSC myogenic differentiation was characterized at the gene level by real-time RT-PCR, and at protein level by immunohistology. At each time point, cell/hydrogel samples were immersed in TRIzol (Sigma). RNA was isolated following manufacture's protocol and total RNA was quantified by Nanodrop (Thermo). Approximately ~1 μg of RNA was used for cDNA synthesis. Real-time RT-PCR was performed using SYBR Green/fluorescein (Fermentas) and primers listed in Table 3.1. A standard ΔΔCt method was employed to calculate fold difference, and β-actin was used as the housekeeping gene.
Table 3.1. Primers used for real time RT-PCR to study the myogenic differentiation of MSCs in hydrogels.

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
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<td>GGACTCAGTACTGACTCCTCCTG</td>
</tr>
<tr>
<td>MEF2</td>
<td>TGCTGCTCTCAGTCGTACAC</td>
<td>TTCACGACTTGGGACACTG</td>
</tr>
<tr>
<td>Myogenin</td>
<td>TGAGAGAGAAGGGAGGGAAC</td>
<td>ACAATACAAGAGCAGTTGGGAA</td>
</tr>
<tr>
<td>MHC</td>
<td>TGACTTCTGGCAAAATGCAG</td>
<td>CCAAAGCGAGAGGAGTTGGTC</td>
</tr>
<tr>
<td>MyoD1</td>
<td>AGAGGGAAGGGAGGAGCAGAAG</td>
<td>GCAGCAGCAAACAACAACCAG</td>
</tr>
</tbody>
</table>

For immunohistological study, cell/hydrogel samples were fixed in 4% paraformaldehyde for 1 h, embedded into OCT and cyrosectioned at 10 µm. The sections were first blocked by 10% goat serum with 0.3% Triton X-100 for 1 h. After washing with DPBS, the sections were incubated with primary antibodies of mouse anti myosin heavy chain (MHC, Abcam) and rabbit anti MyoD1 (Proteintech) overnight at 37°C, respectively. Dylight488-anti mouse and 648-anti rabbit secondary antibodies were then conjugated with primary antibodies for another 1 h, respectively. The cell nuclei were stained by Hoechst. Immunofluorescence images were recorded by Olympus FV1000 confocal microscope.

3.2.7. Statistical analysis

Data are reported as mean ± standard deviation. Statistical difference was determined by ANOVA test with Tukey HSD group-wise comparison using JMP. A statistical significance is defined as p < 0.05.
3.3 Results

3.3.1. MSCs survival and proliferation in hydrogels

MSCs were encapsulated in hydrogels with oligoHB as side chain. These hydrogels had the same chemical structure, composition and similar water content but largely different elastic moduli, thus can be used to study the effect of hydrogel modulus on cell proliferation and differentiation. MSC survival and proliferation in vitro was quantified by dsDNA content (for live cells). MSCs survived and proliferated in all three hydrogels during the 2-week culture period (Figure 3.1). The proliferation was fast during the first 7 days, and then slowed down. The most significant cell growth was observed in the hydrogel PNAH-oligoHB(2) with the highest elastic modulus (40.1 kPa) compared with the softer gels PNAH-oligoHB(4) and PNAH-oligoHB(6) (with matrix modulus of 20kPa and 11kPa, respectively). Consistent with dsDNA content results, live cell staining results (Figure 3.2) also demonstrated that cell density increased in all three hydrogels during the culture.
Figure 3.1. dsDNA content of MSCs in hydrogels PNAH-oligoHB(2), PNAH-oligoHB(4), and PNAH-oligoHB(6) after 1, 7 and 14 days of culture (*p<0.05, **p<0.01).

3.3.2. MSCs myogenic differentiation in hydrogels

MSC myogenic differentiation was determined at the gene level by real-time RT-PCR and protein level by immunohistochemistry. Myogenic markers myosin heavy chain (MHC), Myogenin, MyoD1, and MEF2 were used to characterize myogenic differentiation of encapsulated MSCs. Figure 3.3 demonstrated that no obvious myogenic differentiation was occurred in all three hydrogels after 7 days of culture, while significant differentiation was observed after 14 days of culture. MSCs in the hydrogel with elastic modulus of 20 kPa exhibited significantly higher expressions of all the checked markers MHC, Myogenin, MyoD1 and MEF2 than those in hydrogels with elastic moduli of 11 and 40 kPa. These results were consistent with protein expression results (Figure 3.4). At day 14, MSCs in the hydrogel with elastic modulus of 20 kPa
were positive to MHC (Figure 3.4A). In contrast, MSCs in the hydrogels with elastic moduli of 11 and 40 kPa were negative to MHC. Similar trend was observed for MyoD1 expression (Figure 3.4B). Overall, MSCs in the hydrogel with elastic modulus of 20 kPa showed the highest expressions of myogenic differentiation.

Figure 3.2. Live cell images of MSCs in hydrogels PNAH-oligoHB(2), PNAH-oligoHB(4), and PNAH-oligoHB(6) after 1, 7 and 14 days of culture. Cells were stained with CM-Dil.
Figure 3.3. Myogenic gene expressions of MSCs in hydrogels PNAH-oligoHB(2), PNAH-oligoHB(4), and PNAH-oligoHB(6) after 7 and 14 days of culture (*p<0.05, **p<0.01).
Figure 3.4. Immunohistochemical staining of MSCs in hydrogels PNAH-oligoHB(2), PNAH-oligoHB(4), and PNAH-oligoHB(6) after 14 days of culture. (A) MHC; and (B) MyoD1.
3.4 Discussion

The focus of this chapter is to use hydrogels with different elastic modulus but the same chemical structure, composition and water content for the study of the decoupled effect of matrix elastic modulus on MSC myogenic differentiation. Matrix modulus has been demonstrated to direct stem cell differentiation [42, 53, 54]. Yet it is difficult to decouple the effect of matrix modulus from other parameters that can potentially affect stem cell differentiation, such as matrix composition and water content [45]. Majority of current approaches used to modulate matrix modulus simultaneously change these properties. For example, when tailoring elastic modulus of poly(ethylene glycol) (PEG) hydrogels, varying monomer concentrations and crosslinking densities is often used. This approach not only changes hydrogel modulus, but also alters hydrogel water content [45].

In earlier works of our lab, Z. Li et al. used the same molar ratio of NIPAAm, AAc and macromer to synthesize thermo-sensitive hydrogels [46, 48, 50]. Thus, the hydrogels in each family (with the same oligomer type) have the same chemical structure and composition. The hydrogel elastic modulus was finely tuned by the length of the oligomer. An increase in number of repeating units in each oligomer type from 2 to 6 significantly decreased hydrogel elastic modulus. Meanwhile, hydrogel water content did not significantly change. Furthermore, the hydrogels in each family had the same degradation product, and hydrogel water content did not significantly change during degradation. Therefore, the developed hydrogels are suitable for soft tissue engineering such as skeletal muscle regeneration as well as investigation of the effect of elastic modulus alone on encapsulated cell fates.
3.4.1. Hydrogel property on MSCs proliferation and differentiation

According to the results demonstrated above, MSCs were able to survive and grow in all three chosen hydrogels (PNAH-oligoHB(2), PNAH-oligoHB(4) and PNAH-oligoHB(6)). MSCs seeded in PNAH-oligoHB(6) hydrogel with 40 kPa showed significant faster growth compared with those in softer hydrogels (PNAH-oligoHB(2) and PNAH-oligoHB(4)). Similar results were also demonstrated by other works [55, 56]. Rowlands et al, for example, found that MSCs followed a general trend of increasing proliferation capability with the increase of substrate rigidity, which may attribute from the activation of certain pathways such as ERKs through the cell-matrix focal adhesion complexes [56]. MSCs differentiated into myogenic lineage in the hydrogels determined by both gene and protein expressions (Figures 3.3 and 3.4). The hydrogel with elastic modulus of 20 kPa showed the highest myogenic expression. This modulus matches that of the skeletal muscle tissues (~17 kPa [42]). These results highlighted the fact that designing and generating a proper mechanical environment may be critical for providing insights into fundamental cellular processes such as cell proliferation, differentiation, or even wound healing and disease modeling in vivo.

Matrix modulus has been widely recognized as a biomechanical cue to guide stem cells differentiation. Engler found that MSCs differentiated into neural, myogenic and osteogenic phenotypes when cultured on polyacrylamide surface with compressive elastic moduli of 0.1-1 kPa, 8-17 kPa, and 25-40 kPa, respectively [42]. These results demonstrated that matrix with native tissue-mimicking modulus can better differentiate stem cells into corresponding cell types [57]. Compared to 2D surfaces, 3D matrices can
better mimic the in vivo cellular environment. In natural skeletal muscle tissues, for examples, each skeletal muscle fiber is a single cylindrical muscle cells, and an individual skeletal muscle could be made up of hundreds or thousands of muscle fibers bundled together. Thus a cell within the skeletal muscles is surrounded by 3D matrix with ECM and other cells in it. Pek et al. encapsulated MSCs in PEG/silica hydrogels with different moduli and found that cell differentiation was modulus-dependent [55]. MSCs underwent neural, myogenic and osteogenic differentiation when the storage moduli were 7, 25, and 75kPa, respectively. Huebsch et al. demonstrated that the MSC osteogenic differentiation can be occurred in agarose, poly (ethylene dimethacrylate) and alginate hydrogels with modulus between 11-30 kPa [58]. In these studies, the effect of matrix modulus on cell differentiation was not decoupled from other parameters that may also affect cell differentiation, like chemical structure, composition and water content [45]. In this chapter, we successfully decoupled matrix modulus with these parameters. While our results demonstrate that similar modulus as shown in the above mentioned studies can differentiate MSCs into myogenic lineage, we decoupled the effect of matrix modulus in this work.

3.5 Conclusions

In this chapter, thermo-sensitive, injectable and biodegradable hydrogels with tunable elastic modulus but similar chemical structure, composition and water content were applied to study the matrix elasticity effect on stem cell fates. MSCs proliferated best in the hydrogel with matrix tensile modulus of 40 kPa. MSCs differentiated into myogenic
lineage in the hydrogels. The most significant differentiation occurred in the hydrogel with elastic modulus of 20 kPa. These hydrogels can be a good candidate to serve as a stem cell carrier for skeletal muscle regeneration.
3.6 References


[10] Conte MS. Bypass versus Angioplasty in Severe Ischaemia of the Leg (BASIL) and


[49] Guan J, Hong Y, Ma Z, Wagner WR. Protein-reactive, thermoresponsive copolymers


4.1 Introductions

Mature skeletal muscle cells are one of the cell types that generally lack the capacity of regeneration. Thus when severely injured or damaged by diseases such as tissue ischemia, trauma or genetic defects [1, 2], their supplement from local stem cells, which could differentiate into skeletal muscle cells and fuse to form new myofibers, provides an important clinical application [1]. Given that 6 million Americans are diagnosed with musculoskeletal diseases every year, the potential of improved skeletal muscle repair strategies is significant [3-5].

Currently, the main approaches to treat skeletal muscle diseases especially critical limb ischemia (CLI) consist of drug delivery [3], cell therapy [6-11] and the combination of both [12-14]. The former involves delivery of growth factors that either stimulate vascularization or promote muscle regeneration. For example, VEGF, PDGFBB and bFGF have been used for vascularization [15-17], while IGF-1 has been employed for muscle regeneration [18,19]. Different studies have demonstrated that delivery of two or more types of growth factors with different functions can result in a greater therapeutic efficacy than delivery of single growth factors [3]. Efficacy of the drug delivery approach
is dependent on the amount of bioactive drugs released to the ischemic muscle tissues, duration of the drug release, and sequence of the release. Overall, current drug delivery approach shows limited success due to the rapid release of delivered drugs, loss of bioactivity, inadequate amount of drug released to the tissue, and inappropriate drug gradients and release sequence [3]. Cell therapy is an alternate approach. It includes direct injection of stem cells into the tissue or encapsulating cells in constructs followed by injection [20-22]. The transplanted stem cells serve two purposes: differentiation to replace damaged host cells, and providing paracrine effects for vascularization and muscle repair and regeneration.

Various stem cell types including ESCs [23], MSCs [8, 21-22, 24-25], adipose tissue-derived cells [26-29], cell lines selected based on the presence of specific markers [30-31], and iPSCs [10] have been used for skeletal muscle regeneration. Among them, MSCs stand out because of their ability to differentiate into myogenic lineages for muscle regeneration, and into endothelial cells for vascularization [32, 33]. MSCs also provide paracrine effects for ischemic limb regeneration. Among the number of different growth factors they secrete, IGF-1 promotes myoblast proliferation and satellite cell differentiation [3, 34-35], and bFGF, VEGF and PDGFBB stimulate angiogenesis. Therefore, MSCs have the potential to induce not only vascularization, but also muscle repair in the ischemic limb through direct and indirect effects.

Various animal studies have demonstrated that stem cell therapy could, to some extent, improve blood perfusion in the ischemic limb and even promote muscle repair in some cases [20, 36-37]. However, clinical trials have shown only a transient therapeutic benefit
Overall, current stem cell therapy has a low efficacy in improving blood perfusion and muscle repair [20, 38]. One of the key causes is the poor survival rate of transplanted cells [20, 37-39]. Several studies have demonstrated that only ~20% of cells remained in the tissue 24 h after transplantation, and only ~3% remained after 30 days [39-42]. Among the possible triggers for cell death, such as ischemia, immune response, inflammation and oxidative stress, ischemia represents the most significant problem to be solved [20, 37-39].

Promoting high rates of both short- and long-term cell survival under ischemic conditions is one of the key steps to significantly increase therapeutic efficacy of stem cell therapy for diseases like CLI [43]. To improve cell survival under ischemia, approaches have been used including blocking the apoptotic signaling pathways of cells [44, 45], preconditioning cells before transplantation [46, 47], co-transplanting with cells rich in paracrine effects [48, 49], using prosurvival cocktails [50], promoting angiogenesis [48], and co-transplanting with hydrogels [51-54]. While these approaches increased cell survival to an extent, achieving high rates of both short- and long-term cell survival remains challenging and insignificantly addressed. Those approaches that induce endogenous cellular survival mechanisms, co-transplant stem cells with other cells, and use prosurvival cocktails, can only temporarily improve cell survival, as adequate vascularization cannot be readily achieved to relieve ischemia for long-term cell survival. The approaches focusing on promoting angiogenesis show significant cell death before angiogenesis can occur. While using hydrogels, such as collagen and hyaluronic acid, can
decrease cell apoptosis, vascularization around matrices for long-term cell survival is challenging [51-54].

In this chapter, we hypothesized that a stem cell delivery system that can continuously release a prosurvival and proangiogenic growth factor will promote both short- and long-term cell survival in the ischemic limbs. The prosurvival effect could promote cell survival before vascularization is established, while the proangiogenic effect could stimulate quick angiogenesis to promote long-term cell survival. Meanwhile, the differentiation of MSCs into endothelial and myogenic lineages, and MSC paracrine effects will enhance vascularization and muscle regeneration. bFGF was used due to its prosurvival and proangiogenic effects [16-17, 56-60] based on some previous works. J.P. Lefaucheur et al., for example, injected bFGF into the tibialis anterior (TA) muscles of mdx mice, which is a mutant from the wild C57BL/10ScSn stain and presents an X-linked dystrophin deficiency homologous to that of patients suffering from Duchenne muscular dystrophy (DMD), to determine the in vivo potential efficacy of bFGF in promoting the regeneration of dystrophin-deficient muscles [56]. They observed a significant increase of regenerated myofiber numbers and a dose-related promoting effect 7 days after bFGF delivery. In another study, K. Brimah et al. studied human muscle formation in vivo from implanted human muscle precursor cells (MPCs) by transplanting donor MPCs into immune-deficient host mice [60]. They found the amount of donor muscle formation was significantly increased when treating the fetal MPCs with IGF-1, bFGF and plasmin, indicating the potential of growth factors such as bFGF on activating muscle cells and improving muscle regeneration. We also investigated how the controlled
release of bFGF affected MSC survival and paracrine effects in vitro under low nutrient and oxygen conditions, and how the delivery system enhanced MSC survival and differentiation, muscle regeneration and blood perfusion recovery in ischemic limbs.

4.2 Experimental materials and methods

4.2.1. Materials
All chemicals were purchased from Sigma-Aldrich unless otherwise stated. 2-hydroxyethyl methylmethacrylate (HEMA) was purchased from TCI and passed through an inhibitor remover column to eliminate inhibitor. N-isopropylacrylamide (NIPAAm, Alfa Aesar) was purified by recrystallization for 3 times using hexane. 3,6-Dimethyl-1,4-dioxane-2,5-dione, acryloyl chloride, sodium methoxide and chondroitin sulfate were used as received.

4.2.2. Synthesis of hydrogel polymer
The hydrogel macromer AA-oligoLA was synthesized by a two-step method [61]. In the first step, OligoLA was generated by ring-opening polymerization of lactide using NaOCH$_3$ as an initiator. In the second step, oligoLA was esterified using acryloyl chloride.

Poly (NIPAAm-co-HEMA-co-AA-oligoLA) was synthesized via free radical polymerization similar to the methods described in our earlier works [62]. In general, stoichiometric amounts of NIPAAm, HEMA and AA-oligoLA (molar ratio 86/10/4) were dissolved in 100 mL of dioxane in a 250 mL three-necked flask. The initiator benzoyl
peroxide was then added. The polymerization was conducted at 60°C overnight. The mixture was precipitated in hexane. The polymer was then purified twice using THF/ethyl ether.

The aqueous hydrogel solution had a sol-gel transition temperature (SGTT) of 26.5 °C as determined by DSC. This allows the hydrogel to solidify at body temperature. The solid hydrogel obtained from the 10 wt% solution had a breaking strain >300% and Young’s modulus of 17.1±3.4 kPa at 37°C. Its final degradation product poly (NIPAM-co-HEMA-co-Acrylic acid) had a SGTT of 41.2°C. It can thus dissolve in the body fluid and removed by circulation system.

4.2.3. bFGF loading into hydrogels

Hydrogel solution (10% w/v) was prepared by dissolving the synthesized copolymer in DubucCEO’s modified phosphate buffer saline (DPBS, pH=7.4). To load bFGF into the hydrogel, heparin was first added to the hydrogel solution at the concentration of 1 mg/mL. The function of heparin is to bind to bFGF to preserve its bioactivity [63]. bFGF was then added to the mixture to reach a final concentration of 50 μg/mL. After mixing completely, the bFGF loaded hydrogel solution was incubated at 37°C for around 30 min to achieve gelation.

4.2.4. bFGF release kinetics

To measure the release kinetics of bFGF from hydrogel, bFGF loaded hydrogel solution (200 μL) was placed in a 2mL micro-centrifuge tube, and incubated for gelation. The supernatant was then removed and 200 μL of release medium DPBS was added. The
bFGF release study was conducted in the 37°C water bath for 4 weeks. At predetermined time points, the release medium was collected and bFGF concentration was measured using a BFGF ELISA kit (PEPROTECH) following the provided protocol.

4.2.5. Bioactivity of released bFGF

The bioactivity of released bFGF was assessed in terms of its stimulative effect on rat fibroblast proliferation [64]. The cells were seeded into a 96-well plate at a density of 2×10^5 cells/mL. After 24 h, the culture medium (Dulbecco’s Modified Eagle Medium supplemented with 10% FBS) was removed and replaced by the collected bFGF release medium supplemented with 0.5% FBS. Cell viability was measured by MTT assay after 48 h of culture [65]. The release medium collected from the hydrogel without bFGF, and 1 ng/mL bFGF solution were used as controls. Relative cell viability was determined by normalizing MTT absorbance of the release medium from the hydrogel with bFGF to that of the release medium from the hydrogel without bFGF.

4.2.6. Mesenchymal stem cell (MSC) culture

Rat mesenchymal stem cells (MSCs) were cultured in 10% fetal bovine serum (FBS) supplementing alpha modified minimum essential medium (αMEM) media with 1% antibiotics in a humidified incubator with 5% carbon dioxide and 21% oxygen. Medium was changed every other day. Cells were passaged when reached 90% confluences. Cells at passages between 11 and 14 were used in this chapter. Our previous works demonstrated that MSCs within these passages remained multipotency [66].
4.2.7. Encapsulation of bFGF and MSCs into hydrogel

Before encapsulation, cells were labelled with live cell tracker CM-Dil (Life Technologies) following the protocol provided by the manufacture. Hydrogel solutions were prepared as stated above in section 4.2.4. Before encapsulation of bFGF or MSCs, hydrogel solution was sterilized under UV light in a laminar flow hood for 30 min. After loading heparin/bFGF into the hydrogel solution as described above in section 4.2.4, cell suspension was then added into the mixture, and the final cell concentration was controlled as 10 million/mL. 200 µL of the mixture was then transferred into each Eppendorf tube followed by incubation in a 37°C water bath for gelation. After removing supernatant, 200 µL of low nutrient culture medium (αMEM without FBS) was added to each tube and incubated under hypoxic culture condition (1% O₂, 5% CO₂, labeled as bH group). At day 1, 7 and 14, pellets were randomly selected for dsDNA test, live cell tracker observation, real time RT-PCR test and immunohistology analysis. Cell/hydrogel mixture without bFGF incubated under ischemic (0% FBS, 1% O₂, 5% CO₂, labeled as AH group) and normal culture condition (10% FBS, 21% O₂, 5% CO₂, labeled as AN group) was used as controls.

4.2.8. MSCs survival, myogenic differentiation and paracrine effect in hydrogel in vitro

The survival of MSCs in hydrogel with or without bFGF was assessed by double-strand DNA (dsDNA, for live cells) content. In brief, samples were collected at day 1, 7 and 14, washed with DPBS for 3 times and treated with papain solution at 60°C overnight. The dsDNA concentration in the digested solution was then measured by PicoGreen assay (Invitrogen), and normalized to the dry weight of the samples. To understand underlying
signaling pathways that control bFGF-induced cell survival, Western Blotting was performed following standard protocols [67]. In brief, after 7 days of culture, proteins were extracted from the AH and bH groups using lysis buffers. The protein samples (50 μg/lane) were then separated by SDS-PAGE followed by transferring to PVDF membranes through electroblotting. The blots were probed with antibodies against protein kinase B (Akt), signal transducer and activator of transcription 3 (STAT3), extracellular-signal-regulated kinases 1/2 (ERK1/2), Kruppel-like factor 4 (KLF4), and β-actin. The blots were then developed using enhanced chemiluminescence.

As for the myogenic differentiation of the seeded cells in hydrogels, real time RT-PCR and immunohistology staining were conducted to detect MSC gene and protein expressions, respectively. After 14 days in vitro culture, cell/hydrogel samples with or without bFGF were immersed in TRIlzol (Sigma) and processed by the manufacture's protocol to isolate and purify RNA. Isolated total RNA was quantified by Nanodrop (Thermo) and approximate 1 μg of RNA was used. cDNA was reverse transcribed from RNA by using cDNA high capacity transcription kit (ABI). A real-time RT-PCR process was conducted by using SYBR Green/fluorescein (Fermentas). Myogenic markers MEF2, Myogenin and Desmin were checked and β-actin was used as the housekeeping gene. A standard ΔΔCt method was used to process the data. For protein expression analysis, samples were fixed in 4% paraformaldehyde for 1 h, washed with DPBS, embedded into OCT and cyrosectioned at 10μm. The sections were then blocked by 10% goat serum with 0.3% Triton X-100 for 1 h. Primary antibody of mouse anti myosin heavy chain (MHC, Abcam) was then diluted 100 times and applied to the sections. After
incubation overnight at 37°C, Dylight488-anti mouse secondary antibody were used at 1:500 dilution for another 1 h. Hoeschst 33342 was then applied to counterstain the nucleus. Immunofluorescence images were recorded by Olympus FV1000 confocal microscope.

The potential paracrine effect of the encapsulated MSCs was also detected by real time RT-PCR (IGF-1 and Angpt1). All the primers used in this chapter were customized designed and listed in Table 4.1.

Table 4.1. Primers used for real time RT-PCR to detect the myogenic differentiation and paracrine effect of MSCs in hydrogel with or without bFGF.

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<thead>
<tr>
<th>Name</th>
<th>Forward</th>
<th>Reverse</th>
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</thead>
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<td>TGCTGCTCTCACTGTCACTAC</td>
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</tr>
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</tr>
<tr>
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</tbody>
</table>
4.2.9. Animal surgical procedures

Animal surgeries was performed in compliance with National Institutes of Health and institutional guidelines. Male wild type C57BL/6 mice (aged 8 to 10 weeks) were anesthetized with an i.p. injection of ketamine 80 mg/Kg and xylazine 5 mg/Kg before all surgical procedures. Hindlimb ischemia was induced by unilateral femoral artery and vein ligation as indicated by Scheme 4.1. The contralateral hindlimb was used as control. Thirty minutes after ligation, mice were injected with a total volume of 200 μL of gel-only (Gel), gel with MSCs (Gel+MSCs), gel with bFGF (Gel+bFGF) or gel with MSCs and bFGF (Gel+MSCs+bFGF) into the muscles in between the ligation points through four injections (2 injections on each side). Open surgery group (Open surgery) with ligation-only were used as the negative control, and non-surgery group (Normal) without ligation or treatment was used as the positive control. For cell tracking purpose, MSCs were labelled with CM-Dil before injection.

Scheme 4.1. Animal surgical procedures.
4.2.10. MSCs survival, proliferation and differentiation in vivo

To assess cell survival in ischemic limbs, frozen muscle tissues were sectioned into 10 μm thick slices. The CM-Dil labelled cells were imaged by Olympus FV1000 filter confocal microscope. Cell density was quantified from 20 randomly selected tissue slices. To determine cell proliferation, MSC myogenic and endothelial differentiation, immunohistochemical analysis was performed based on paraffin-fixed muscle tissue slices. Immunofluorescence images were recorded by an Olympus FV1000 confocal microscope. Density of the Ki67+ cells was quantified from these images. MSC myogenic and endothelial differentiation was identified as MHC+/CM-Dil+ and vWF+/CM-Dil+ cells, respectively. Blood vessels were identified as tubular structure positively stained for vWF.

4.2.11. Ischemia and perfusion

Blood perfusion in ischemic limbs was monitored after induction of ischemia, 2 and 4 weeks post-surgery using a laser Doppler perfusion imager (Perimed). The ischemic/normal limb blood flow ratio was obtained by dividing the blood perfusion intensity in the ischemic region to the same area in the un-operated contralateral hindlimb [35].

4.2.12. Histologic assessment of skeletal muscle tissues

4 weeks after injection, muscle tissues (n=8 per time point per injection group) were collected and processed for histological analyses. The samples were fixed with 4% paraformaldehyde, chemically dehydrated, embedded in paraffin and sectioned into 4 μm
thick slices in sequence. The samples were then stained with Hematoxylin and Eosin (H&E), and Masson’s Trichrome, respectively. Muscle fiber diameter was quantified from the H&E images by analysis of 10 random fields of regenerating muscle in the muscle defect using Image J software. Interstitial fibrosis was morphometrically assessed in Masson’s Trichrome staining images.

4.2.13. Statistical analysis

Data were reported as mean ± standard deviation. Multivariate repeated-measures ANOVA were performed to test the interactions between different tested groups. A statistical significance was considered when p < 0.05.

4.3 Results

4.3.1. Release kinetics of bFGF from hydrogel

The encapsulated bFGF was sustainably released from the hydrogels during a 28-day test period. An initial burst release occurred in the first 5 days, followed by a slower and sustained release until day 28 (Figure 4.1). Interestingly, linear release kinetics was observed after day 7.
4.3.2. Bioactivity of the released bFGF

The bioactivity of the released bFGF was evaluated in terms of its stimulatory effect on fibroblast growth. Standard 1 ng/mL bFGF solution was used as the control, as this concentration significantly stimulated fibroblast growth. As Figure 4.2 demonstrated, fibroblasts cultured in the release medium collected from the hydrogels loaded with bFGF had viability similar to or greater than those cultured in 1 ng/mL bFGF, and significantly higher than those cultured in release medium from hydrogels without bFGF at all chosen time points. This result demonstrated that all the collected bFGF released at different time points up to 28 days remained bioactive.
4.3.3. MSCs survival and proliferation under ischemic condition in vitro

The MSCs encapsulated hydrogels with or without bFGF were cultured under ischemic culture conditions (0% FBS, 1% O₂, 5% CO₂, 37°C) to investigate how the low nutrient and low oxygen condition affects MSC survival, proliferation and myogenic differentiation. As demonstrated in Figure 4.3, under normal culture conditions (10% FBS, 21% O₂, 5% CO₂, 37°C), MSCs showed remarkable survival and proliferation capability during a 14-day culture period in vitro. Under ischemic culture condition, dramatic cell death was observed for samples without bFGF, with 60.7% and 35.1% survival rate after 7 and 14 days of culture, respectively. However, almost all of the MSCs survived in the hydrogel with bFGF after 7 days. Interestingly, the cells even proliferated after 14 days. These results indicated that, while low nutrient and low oxygen
conditions caused significant death of encapsulated MSCs, the addition of bFGF overcame the harsh environment effect and improved MSC survival and proliferation. Live cell tracker images (Figure 4.4) demonstrated homogenous distribution of seeded cells in hydrogels, and consistent result of cells survival as dsDNA test.

![Graph showing dsDNA concentration](image)

Figure 4.3. dsDNA concentrations of MSCs-gel under ischemic culture condition (AH, 0% SFB, 1% O₂, 5% CO₂) , MSCs-gel-bFGF under ischemic culture condition (bH) and MSCs-gel under normal culture condition (AN) on 1ˢᵗ, 7ᵗʰ, 14ᵗʰ day (*p<0.05, **p<0.01).
Figure 4.4. Live cell tracker images of MSCs-gel under ischemic culture condition (AH, 0% SFB, 1% O₂, 5% CO₂), MSCs-gel-bFGF under ischemic culture condition (bH) and MSCs-gel under normal culture condition (AN) on 1ˢᵗ, 7ᵗʰ, 14ᵗʰ day.

Figure 4.5. Western blotting result of FLK4 expression of encapsulated MSCs after 7 days in vitro culture (AH: MSCs-gel under ischemic culture condition, bH: MSCs-gel-bFGF under ischemic culture condition).
To understand the underlying mechanism that bFGF enhanced MSC survival under the low nutrient and oxygen conditions, Western blotting was conducted for MSCs cultured in the hydrogels with and without bFGF. Among the different prosurvival signaling pathways such as Akt, Erk1/2, KLF4, and STAT3, KLF4 was found to be upregulated (Figure 4.5).

4.3.4. Paracrine effect of encapsulated MSCs

Real time RT-PCR results demonstrated that MSCs encapsulated in hydrogel with bFGF exhibited higher gene expressions of angiogenic growth factor Angpt 1 as well as prosurvival and promyogenic growth factor IGF-1 (Figure 4.6) compared to those seeded in gel without bFGF under ischemic culture conditions.
4.3.5. MSCs myogenic differentiation under ischemic condition in vitro

The myogenic differentiation of seeded MSCs in hydrogel under ischemic culture condition with and without bFGF was evaluated at the mRNA level by real-time RT-PCR, and at the protein level by immunohistochemistry. And MSCs in hydrogels cultured
in normal culture condition were used as the control. Myogenic markers Desmin, Myogenin, MEF2 and beta-actin as endogenous control were used. MSCs cultured on TCPS were used to calculate the fold increase. As results shown in Figure 4.7, under normal culture condition, myogenic markers MEF2, Desmin and Myogenin were up-regulated more than 57, 115 and 130000 times respectively in MSCs encapsulated within hydrogel compared to those cultured on TCP, illustrating their successful myogenic differentiation by hydrogel properties. Similar results were also found in previous works (Chapter 3). In contrast, under ischemic culture condition, the differentiations were dramatically depressed. Whereas together with the delivery of bFGF under ischemic culture condition, the myogenic differentiation of MSCs was significantly improved for all the tested markers. The MEF2 and Myogenin expressions, for instance, were recovered up to 38 and 10513 times, respectively. The myosin heavy chain (MHC) staining result (Figure 4.8) also indicated that ischemic conditions dramatically depressed the seeded MSC’s myogenic differentiation at the protein level as well, whereas bFGF overcame this environmental effect and recovered the differentiation significantly.
Figure 4.7. Gene expressions (a.MEF2, b. Myogenin, c. Desmin) of cells in MSCs-gel under ischemic culture condition (AH), MSCs-gel-bFGF under ischemic culture condition (bH) and MSCs-gel under normal culture condition (AN) on 14\textsuperscript{th} day (*p<0.05, **p<0.01).
4.3.6. Muscle regeneration and fibrosis

Induction of ischemia in the hindlimbs led to remarkable muscle degeneration as muscle fibers became largely separated (Figure 4.9). Injection of hydrogel (Gel-only group), hydrogel with bFGF (Gel+bFGF group), and hydrogel with MSCs (Gel+MSCs group) reduced the degree of muscle fiber separation. Interestingly, injection of hydrogel with both bFGF and MSCs (Gel+bFGF+MSCs group) completely averted muscle fibers from separating. Muscle fiber size changed during the degeneration (Figure 4.9.A). The mean muscle fiber diameters determined from H&E images were used to quantify the change.
(Figure 4.9.B). The non-injection group had remarkably lower diameter than the normal muscle in the non-surgery group (p<0.01). Injection of Gel-only slightly increased the diameter (p>0.5, Gel-only vs. Open surgery). In contrast, injection of Gel+MSCs and Gel+bFGF significantly elevated the muscle fiber diameter (p<0.01), with 35% and 44% of increase, respectively. The most significant increase was found for Gel+MSCs+bFGF group where muscle fiber diameter was similar to that of the normal muscle (p>0.1), and significantly greater than that of the Gel+MSCs or Gel-only groups (p<0.05).

The interstitial fibrosis was associated with the injured muscles (Figure 4.9.C). In the non-injection group, the muscles developed substantial fibrosis. The muscles treated with Gel+MSCs and Gel+bFGF groups showed decreased fibrosis. An even more-pronounced reduction of fibrosis was observed in the Gel+MSCs+bFGF group.

Continued

Figure 4.9. A. H&E images; B. average muscle fiber diameter collected from histology images (*p<0.05, **p<0.01); C. Mason trichrome images of mouse tissues around the injection points 4 weeks after surgery.
Figure 4.9 Continued

B

![Bar chart showing muscle fiber diameter (µm) for different groups.](image)

- Open surgery
- Gel
- Gel+MSCs
- Gel+bFGF
- Gel+bFGF+MSCs
- Normal

C

![Images showing tissue sections for different treatments.](image)

- Open surgery
- Gel-only
- Gel+bFGF
- Gel+MSCs
- Gel+MSCs+bFGF
4.3.7. Cell survival and proliferation in vivo

To determine whether bFGF release can enhance MSC survival in vivo, the hydrogel encapsulated with CM-Dil-labeled MSCs (Gel+MSCs group), and hydrogel encapsulated with bFGF and CM-Dil-labeled MSCs (Gel+MSCs+bFGF group) were injected into hindlimb after induction of ischemia. Live cell images showed that a greater density of CM-Dil+ live cells appeared in the Gel+MSCs+bFGF group (Figure 4.10.A). The live cell density in the Gel+MSCs+bFGF group was ~3.6 times of that in the Gel+MSCs group (p<0.01, Figure 4.10.B). These results demonstrated that MSC transplantation with bFGF release significantly increased cell survival in the ischemic limbs.

To determine how the injection of hydrogel (Gel-only group), hydrogel with bFGF (Gel+bFGF group), hydrogel with MSCs (Gel+MSCs group), and hydrogel with both bFGF and MSCs (Gel+bFGF+MSCs group) affects the cells in the host tissues, immunohistochemical staining of proliferation-associated protein Ki67 was performed (Figure 4.11). Abundant expression of Ki67 was detected in tissues injected with Gel+MSCs, Gel+bFGF, and Gel+MSCs+bFGF after 4 weeks, while a less-pronounced increase was observed in the tissue injected with Gel-only. The injection of Gel+MSCs+bFGF resulted in the most significant Ki67 expression compared to the Gel+MSCs and Gel+bFGF groups (p<0.05 to Gel+MSCs or Gel+bFGF).
Figure 4.10. Cell survival (A. CM-Dil images; B. cell densities in vivo) 4 week post-surgery (*p<0.05).
Figure 4.11. Ki67 expression of cells in vivo 4 weeks post-surgery (A. Ki67 staining images; B. quantitative Ki67+ cell densities, *p<0.05, **p<0.01).
4.3.8. MSCs differentiation in vivo

The differentiation of transplanted MSCs in vivo was detected by immunohistochemistry. As Figure 4.12.A showed, above 90% of the transplanted MSCs left inside injection gels

![Image](image.png)

Figure 4.12. Differentiation of transplanted cells in vivo (A. MSCs in the gel; B. MSCs out of the gel) 4 weeks after surgery.
4 weeks after injury/treatment had successful myogenic differentiation in vivo based on their positive expression of MHC. For the cells migrated out from the injection points, some of them differentiated and formed blood vessels (as indicated by vWF staining in Figure 12.B), others differentiated into skeletal muscle cells, integrated with host muscle tissues and exhibited a distinctive banding pattern (as indicated by MHC staining in Figure 12.B), and it’s possible that they formed functional myofibers together with the host cells.

4.3.9. Muscle vascularization and blood perfusion recovery

A laser Doppler perfusion imaging (LDPI) system was used to quantify blood perfusion of ischemic limbs (Figure 4.13.A). As Figure 4.13.B showed, the regional blood flow was reduced dramatically after ligation to around 11% of normal limbs, as expected. Gel-only and Gel+MSCs treatments led to a slow increase in blood perfusion over the 4-week experimental time period. While Gel+bFGF group increased blood perfusion significantly 4 weeks after surgery up to 84% compared with 45% recovery in non-treatment (Open surgery) group. The Gel+MSCs+bFGF group led to a final complete recovery of blood flow, 102% of normal limbs. In particular, the mice treated with Gel+MSCs+bFGF showed a marked increase and full recovery of blood flow (106% of normal limbs) as early as 2 weeks after the injury, and this recovery remained up to 4th week.

Blood vessel density in the muscle was quantified to further determine tissue vascularization with or without treatment (Figure 4.14). 4 weeks after injection, vessel density in the Gel group was substantially greater than the non-injection group (p>0.5).
Injection of Gel+bFGF groups significantly increased vessel densities (p<0.05), and the Gel+bFGF group demonstrated significantly greater vessel density than the Gel-only group (p<0.05). Consistent with the blood perfusion results, the vessel density in the Gel+MSCs+bFGF group was similar to that in the normal muscles after 4 weeks (p>0.1).

Figure 4.13. Blood flow (A. Laser Doppler images; B. quantitative values of blood flow) 0 day, 2 weeks and 4 weeks after surgery (*p<0.05, **p<0.01).
Figure 4.13 Continued

Figure 4.14. Blood vessel densities 4 weeks after surgery (based on vWF staining images, *p<0.05, **p<0.01).
4.4. Discussion

The objective of this chapter was to develop a stem cell delivery system that augments cell survival under ischemic conditions, thus enhancing ischemic limb regeneration. Ischemic limbs are characterized by a low nutrient and oxygen, and poorly vascularized environment. Inferior cell survival in this environment is one of the key causes that are responsible for the low therapeutic efficacy of stem cell therapy. Augmentation of cell survival under these harsh conditions represents a critical need to improve the efficacy. In this chapter, we hypothesized that a stem cell delivery system with the following properties will significantly augment cell survival: 1) a prosurvival environment to promote cell survival before vascularization is established, and 2) a proangiogenic environment to quickly vascularize the ischemic limb to improve long-term cell survival.

The created delivery system was based on MSCs, a biodegradable and thermosensitive hydrogel, and bFGF. MSCs have been shown to differentiate into skeletal muscle and endothelial cells necessary for ischemic limb regeneration [36, 37, 68]. The degradable and thermosensitive hydrogel served as cell and biomolecule carrier. The benefit of using this type of hydrogel is that gelation is controlled simply by temperature without using crosslinkers that may initiate toxicity. In addition, the fast gelation rate enables the hydrogel to quickly immobilize in the tissue after injection so as to efficiently hold cells, leading to high cell retention [62]. bFGF is a prosurvival and proangiogenic growth factor [15-16, 59, 63, 69]. In this system, bFGF is encapsulated in the hydrogel. It can thus gradually release from the hydrogel (Figure 4.1 and Figure 4.2). The released bFGF not only promotes stem cell survival but also stimulates angiogenesis.
4.4.1. Cell survival and proliferation under ischemic conditions

The MSCs encapsulated in the hydrogel experienced extensive death when cultured in the low nutrient and oxygen conditions in vitro with 60.7% and 35.1% survival after 7 and 14 days, respectively (Figure 4.3 and Figure 4.4). Addition of bFGF in the hydrogel significantly increased MSC survival in the first 7 days as no significant cell death was found (Figure 4.3 and Figure 4.4). The cells even proliferated afterwards. Consistent with in vitro results, the released bFGF significantly enhanced MSC survival in the ischemic limb (Figure 4.10). The density of surviving MSCs was increased by ~3.6 times over the MSC transplantation without bFGF (Gel+MSCs group). Besides promoting MSC survival and proliferation, the released bFGF stimulated host cell proliferation in the ischemic limb as the density of Ki67+ cells in the Gel+bFGF group was significantly higher than that in the Gel-only and Open-surgery groups (Figure 4.11). These results are consistent with those reported previously that bFGF can inhibit apoptosis of osteoblasts [71] and endothelial cells [72] under ischemic conditions. In these reports, the improved cell survival is a result of bFGF activating osteoblast PI3k/Akt pathway [71] and endothelial cell hypoxia-induced factor-1 (HIF-1) pathway [72], respectively. In this chapter, we demonstrated that the prosurvival effect on MSCs is due to bFGF activating KLF4 pathway, an important protective factor in disease states and mediator to nitric oxide in ischemic tissues (Figure 4.5).

4.4.2. bFGF effect on vascularization and regeneration of ischemic limbs

Release of bFGF alone without using MSCs (Gel+bFGF group) had a significant influence on ischemic limb vascularization (Figure 4.13 and Figure 4.14). After 2 and 4
weeks of injection, blood perfusion was dramatically increased compared to the hydrogel only group (Gel-only group). Consistent with blood perfusion results, the blood vessel density of the Gel+bFGF group was significantly higher than that of the Gel-only group (Figures 4.14). These results are in accord with previous report that bFGF releasing gelatin-PLGA hydrogels stimulated blood reperfusion recovery in a murine critical limb ischemic model [58]. The enhanced vascularization is likely attributed to the angiogenic effect of bFGF. Controlled release of bFGF from the hydrogel has the advantage of continuously supplying bioactive bFGF for angiogenesis without the need for repeated administration due to its short half live. It is also possible that bFGF salvaged the host vascular cells as it did for MSCs. Besides promoting vascularization, the released bFGF enhanced muscle regeneration as demonstrated in Figure 4.9, where the average muscle fiber diameter was increased by 20% compared to the Gel-only group. bFGF has been shown to promote skeletal muscle regeneration by stimulating skeletal muscle cell proliferation [55, 59]. Our results in Figure 4.11 indirectly confirmed this effect as the number of proliferating cells were significantly higher in the Gel+bFGF group.

4.4.3. MSCs effect on vascularization and regeneration of ischemic limbs

Delivery of MSCs with hydrogel (Gel+MSCs group) slightly enhanced blood perfusion recovery and muscle regeneration compared to the delivery of hydrogel alone (Gel-only group). This is judged from the substantially increased blood perfusion (Figure 4.13), blood vessel density (Figure 4.14), and average muscle fiber diameter (Figure 4.9). The improvement is likely the contribution of MSCs survived in the ischemic limb. These cells can differentiate into skeletal muscle cells or endothelial cells (Figure 4.12). They
may also provide paracrine effects for vascularization and muscle regeneration. MSCs are known to secrete angiogenic growth factors like VEGF, bFGF and PDGF for vascularization, and IGF-1 for promyogenesis [73, 74]. However, since the cell survival in this group is low (Figure 4.10), the improvement on blood perfusion recovery and muscle regeneration was limited.

4.4.4. Combined delivery of MSCs and bFGF on the vascularization and muscle regeneration of ischemic limbs

The remarkable blood perfusion recovery and muscle regeneration were found for Gel+MSCs+bFGF group. The blood perfusion was fully recovered only 2 weeks after injection (Figure 4.13), suggesting that the ischemia was quickly and completely alleviated. This result was validated by blood vessel densities in the recovered and normal limbs, where both limbs had almost the same density (Figure 4.14). The Gel+MSCs+bFGF group also exhibited remarkably improved muscle regeneration. The mean muscle fiber diameter was similar to that of the normal limbs after 4 weeks. The enhanced blood perfusion recovery and muscle regeneration over Gel+MSCs and Gel+bFGF groups can be attributed to the increased MSC survival, differentiation, and paracrine effects. We have demonstrated in Figure 4.10 that MSC survival was significantly augmented with the encapsulation of bFGF in the hydrogel. This allowed a greater number of MSCs to differentiate into skeletal muscle and endothelial cells to participate in muscle regeneration and vascularization, respectively (Figure 4.12). Delivery of MSCs with bFGF can increase cell paracrine effects. Figure 4.6 showed that the expression of angiogenic factor Angpt-1 and promyogenic factor IGF-1 was
significantly upregulated. These growth factors promote both vascularization and muscle regeneration. Furthermore, the enhanced paracrine effects can rescue the host cells and increase their proliferation (Figure 4.11), which may further facilitate vascularization and muscle regeneration. Collectively, this chapter demonstrates that a stem cell delivery system that augments both short- and long-term cell survival under ischemic conditions can quickly recover blood perfusion and promote skeletal muscle regeneration in the ischemic limbs.

4.5 Conclusions

In this chapter, by using an injectable biodegradable thermo-sensitive hydrogel, bFGF was successfully gradually released and together with MSCs were delivered into the ischemic limbs. In vitro results showed the myogenic differentiation of MSCs could be successfully recovered when they were encapsulated in a hydrogel with similar matrix modulus as native skeletal muscle under ischemic conditions with the delivery of bFGF. In vivo results indicated that cell survival and engraftment with host tissues were significantly improved when delivering MSCs within hydrogel containing bFGF. In addition, the average muscle fiber diameter and blood vessel densities were significantly increased in ischemic limbs injected with Gel+MSCs+bFGF compared with non-treatment (Open surgery) or other treatment (Gel-only, Gel+MSCs, Gel+bFGF) groups. This dramatic improvement may result from 1). significantly increased cell survival and integration (Figure 4.10 and Figure 4.12); 2). significantly increased cell proliferation (Figure 4.11); 3). MSC myogenic differentiation to supply skeletal muscle cells, and
endothelial differentiation for blood vessel formation (Figure 4.12); 3). bFGF function to induce fast angiogenesis (Figure 4.13 and Figure 4.14); 4). paracrine effect of transplanted MSCs (Figure 4.6).

This strategy to enhance skeletal muscle regeneration and recover blood perfusion may provide a therapeutic option for treatment of muscle damages caused by a variety of diseases. In the future, additional factors that play roles in regulating cell migration, proliferation and differentiation could also be added and delivered using this system. This system may further be applied for the regeneration of other ischemic tissues.
4.6 References


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Chapter 5: Oxygenation Augments Functional Muscle Regeneration of Ischemic Limb

5.1 Introductions

As mentioned in Chapter 3 and Chapter 4, for the treatment of critical limb ischemia (CLI), one of the most severe forms of PAD, it’s crucial to promote the muscle repair/regeneration. One of the most promising approaches to reach this treatment goal is by stem cell transplantation. Current strategies of cell delivery are typically limited by low cell retention and survival, as well as poor cell integration with the host tissues due to the ischemic local environment [1]. In Chapter 4, we successfully introduced a cell delivery system to gradually release angiogenesis growth factor bFGF for fast blood vessel formation to conquer the harsh local environment. On the other hand, approaches aimed at dealing with the hypoxia may also be promising. It’s known that after the happen of ischemia, low oxygen level in the limbs can trigger anaerobic glycolysis and dysregulation of ions such as potassium and calcium in delivered stem cells. The resulting acidosis and lack of homeostasis cause an osmotic pressure that leads to the swelling of cells, which would ultimately cause cell death. Thus hypoxia could result to dramatic inferior cell survival, growth and differentiation.

Many approaches were proposed to address these issues. A proper delivery vehicle with sufficient protection from cytotoxic local environment and suitable matrix for cell
adhesion, survival and differentiation can be used to improve stem cell survival. As results indicated in Chapter 3, an ideal stem cell carrier for skeletal muscle regeneration should have skeletal muscle mimicking mechanical properties (especially matrix elasticity) to promote myogenic differentiation of encapsulated stem cells (around $10^1$ kPa [2]).

To further address the cell survival issue under hypoxic local condition in CLI, approaches such as transfecting cells with pro-survival gene like Akt [3] and hyperbaric oxygenation [4-10] were also introduced. Gene transfection of Akt contributed to a better cytoprotective effect and higher resistance of cells to anoxia [3]. However, the virus used during the transfection process may bring up safety concerns. Use of mild hyperbaric oxygen less than 2 atms absolute with normal air is a common emerging complementary treatment for severe muscle injury. Best et al. observed an increased morphological muscle regeneration and decreased functional deficit in an acute muscle stretch injury model [6] when treated with hyperbaric oxygen at 2.5 atms absolute with 100% $\text{O}_2$ 7 days after injury (percent ankle isometric torque was decreased significantly from 47.5%+/- 5.4% in untreated group to 14.9%+/- 5.5% in experimental group). Gregorevic et al. found that hyperbaric oxygen at 3 atms with 100% $\text{O}_2$ upregulated the contractile properties of regenerating rat soleus muscles after myotoxic injury [7]. In another work, Fujita et al. investigated the impact of hyperbaric oxygen at 1.25 atms absolute with normal air on the muscle regeneration after injury caused by bupivacaine hydrochloride. Their results demonstrated that both the cross-section area of centrally nucleated muscle fibers and the mature skeletal muscle cells density were significantly higher in rats.
exposed to hyperbaric oxygen than in non-hyperbaric oxygen control group, indicating mild hyperbaric oxygen’s effect on skeletal muscle regeneration in the early phase after injury [4]. All of these results demonstrated oxygen’s functions on helping stem cell survival and/or differentiation, thus enhancing angiogenesis and functional recovery of damaged muscle tissues. However, hyperbaric oxygenation may induce issues such as the generation of reactive oxygen species (ROS) and lack of continuity for long-term muscle regeneration and functional recovery. To address these limitations, we used core-shell oxygen release microspheres to gradually and continuously deliver oxygen [11] to cells. Here in this chapter, our hypothesis is that localized and sustained delivery of oxygen, together with stem cells that could differentiate into mature skeletal muscle cells/endothelia cells, can stop or reverse muscle injuries resulting from tissue ischemia and contribute to a fast muscle regeneration as well as blood perfusion and functional recovery.

This chapter investigated a potential interplay between released oxygen from the oxygen release microspheres and MSCs in ischemic muscle tissues, and the possibility that combined delivery of stem cells and oxygenation microspheres could induce the functional muscle formation in ischemic hindlimbs. As targets for these experiments, we chose the muscle tissues where MSCs and/or oxygen release microspheres were injected. The ultimate goal of this approach is to activate the survival/proliferation phase of the local progenitor cells and transplanted stem cells as well as induce their differentiation into contractile muscles fibers to regenerate functional tissues. At the same time,
angiogenesis of the local tissues may be promoted by providing a proper high oxygen environment for the cell proliferation and differentiation processes.

5.2 Materials and methods

5.2.1. Materials & synthesis of polymers

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. N-isopropylacrylamide (NIPAAm, Alfa Aesar) was purified by recrystallization using hexane three times and lyophilized overnight before use. 2-hydroxyethyl methylmethacrylate (HEMA, TCI) was passed through a column packed with inhibitor remover to remove inhibitor. Acryloyl chloride, 3,6-Dimethyl-1,4-dioxane-2,5-dione, chondroitin sulfate and sodium methoxide were used as received.

The APLA macromer was synthesized through a two-step method [12] as described in Chapter 4, which includes a ring opening polymerization of lactide with NaOCH₃ as the initiator followed by an esterification process of the products from previous reaction with acryloyl chloride. APLA hydrogel polymer (Poly (NIPAAm-co-AA-oligoLA-co-HEMA)) was then synthesized via free radical polymerization [13] as described in section 4.2.2.

The synthesized APLA hydrogel had a SGTT of ~26 °C as determined by DSC. Its degradation product has a SGTT above body temperature, so that it can be dissolved in the body fluid and further removed. The hydrogel was highly flexible with a breaking
strain above 300% and Young’s modulus of 17.1±3.4 kPa (tested in aqueous solution at 37 °C after gelation, gel solution was prepared as 10% w/v in DPBS).

5.2.2. Fabrication of core-shell oxygen release microspheres (ORM)

To fabricate the oxygen release microspheres, H$_2$O$_2$ was first mixed with PVP to form a complex. The molar ratio of H$_2$O$_2$ and repeating unit N-vinylpyrrolidone (VP) was controlled at 4.5/1. To ensure uniform thorough mixing, the mixture was stirred overnight at 4°C. The core-shell oxygen release microspheres (with PLGA as shell material and H$_2$O$_2$/PVP complex as core) were fabricated by a co-axial electrospraying-electrospinning device [11]. In brief, as indicated by Scheme 5.1, the inner and outer tubes of the device were charged with H$_2$O$_2$/PVP complex and PLGA solution (5 wt% in dichloromethane), respectively. And they were connected to a positive transformer at a voltage of +17 kV. At the same time, a tube with PEG solution (molecular weight of 10000, 10 wt% in dichloromethane) was pumped and charged by a voltage of +15 kV to produce a fibrous network to support the electrosprayed microspheres. The collector was connected to a negative transformer at a voltage of -10 kV and rotating at a speed of 1000 rpm. The infusion speeds of the H$_2$O$_2$/PVP complex, PLGA solution and PEG solution were 0.2, 1 and 17 mL/h, respectively. After fabrication, the fiber-microsphere mixture was dispersed within DI water to dissolve the PEG/collct the microspheres. The solution was then centrifuged. The microspheres precipitate was collected and freeze-dried for 1-2 hours and stored at -20°C for future use.
5.2.3. Oxygen permeability of hydrogel

Oxygen permeability of the synthesized APLA hydrogel was tested by electron paramagnetic resonance (EPR) spectroscopy by using LiNc-BuO as the oxygen probe [14]. The hydrogel solution (10% w/v in DPBS) was first thoroughly mixed with the probes, and then incubated at 37°C to form a solid hydrogel. The gel was then placed into a gas-permeable EPR tube, and flushed with nitrogen for around 20 min to fully remove the oxygen within the gel. After complete removal of oxygen validated by EPR spectrum, medical air was subsequently used to flush the gel to diffuse oxygen into the sample. ERP spectrum was recorded using an L-band EPR spectrometer (Magnettech, Germany), and the peak-to-peak width of the spectrum was collected to calculate the oxygen partial...
pressure within the gel using a standard calibration curve collected from DPBS buffer [15] following the same procedure.

5.2.4. Oxygen release kinetics

Oxygen release kinetics from the microspheres was conducted in DPBS supplemented with catalase. An oxygen-sensitive luminophore Ru(Ph$_2$phen$_3$)Cl$_2$ was used to detect the oxygen concentration and an oxygen-insensitive fluorescence rhodamine-B was used as the control. These two dyes were first mixed with poly-dimethylsiloxane (PDMS) precursor, and then a curing agent was added to form PDMS membrane. Here PDMS was chosen due to its high oxygen permeability and hydrophobicity to prevent water from penetrating inside interacting with Ru(Ph$_2$phen$_3$)Cl$_2$ and/or rhodamine-B. The produced fluorescence-mixed PDMS membrane was punched into disks (6mm in diameter) and placed into the wells of a 96-well plate. The plate was set in 1% oxygen incubator to balance the oxygen pressure for 24 h before quickly added with hydrogel solution (10% w/v prepared using 1% oxygen pressure balanced DPBS) with 50 mg/mL of oxygen release microspheres and 1 mg/mL of bovine catalase into it. The plate was then sealed and incubated at 37°C with 1% O$_2$ and 5% CO$_2$. Fluorescence intensity was tested on day 1, 2, 3, 4, 7, 10 and 14 at 610 nm for Ru(Ph$_2$phen$_3$)Cl$_2$ (excitation at 470nm) and at 576 nm for rhodamine-B (excitation at 543nm). The collected fluorescence intensity was then converted to oxygen concentration according to a standard calibration curve obtained by measuring the fluorescence intensity of DPBS buffer balanced under conditions of 1, 5, and 21% oxygen.
5.2.5. Encapsulation of oxygen release microspheres and MSCs into hydrogel

A 10% (w/v % in DPBS) hydrogel solution was used to encapsulate MSCs and oxygen release microspheres. Rat bone marrow-derived mesenchymal stem cells (MSCs) were cultured in 10% fetal bovine serum (FBS) supplementing alpha modified minimum essential medium (αMEM) media with 1% antibiotics at 37°C with 5% CO₂ and 21% O₂. Cells at passages 11-14 were used in this chapter. Our previous works demonstrated that MSCs within these passages remained multipotency [16]. MSCs were trypsinized first from the tissue culture plates (TCP) and re-suspended in culture media. Before encapsulation, hydrogel solution and oxygen release microspheres was sterilized under UV light in a laminar flow hood for 20-30 minutes. After loading the bovine catalase (1mg/mL) and oxygen release microspheres (ORM, 50mg/mL) into the hydrogel solution, cell suspension was then added into the mixture, and the final cell concentration was controlled at 10 million/mL. 0.2 mL MSCs/APLA hydrogel solution with or without ORM was dispensed to each Eppendorf tube and cultured under ischemic culture condition (0% FBS, 1% O₂, 5% CO₂) (labeled as OH and AH groups, respectively). MSCs-hydrogel mixture cultured under normal culture condition (10% FBS, 21% O₂, 5% CO₂) (labeled as AN group) was used as a control. At day 1, 7 and 14, pellets were randomly selected for dsDNA test, live cell tracker observation, real time RT-PCR test and immunohistology analysis.

5.2.6. Oxidative pressure of MSCs in vitro

The oxidative pressure of encapsulated MSCs in vitro was tested by using general oxidative stress indicator CM-H2DCFDA (C6827, Invitrogen) following the standard
protocol. In brief, cells were trypsinized and re-suspended in pre-warmed PBS buffer containing the indicator at a concentration of 5μM. After 30 min incubation, cells were collected from the loading buffer after centrifuge and encapsulated into the hydrogel solution with or without ORM. After 3 days ischemic incubation (0% SFB, 1% O₂ and 5% CO₂) in vitro, samples were collected for fluorescence microscopy imaging and fluorescence intensity reading at 527nm (excitation at 495nm). Cell-free samples were used as the control, and the fluorescence intensity was normalized by cell numbers (based on dsDNA test) within the sample.

5.2.7. MSCs survival, myogenic differentiation and paracrine effect in hydrogel in vitro

The survival of MSCs in hydrogel with or without ORM was assessed by double-strand DNA (dsDNA, for live cells) content using the same method as described in section 4.2.8.

Real time RT-PCR and immunohistology staining were done for gene and protein expressions respectively to detect the myogenic differentiation condition of the seeded cells in hydrogels. After 14 days in vitro culture, cell/hydrogel mixtures with or without ORM were immersed in TRIzol (Sigma) and processed by the manufacture's protocol to isolate and purify RNA. cDNA was reverse transcribed from RNA by using cDNA high capacity transcription kit (ABI). A real-time RT-PCR process was conducted as described in section 4.2.8. A standard ΔΔCt method was used to process the data. Myogenic markers MEF2, Myogenin, Myosin heavy chain (MHC), Desmin and MyoD1 are checked and β-actin was used as the housekeeping gene. For protein expressions analysis,
samples were fixed in 4% paraformaldehyde for 1 h, embedded into OCT and
cyrosectioned at 10µm. The staining and imaging was conducted using the same method
as described in section 4.2.8. The potential paracrine effect of the encapsulated cells was
also detected by real time RT-PCR (Pdgfb, IGF-1 and Angpt1). All the primers
information was listed in Table 5.1.

Table 5.1. Primers used for real time RT-PCR to detect the myogenic differentiation and paracrine
effect of MSCs in gel with or without ORM.

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
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<tr>
<td>β-actin</td>
<td>AAGATCAAGATCATTGCTCCTC</td>
<td>GGACTCATCGTACTCCTG</td>
</tr>
<tr>
<td>MEF2</td>
<td>TGCTGCTCTCACTGTCACTAC</td>
<td>TTCACGACTTGGGGACACTG</td>
</tr>
<tr>
<td>Myogenin</td>
<td>TGAGAGAGAGGAGGAGGAAC</td>
<td>ACAATACACAAAGCAGCTGGAA</td>
</tr>
<tr>
<td>MHC</td>
<td>TGACTTCTGGCAAAATGCAG</td>
<td>CCAAGCGAGAGGAGTTGTC</td>
</tr>
<tr>
<td>Desmin</td>
<td>ATACCGACACCAGATCCAGTCC</td>
<td>TCCCTCATCTGCTCATCAAGG</td>
</tr>
<tr>
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<td>GCAGCAAGCAACACAAACCAG</td>
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<tr>
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</tr>
<tr>
<td>Angpt1</td>
<td>CCATCTCCGACTTCTATTTTCC</td>
<td>ATGCTCCACACGTGGAGACG</td>
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</table>
5.2.8. Animal surgical procedure

Animal surgery was performed in compliance with National Institutes of Health and institutional guidelines using male wild type C57BL/6 mice (aged 8 to 10 weeks) by unilateral femoral artery and vein ligation with the same method as described in section 4.2.9 (Scheme 4.1). After the vessel ligation, mice were injected with a total volume of 200 μL of gel with MSCs (Gel+MSCs), gel with ORM (Gel+ORM) or gel delivered with MSCs and ORM (Gel+MSCs+ORM) into the muscles in between the ligation points through four injections (50 μL/injection). Open surgery group with ligation-only were used as the negative control, and non-surgery group (Normal) was used as positive control.

5.2.9. MSCs survival, proliferation and differentiation in vivo

MSCs were treated with live cell tracker CM-Dil (Invitrogen) following the standard provided protocol before encapsulated into hydrogels. Fluorescence images were taken by Olympus FV1000 filter confocal microscope of frozen muscle tissue slices prepared as described above, and survived cell numbers/densities were analyzed based on these images by ImageJ. Immunostaining for Ki-67 (Abcam), MHC (Millipore) and vWF (Millipore) were performed based on paraffin-fixed muscle tissue slices to identify cell proliferation, skeletal muscle cells/myogenic differentiation, and endothelial cells/endothelial differentiation, respectively.

5.2.10. Ischemia and blood perfusion

Measurements of the ischemic/normal limb blood flow ratio were performed on
anesthetized animals (n=4) using an LDPI analyzer (Perimed) as described in section 4.2.11.

5.2.11. Histologic assessment of skeletal muscle

Mice hindlimb muscle tissues (n=8 per time point per experimental group) were collected and processed for histologic analyses. The samples were fixed with 4% paraformaldehyde, chemically dehydrated, embedded in paraffin and sectioned to slices of 4 μm in sequence. The samples were then stained with H&E following previous well-defined protocol, and fiber diameters were analyzed using ImageJ based on the H&E stained images. Interstitial fibrosis was morphometrically assessed by Mason Tri-chrome stained sections.

5.2.12. Mechanical measurement of ischemic muscles ex vivo

The contractility and fatigability of the ischemic skeletal muscles were tested following a previous well-defined protocol [17]. In brief, intact soleus and EDL muscles were dissected (n=4/group), and mounted vertically midway between two cylindrical parallel steel wire electrodes by their tendons. The muscles were connected to a force transducer (ADInstruments) and bathed in a physiological saline solution (140 mM NaCl, 5 mM KCl, 10 mM HEPES, 2.5 mM CaCl₂, 2 mM MgCl₂ and 10 mM glucose) in a chamber oxygenated with medical air at 25°C. The muscle length was adjusted carefully until the force does not increase under a chosen stimulation (100Hz for EDL and 60Hz for soleus muscles). The mechanical measurement of the ischemic muscles ex vivo was conducted by applying a square wave pulse (60mA, 0.3ms in duration) using a custom-written
LabView program to the muscles via a purpose-built power amplifier (ADInstruments). An equilibration procedure (80Hz for 30min) was first applied allowing the muscles to adapt to the new environment in chamber. Then pulses with different frequencies (from 1 to 140 Hz) were applied and peak tetanic force ($T_{\text{max}}$) was determined as the difference between the maximum collected force during the contraction test and the baseline level. Specific force was calculated by normalizing the peak tetanic force $T_{\text{max}}$ by muscle weight. After recovering the muscles at $\frac{1}{2} T_{\text{max}}$ for 30min, an additional fatigue test was performed at the frequency of 40Hz for 5min, and fatigue time was calculated as the time when muscle contraction force decreased to 70% of its maximum value.

5.2.13. Running ability of mice

The running ability of the mice was detected by a running test. Mice were first trained for running on a special-designed treadmill (Cambridge Technology Inc., Model Exer-6M) for a week before surgery. 1 day, 2 and 4 weeks after injury/treatment, mice from all groups (n=4/group) were placed onto the running lane at a speed of 120cm/min, and the mice running ability was quantitively evaluated as their running distance per hour.

5.2.14. Statistical analysis

Data were reported as mean ± standard deviation. Multivariate repeated-measures ANOVA were performed to test the interactions between different test groups. A statistical significance was considered when $p < 0.05$.

5.3 Results
5.3.1. Release kinetics of oxygen from hydrogel

Molecular oxygen was obtained when the released H₂O₂/PVP complex from the microspheres due to the degradation of PLGA met the bovine catalase within hydrogel. As indicated by Figure 5.1, under 1% oxygen condition, oxygen was sustainably released from the hydrogel during a 14-day test period. The release was gradually increased during the first four days, and reached its highest level (15%) on day 4, after which the oxygen was continuously released at a constant speed and the oxygen content was successfully kept around 11%.

Figure 5.1. Oxygen release kinetics in APLA hydrogel (prepared as 10% w/v in DPBS) 1, 2, 3, 4, 7, 10, 14 days under hypoxic condition (1% O₂).
5.3.2. Oxygen permeability of hydrogel

Oxygen permeability of hydrogel could significantly influence the diffusion of oxygen released from the microspheres, and affect the cellular activities including cell survival, proliferation and differentiation subsequently. EPR results demonstrated in Figure 5.2 showed that the oxygen partial pressure in the gel (148.6 mmHg) was similar to that in DPBS buffer (159.6 mmHg), indicating the excellent oxygen permeability of the APLA gel.

Figure 5.2. Oxygen permeability in DPBS buffer and APLA hydrogel.
5.3.3. Oxidative stress of cells under ischemic in vitro culture

The oxidative stress of cells in APLA hydrogel with or without ORM under ischemic culture condition was detected by fluorescent reader and confocal microscopy. As indicated in Figure 5.3, no significant difference in the oxidative stress was observed for cells delivered with or without oxygen release microspheres 3 days after in vitro ischemic culture. This result indicated that the oxygen level provided by the oxygen release microspheres was proper, and did not cause the further production of toxic reactive oxygen species (ROS).

![Oxidative stress of cells in APLA hydrogel with or without oxygen release microspheres 3 days after in vitro ischemic culture.](image)

Figure 5.3. Oxidative stress of cells in APLA hydrogel with or without oxygen release microspheres 3 days after in vitro ischemic culture.
5.3.4. **MSCs survival, growth and myogenic differentiation under ischemic condition in vitro**

Under normal culture condition with sufficient oxygen and nutrient, MSCs demonstrated a remarkable survival and proliferation capacity during a 14-day culture period. However, under low oxygen and low nutrient condition (0% SFB, 1% O₂), dramatic cell death was observed for samples without ORM. When cells were delivered with ORM, all of them survived in hydrogels within this two-week culture period. Interestingly, these cells even proliferated (cell numbers increased to 115.0% and 130.4% after 7 and 14 days culture under ischemic condition, respectively) as shown in Figure 5.4. These results indicated that, the released oxygen from ORM was capable of helping encapsulated cells to overcome the low oxygen environment, without producing extra oxidative stress, thus improved MSC survival and proliferation. Live cell tracker images (Figure 5.5) demonstrated homogenous distribution of seeded cells within the hydrogels, and consistent result of cells survival and growth as indicated by dsDNA testing result.

The myogenic differentiation of seeded MSCs in hydrogel under ischemic culture conditions with or without ORM was evaluated at the mRNA level by real-time RT-PCR, and at the protein level by immunohistochemistry. As results shown in Figure 5.6, under ischemic culture conditions, the differentiations of encapsulated stem cells were dramatically depressed for all the tested myogenic markers. In contrast, with the delivery of ORM under ischemic culture conditions, their myogenic differentiations was recovered up to more than 170000, 260000, 83, 743, 26000 times for MHC, MyoD1, MEF2, Desmin and Myogenin expressions, respectively. These values were even
significantly higher than those in normal culture conditions. The MHC staining result (Figure 5.7) also indicated that ischemic conditions dramatically depressed the seeded MSC’s myogenic differentiation, whereas the controlled release of oxygen from ORM supplied the MSCs with a proper amount of oxygen in a durable manner. In conclusion, the oxygenated environment together with the hydrogel matrix properties successfully improved MSC myogenic differentiation significantly under ischemic conditions.

![Figure 5.4. dsDNA concentration of cells in AN (Gel-MSCs under normal culture condition), AH (Gel-MSCs under hypoxic culture condition), and OH (Gel-MSCs-ORM under hypoxic culture condition) on 1st, 7th and 14th day of in vitro culture (*p<0.05, **p<0.01).](image-url)
Figure 5.5. Live cell tracker images of cells in AN (Gel-MSCs under normal culture condition), AH (Gel-MSCs under hypoxic culture condition), and OH (Gel-MSCs-ORM under hypoxic culture condition) on 1st, 7th and 14th day of in vitro culture.
Figure 5.6. Gene expressions (Myogenin, Desmin, MyoD1, MHC and MEF2) of cells in AN (Gel-MSCs under normal culture condition), AH (Gel-MSCs under hypoxic culture condition), and OH (Gel-MSCs-ORM under hypoxic culture condition) on 14th day of in vitro culture (*p<0.05, **p<0.01).
Figure 5.7. MHC expression of cells in AN (Gel-MSCs under normal culture condition), AH (Gel-MSCs under hypoxic culture condition), and OH (Gel-MSCs-ORM under hypoxic culture condition) on 14\textsuperscript{th} day of in vitro culture.

5.3.5. Paracrine effect of encapsulated MSCs

As indicated by real time RT-PCR results (Figure 5.8), MSCs in hydrogel with ORM (OH) exhibited significantly higher gene expressions of angiogenesis growth factors Pdgfb and Angpt 1 as well as pro-survival and pro-myogenic factor IGF-1 compared to those in gel without ORM (AH) under ischemic culture conditions. Interestingly, their expressions were even higher than the cells encapsulated in gel under normal culture condition (AN). All these results indicated a possible higher paracrine effect of the encapsulated MSCs when delivered with ORM.
Figure 5.8. Paracrine effect (Pdgfb, Angpt1 and IGF-1) of cells in AN (Gel-MSCs under normal culture condition), AH (Gel-MSCs under hypoxic culture condition), and OH (Gel-MSCs-ORM under hypoxic culture condition) after 7 days in vitro culture (*p<0.05, **p<0.01).
5.3.6. Muscle fibrosis and regeneration

To directly analyze the muscle regeneration in different experimental groups, the mean diameter of myofibers in ischemic limbs 4 weeks after injury/treatment were quantified based on H&E images. There was no significant difference of muscle fiber diameter in Gel+MSCs group compared with Open-surgery group (p>0.5). In contrast, the mean diameters of muscle fibers were quantitatively greater in ischemic limbs treated with Gel+ORM or Gel+MSCs+ORM. The delivery of ORM alone led to a ~39% increase in average muscle fiber diameter, whereas delivery of MSCs in hydrogels with ORM resulted to a ~45.7% increase in the fiber diameter 4 weeks after surgery when compared with non-treatment group. Furthermore, injured muscles without treatment demonstrated significant interstitial fibrotic tissues, while normal (non-operated) limbs showed little fibrosis, as expected. Limbs treated with Gel+MSCs or Gel+ORM exhibited an obvious decrease in fibrosis as showed in Figure 5.9.C. An even more-pronounced reduction of fibrosis was observed within Gel+MSCs+ORM group, and the muscles were much more compact than those in other experimental groups.
Figure 5.9. A. H&E images; B. Myofiber diameter collected from H&E images (*p<0.05, **p<0.01); and C. Mason tri-chrome images of mouse tissues around the injection points 4 weeks after surgery.
5.3.7. Recovery of muscle mechanical function and mice running ability

One of the most important therapeutic goals for skeletal tissue regeneration is to recover the mechanical properties of the ischemic tissues as well as the running/movement ability of the patients. In order to detect the recovery of muscle mechanical functions, contractile forces and fatigue properties of EDL and soleus muscles as well as the mice running ability (distance per hour) were analyzed. Results (Figure 5.10 and Figure 5.11) indicated that for both EDL and soleus muscles, the single delivery of MSCs or ORM in hydrogel had no significant positive effect on the improvement of muscle contractility or fatigue resistance. While delivered together (Gel+MSCs+ORM), the specific contraction force as well as the fatigue index of both tested muscles were significantly increased, and their values were even comparable with normal non-injured muscles (p>0.05 for all the
mechanical tests between normal muscles and those in Gel+MSCs+ORM group). The mice running ability result was demonstrated in Figure 5.12, and dramatic increase staring from 2 weeks post-surgery was detected when delivering MSCs and ORM together in hydrogel (increase of ~89% and 64% on week 2 and week 4 compared with open surgery group, respectively). Interestingly, the single delivery of MSCs or ORM also led to a modest increase (~30-40%) of running ability of mice starting from week 2 and retained to week 4.
Figure 5.10. Contraction force of mouse muscles (EDL and soleus) collected 4 weeks after surgery (*p<0.05, **p<0.01).
Figure 5.11. Fatigue properties of mouse muscles (EDL and soleus) collected 4 weeks after surgery (*p<0.05, **p<0.01).
Figure 5.12. Running ability of mice 4, 7, 14 and 28 days after surgery (*, **, ***, #, ## demonstrate significant difference between groups according to ANOVA and Tukey HSD statistical analysis).

5.3.8. **MSCs survival and proliferation in vivo**

Ischemia led to the greatest loss of muscle function, as compared with other injury models, and the least spontaneous return of functions [18]. Here in this chapter, mice were treated following the ischemia induction with an injectable, thermo-sensitive and biodegradable hydrogel delivered with MSCs and oxygen release microspheres fabricated by electrospraying method. Live cell tracker CM-Dil (Invitrogen) was used to mark the transplanted cells to detect their survival, proliferation and positions in vivo. As Figure 5.13 showed, there was significantly more cell survival when delivering MSCs in gel with ORM compared with those without ORM. In addition, the degradation of the
hydrogel allowed some of encapsulated cells to migrate out from the injected gels and possibly integrate with the surrounding host tissues. Proliferation-associated protein Ki67 (Figure 5.14) was also performed and quantitatively assessed to determine cell proliferation in tissues. Abundant expression of Ki67 was detected in tissues treated with Gel+MSCs+ORM 4 weeks after injury. A less-pronounced increase was observed in Gel+MSCs and Gel+ORM groups, whereas the least proliferation was observed in open-surgery group.
Figure 5.13. A. Live cell tracker images of mouse tissues collected around the injection points 4 weeks after surgery; B. Survival cell numbers/densities collected from the live cell tracker images (*p<0.05).
Figure 5.14. A. Ki67 expression of cells in vivo 4 weeks after injury/treatment; B. Quantitative numbers of Ki67-positive cells (*p<0.05, **p<0.01).
5.3.9. MSCs differentiation in vivo

The differentiation of transplanted MSCs in vivo was detected by immunohistochemistry. As Figure 5.15.A showed, above 90% of the transplanted MSCs left in/around injection gels 4 weeks after injury had successful myogenic differentiation in vivo based on their positive expression of MHC. For the cells migrated out from the injection points, some of them differentiated into endothelial cells and formed blood vessels (as indicated by vWF staining in Figure 5.15.B), most differentiated into skeletal muscle cells and integrated with host muscle tissues (as indicated by MHC staining in Figure 5.15.B). Some differentiated cells formed aligned structure following the pattern of native muscles, and it’s possible that they produced functional myofibers together with the host muscle cells.
Figure 5.15. Cell differentiation (MHC and vWF) in vivo 4 weeks after injury/treatment: A. Cells within/around the injected gel, B. cells outside/far away from the injected gel.
5.3.10. Muscle vascularization and blood perfusion

A laser Doppler perfusion imaging (LDPI) system was used to quantify blood perfusion of ischemic limbs (Figure 5.16). As the images showed, the regional blood flow was reduced dramatically after ligation to around 11% of normal limbs, as expected. Gel+ORM and Gel+MSCs treatments led to a slow increase in blood perfusion over time. While Gel+ORM and Gel+MSCs groups increased blood perfusion significantly 4 weeks after surgery up to around 68% and 65% respectively compared with 45% recovery in non-treatment (Open surgery) group. The Gel+MSCs+ORM group contributed to an almost complete recovery of blood flow, 94% of normal limbs. In particular, the ischemic limbs treated with Gel+MSCs+ORM showed a remarked increase in blood flow (81.5% of normal limbs) as early as 2 weeks after the injury/treatment, and this recovery remained and kept increasing up to week 4. The vWF staining was also performed and blood vessel densities were quantified based on the images at areas both close and far from the injection points (Figure 5.18). There was significant increase of blood vessel densities at both areas for Gel+ORM group compared with non-treatment (Open surgery) group. Interestingly, the blood vessel density in Gel+MSCs group was significantly increased near the injection points, and the increase was modest far away from the injection points. As for the Gel+MSCs+ORM group, the blood vessel densities at both areas were improved dramatically, and reached a complete recovery as compared with normal limb (p=0.99, p=0.21 for areas close and far from the injection points respectively). This result was consistent with laser Doppler results mentioned above.
Figure 5.16. Blood flow images (from laser Doppler) 0 day, 2 and 4 weeks after surgery.
Figure 5.17. Quantitative blood perfusion recovery collected from laser Doppler result 0 day, 2 and 4 weeks after surgery (*, **, #, ## demonstrate group with significant difference according to ANOVA and Tukey HSD statistical analysis).

Figure 5.18. Blood vessel densities close and far away from the injection points 4 weeks after surgery (*, **, #, ## demonstrate groups with significant difference according to ANOVA and Tukey HSD statistical analysis).
5.4 Discussion

These results above suggested a beneficial interplay between MSCs and released oxygen, when delivered appropriately in an injectable thermo-sensitive hydrogel with proper matrix properties, enhancing skeletal muscle regeneration, mechanical properties and blood perfusion recovery of ischemic muscle tissues. After the happen of ischemia, local hypoxia causes cell death and muscle degeneration, followed by the loss of tissue structure and function. The survival and functional maintenance is of especially great importance before the buildup of new blood vessels. Exposure to oxygen is widely accepted as an approach to promote the healing of many tissue injuries including bone fracture [19], spinal cord injury [8], articular cartilage damage [9] and skeletal muscle injury [4, 5, 7, 10]. However, its impact on salvaging and driving regeneration of ischemic skeletal muscles has not been well addressed. Some preliminary data showed that externally provided oxygen might help promote skeletal muscle regeneration through the acceleration of macrophage infiltration and phenotype transition by reducing local hypoxic conditions [4].

5.4.1. Oxygen delivery in hydrogel

The results in this chapter showed that the sustained delivery of oxygen by ORM alone had a significant influence on muscle regeneration, blood perfusion and mice running ability recovery. As indicated by Figure 5.17 and Figure 5.18, blood vessel densities were significantly increased compared with non-surgery (Open surgery) group both near and far from the injection points, and blood perfusion of the ischemic limbs was recovered to ~68% of normal limbs 4 weeks after injury. In addition, muscle regeneration
was also improved as demonstrated in Figure 5.9, the average muscle fiber diameter was increased by ~18% compared with non-treatment (Open surgery) group 4 weeks post-surgery. All of these results indicated oxygen’s potential function in building up a local environment promoting the survival and proliferation of host cells in the ischemic tissues, which was verified by the increased Ki67 expressions of cells (Figure 5.14), thus helped recovery of blood perfusion and tissue regeneration. Similar results were observed in previous works. For example, Oh et al. found that when incorporating calcium peroxide-based oxygen generating particles into 3D PLGA scaffolds, oxygen was able to be gradually released over the course of 10 days and cell viability were extended under hypoxic conditions [20]. In another work, Lee et al. used perfluorooctane emulsion (oxygen carrier)-loaded hollow microparticles to timely release oxygen, and cells attached were found alive throughout the whole matrix until the infiltration of blood vessels 14 days after implantation [21]. In this chapter, oxygen was successfully gradually released for at least 14 days (Figure 5.1). The APLA hydrogel had excellent oxygen permeability (Figure 5.2), allowing oxygen to be fast diffused through the gel, thus improving the survival and proliferation of both the transplanted cells and host tissue cells (Figure 5.13 and Figure 5.14).

5.4.2. Hydrogel as cell and oxygenation microspheres carrier

Strikingly, delivery of MSCs with ORM in hydrogels showed a remarkable synergetic effect on the muscle regeneration, blood perfusion and functional recovery of ischemic tissues. In particular, the mean muscle fiber diameter was significantly enhanced by ~45.5% with delivery of MSCs and ORM in hydrogels (Figure 5.9). Furthermore, the
contractility and fatigue resistance function of the tested muscles (EDL and soleus) as well as the mice running ability was also dramatically upregulated compared with open surgery group and reached normal level 4 weeks after injury/treatment (Figure 5.10, Figure 5.11 and Figure 5.12). This result was qualitatively validated by an increased number/density of cells (include host cells and transplanted cells) found in an active proliferative state, as shown of the presence Ki67-positive cells (Figure 5.14). These results may suggest an enhancement in myoblast/satellite cell recruitment for new muscle formation. In the other hand, the improved myogenic regeneration in response to Gel+MSCs+ORM could be explained by the effect from transplanted cells. They could on one side, serve as a supply of myoblasts or endothelial cells. This could be validated by the significant increase of cell survival (Figure 5.13), myogenic or endothelial differentiation (Figure 5.15), and successful engraftment of transplanted cells with host muscle tissues (Figure 5.15.B) or their integration with blood vessel cells and form new blood vessels. In addition, the transplanted and survived MSCs may also have a paracrine effect. This could be verified by the gene expression results showing that MSCs delivered with ORM in hydrogel had significantly higher expressions of pro-survival and pro-myogenic factor Insulin-like growth factor 1 (IGF-1), angiogenesis factors Platelet-derived growth factor beta polypeptide (PDGFB) and angiopoietin-1 (Angpt-1) (Figure 5.6), which were even higher than cells cultured in normal culture condition (AN).

Furthermore, the combination of MSCs and ORM delivery with hydrogels was shown to alleviate ischemia, with an almost full return to normal hemodynamic levels. The laser Doppler perfusion result showed the blood perfusion of limbs in Gel+MSCs+ORM group
was recovered to 81.5% of normal limbs as early as 2 weeks after surgery, and this recovery was increased to around 94% on week 4 (Figure 5.16 and Figure 5.17). This result was validated by significantly increased blood vessel densities at both areas close and far from the injection points up to normal limb level (Figure 5.18). These results could be explained by the possible secret of angiogenesis factors PDGFB and Angpt-1 (Figure 5.6) as well as the endothelial differentiation (Figure 5.15.B) of the transplanted MSCs.

5.5 Conclusions

In this chapter, by using an injectable biodegradable thermo-sensitive hydrogel and oxygenation microspheres, oxygen was successfully gradually released from the gel within a 2-week test period. In vitro results showed the myogenic differentiation of MSCs could be successfully achieved when they were encapsulated in a hydrogel with similar modulus as native skeletal muscle under ischemic conditions with oxygen release microspheres, and these expressions were even significantly higher than those cultured in hydrogel under normal culture condition. In vivo results indicated that cell survival and engraftment with host tissues were significantly improved when delivering MSCs within hydrogel containing oxygen release microspheres. In vivo results demonstrated that the average muscle fiber diameter and blood vessel densities were significantly increased in ischemic limbs injected with Gel+MSCs+OMR compared with non-treatment or other treatment groups. In addition, the blood perfusion, mechanical function of the ischemic limbs as well as the running ability of tested mice (Gel+MSCs+ORM group) were also
dramatically increased and completely recovered. This strategy may provide a therapeutic option for the treatment of muscle damages caused by a variety of diseases.
5.6 References


Chapter 6: Correlation of Matrix Elasticity-mediated Cellular Traction with Stem Cell Fates in Hydrogels

6.1 Introductions

When seeded in matrices such as natural-derived proteins collagen, fibronectin or synthesized hydrogels (such as PEG, NIPAAm-based thermo-sensitive hydrogels), dynamic protein complexes known as focal adhesions (FAs) will form in the cell-matrix interface linking the actin-myosin cytoskeleton within the cell to the special ligands in the matrices. It’s through these focal adhesion sites that cells can exert traction forces to their surrounding materials. The physical properties of the matrices, especially the matrix elasticity $E$, can therefore be probed by cells through these actin-myosin stresses. This cell-generated traction force was found to be of great importance on controlling stem cell fates including cell migration [1], proliferation [2] and differentiation [3] by some experimental works. On one hand, researchers found that these forces applied on focal adhesion sites could help stimulate integrin clustering and activate integrin-associated signal cascades such as focal adhesion kinase (FAK), Shc, ERK, mDia1 and caveolin-1 (cav-1) [4-6] to effect cellular activities. On the other hand, these forces are also connected to microfilaments and microtubules directly or indirectly through the cytoskeletal linker proteins (vinculin, paxillin and tallin). Thus they may be delivered to
deform the cell nuclei through the link between cytoskeletal components with nesprins, which spans outside the nuclei membrane and bind to the inner nuclei membrane proteins such as SUN proteins [7]. Given the fact that genes change positions within the cell nuclei during the stem cell differentiation process [8], there is a possibility that the matrix elasticity is sensed by cells and influences the cellular activities through the interplay of cell tractions, cytoskeleton tension and nuclei deformation.

As the previous results indicate in Chapter 3, Chapter 4 and Chapter 5, when seeding stem cells (CDCs or MSCs, for example) in hydrogels, the matrix modulus could influence cell spreading, proliferation and differentiation even in the absence of soluble chemical factors. Other than the mechanical properties, some researchers have found that stem cell fates can also be controlled by geometric constraints, which limited cell adhesions and further traction generation. Despite of these experimental advances, little are known on how these biophysical properties are related to cell traction force generation and further to stem cell fates.

These cell-generated tractions were recently quantitatively measured by 2D/3D traction force microscopy, in which fluorescent micro-beads were seeded in the vicinity of each cell and their displacements were tracked using microscopy and used to calculate the cellular tractions [9]. This method allows precise measurement of the spatial and temporal nature of these cell-generated tractions, providing a method to understand where and when the mechanical events come to play in both pathological and physiological settings. However, it’s generally limited by the necessary use of transparent
materials/matrices and small imaging/measurement scope (length scale of several hundred microns). When considering the stem cell differentiation process, for example, it is difficult to detect experimentally the extent to which a single cell is differentiated. Alternatively, the differentiation condition of large numbers of cells (thousands or even millions) can be assessed easily by well-defined techniques. In order to solve these problems, we introduced a novel method using finite element modeling (FEM) combined with digital image correlation (DIC) to obtain the cell-exerted tractions, especially the traction history.

Digital image correlation (DIC) technique is a non-contact tool to obtain full-field deformation measurement of materials and structures subjected to various loadings [10-14]. By comparing the digital images of specimen surface/volume in the un-deformed and deformed states, 2D/3D full-field deformation information can be collected based on a correlation criterion [12]. Commonly used criteria include cross-correlation (CC) criterions and sum-squared difference (SSD) correlation criteria.

Given the specimen deformation caused by cellular tractions, and the mechanical properties of the matrix, cell-generated traction history could be obtained through a finite cell-matrix interaction model. By correlating the cell traction history with the cell differentiation result, we may be able to understand better on how the cell-generated traction relate to stem cell differentiation condition.

6.2 Materials and methods
6.2.1. Synthesis of polymers

The APLA macromer was synthesized through a two-step method and the copolymer was synthesized via free radical polymerization using the same method as previously described in Chapter 4.

The synthesized hydrogel is thermo-sensitive (SGTT of ~26 °C), biodegradable (its degradation product has a SGTT above body temperature, so that it can be dissolved in the body fluid and further removed by circulation system), flexible (with its fracture strain over 300%) and soft. Here in this chapter, the matrix elasticity of the hydrogels was modified by using different gel concentrations in DPBS (5%, 10% and 20% w/v).

6.2.2. Hydrogel preparation and mechanical characterization

To prepare a hydrogel solution, the above synthesized copolymer was dissolved in Dulbecco's modified phosphate buffer saline (DPBS) at 4°C to form a 5%, 10% or 20% (w/v) solution. The solution was then incubated at 37°C for 20-30 minutes to form a solid hydrogel.

The tensile Young’s moduli of the hydrogels were collected through a uniaxial tensile test by a Instron tensile tester. An elongation speed of 50 mm/min was used. The elastic moduli were determined based on the linear part of tensile stress-strain curves (~ 30%). In order to determine the time dependent stress-strain state of the hydrogel under an arbitrary loading process, the deformation history must be considered. The viscoelastic properties of the hydrogels were assessed by a creep & relaxation test as demonstrated in Scheme 6.1. This test was conducted using a custom-designed rig shown in Scheme 6.2.
A constant tensile engineering stress \( \sigma = \frac{F}{A_0} = \frac{F}{\omega_0 T_0} \), around 3.3kPa, was applied first for 1-3 minutes, and then the stress was quickly removed, allowing samples to relax for another 1-3 minutes. The strain creep & relaxation curves were collected by a remotely controlled camera system (Nikon D3000 with the AF-S 18-55mm lens and a PROMASTER 52mm close-up+4 micro lens), and this data was used to derive Prony series parameters (as inputs for finite element analysis). All the mechanical tests in this chapter were conducted in a water environment at 37°C. Samples were prepared freshly before tests by adding gel solutions (5%, 10% or 20%) into a custom-designed mold (dimensions as demonstrated in Scheme 6.3, 1.2 mL of gel solution was used for each sample) and set for gelation at 37°C for 20-30 min. Dimensions of samples were measured and collected before test.

Scheme 6.1. Creep & relaxation test to assess the viscoelastic properties of the hydrogel.
6.2.3. MSC encapsulation, proliferation and myogenic differentiation in hydrogels

A 5%, 10% or 20% (w/v % in DPBS) hydrogel solution was used to encapsulate MSCs. Rat bone marrow-derived mesenchymal stem cells (MSCs) were cultured in 10% fetal
bovine serum (FBS) supplementing alpha modified minimum essential medium (αMEM) media with 1% antibiotics in a normal culture condition. Cells at passages 11-14, which remained multipotency [15], were used. Before encapsulation, MSCs were trypsinized first from the tissue culture plates (TCP) and re-suspended in culture media. Hydrogel solution was sterilized under UV light in a laminar flow hood for 30min first. Cell suspension was then added into the gel solution, and the final cell concentration was controlled as 10 million/mL. 0.2 mL cell/hydrogel mixture was dispensed to each Eppendorf tube and cultured under the normal culture condition (10% FBS, 21% O₂, 5% CO₂, 37°C). At 1 and 7 days in vitro culture, pellets were randomly selected for dsDNA and real time RT-PCR testing.

The survival and proliferation of MSCs in hydrogel was assessed by double-strand DNA (dsDNA, for live cells) content using the same method as described in Chapter 3. As for the myogenic differentiation of the seeded MSCs in hydrogels, real time RT-PCR was conducted for cell gene expression. After 7 days culture, cell encapsulated gels were immersed in TRIzol (Sigma) and processed by the manufacture's protocol to isolate and purify RNA. Isolated total RNA was quantified by Nanodrop (Thermo) and approximately 1 µg of RNA was used. cDNA was reverse transcribed from RNA by using cDNA high capacity transcription kit (ABI). A real-time RT-PCR process was conducted by using SYBR Green/fluorescein (Fermentas) using the same method as described in Chapter 4. Primers were customized designed and listed in Table 6.1.
Table 6.1. Primers used for real time RT-PCR to detect the myogenic differentiation of MSCs in APLA hydrogel with different concentrations.

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
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<tbody>
<tr>
<td>β-actin</td>
<td>AAGATCAAGATCATTGCTCCTC</td>
<td>GGACTCATCGTACTCCTG</td>
</tr>
<tr>
<td>MEF2</td>
<td>TGCTGCTCTCAGTCACTAC</td>
<td>TTCAGGACTTGGGGACACTG</td>
</tr>
<tr>
<td>Myogenin</td>
<td>TGAGAGAGAAGGGAGGGAAC</td>
<td>ACAATACACAAAGCCTGGAA</td>
</tr>
<tr>
<td>MHC</td>
<td>TGACTTCTGGGAAAATGCAG</td>
<td>CCAAGCGAGAGGAGTTGTC</td>
</tr>
<tr>
<td>Desmin</td>
<td>ATACCGACACCAGATCCAGTC</td>
<td>TCCCCTCATCTGCTCATCAGG</td>
</tr>
<tr>
<td>MyoD1</td>
<td>AGAGGGAAGGGAAGGAGGAGAGAAG</td>
<td>GCAGCAGCAAAACACACCAG</td>
</tr>
</tbody>
</table>

6.2.4. Speckle pattern production

Random speckle patterns were produced on the cell-encapsulated hydrogel samples by

1). Random seeding of silicon carbide micro powders through filter or air flush method (Scheme 6.4);

2). Random seeding of charcoal micro powders through filter method (Scheme 6.4.a);

3). Spray coating with paint (Tamiya Polycarbonate PS-5 Black Spray Paint 86005).

The powders information was listed in Table 6.2, and custom-designed facilities as shown in Scheme 6.4 were used to apply the powders onto the sample surface. In brief, for filter method, powders were applied through a filter (with pore size of 30 μm) to sample surface. In air flush method, powders were first set in a container, air was then flushed into the container continuously and some of the powders were activated by the
flow and applied onto the sample surface through a tube connecting the container with the outside (to sample surface). For the spray approach, after shaking thoroughly, the paint was sprayed to the sample surface for around 3s at a distance of 40-50 cm, and images were taken after thorough drying (10s at 37°C).

Table 6.2. Powders used for speckle pattern production.

<table>
<thead>
<tr>
<th>Powder Type</th>
<th>Diameter (μm)</th>
<th>Density (g/cm³)</th>
<th>Pros</th>
<th>Cons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silicon Carbide</td>
<td>$16 \pm 7$</td>
<td>3.21</td>
<td>- High density to avoid float of powders</td>
<td>- Comparatively low contrast and potential reflection problem</td>
</tr>
<tr>
<td>Charcoal</td>
<td>$10 \pm 5$</td>
<td>0.4</td>
<td>- High contrast without reflection issue</td>
<td>- Low density, easier to float</td>
</tr>
<tr>
<td>Spray</td>
<td>-</td>
<td>-</td>
<td>- High contrast</td>
<td>- Adhesion problem</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Can adhere to gel surface</td>
<td>- Unknown component, potential toxicity to cells</td>
</tr>
</tbody>
</table>
6.2.5. Toxicity of produced speckle patterns

To investigate the potential toxicity of the produced speckle patterns, 4 different concentrations of powders/spray paint were tested by MTT assay according to the standard protocol [16]. In brief, MSCs were trypsinized first from the TCP, re-suspended in culture media, and seeded into wells of a 96-well plate at the concentration of $2.5\times10^4$ cells per well. After 24 hours culture to allow cells complete attachment, cell culture medium was replaced by fresh media (200μL) supplied with 4 different concentrations of powders/spray paint (0.001g, 0.005g, 0.01g and 0.02g per well). Powder/spray-paint only (without cells) was used as negative control, and MSCs-only was used as positive control.

6.2.6. Displacement/strain measurement accuracy effected by patterning and DIC parameters

After the production of speckle patterns according to the methods described above,
images were taken under optical microscopy (OLYMPUS) and analyzed by Correlated Solutions VIC-2D digital image correlation software. To assess the displacement/strain measurement accuracy of different patterning and DIC parameters, two sets of experiments were conducted. Firstly, using the same DIC parameters, patterning effect on displacement/strain measurement accuracy can be detected by comparing the images based on speckle patterns produced by different methods and different transformation methods (digital and experimental transformation, uniform and non-uniform transformation). Secondly, DIC analysis parameters were optimized for the chosen speckle pattern and image acquisition settings, where the DIC parameters were systematically varied from 31 pixels to 151 pixels for subset size, and 1 pixel to 20 pixels for step size. This analysis was conducted on a sample under zero loading condition to avoid any other potential error resulted from the system.

6.2.7. Images processing using digital image correlation (DIC)

After 7 days in vitro culture under normal culture condition (21% O₂, 5% CO₂, 37°C), the cell-hydrogel samples were sprayed on the surface to produce random speckle patterns as described above in section 6.2.6. Samples were punched (6mm in diameter) and then set in the wells of 6-well plates in a water bath (kept at 37°C) under an optical microscope for imaging. Images were taken before and 60 min after trypsin-EDTA treatment (5mL of trypsin-EDTA were added into each well, and the whole sample was immersed in trypsin buffer during the whole imaging process). Here trypsin-EDTA was used to release the cell-generated traction force to the surrounding matrix. Correlated Solutions VIC-2D digital image correlation software was used to analyze the local deformation fields caused
by cellular traction force by comparing images before and after trypsin-EDTA treatment. Optimal DIC parameters (subset size of 61 and step size of 3, determined as described in section 6.2.8) were used for this deformation analysis. Gel-only samples were used as a control to eliminate the trypsinization effect on hydrogels.

While dealing with the deformation information of the cell-hydrogel/hydrogel-only samples, we assumed:

1. Cells were homogenously distributed within the whole sample;
2. The hydrogel was homogenous and isotropic;
3. There was no translation or rotation of the samples during the test process.

Data was analyzed using Matlab and presented as the principle displacement/strain at the radial direction versus radial distance under the cylinder polar coordinate system. The original point of the system was set as the point where minimum magnitude of displacement happened.

6.2.8. Prony series parameter collection

Prony series model is as demonstrated in Scheme 6.5. Its general form of constitutive relation for solids is given by:
\[ \sigma + \sum_{n=1}^{N} \left( \sum_{i_1=1}^{N-n+1} \ldots \left( \sum_{i_a=i_{a-1}+1}^{N-(n-a)+1} \ldots \left( \sum_{i_n=i_{n-1}+1}^{N} \left( \prod_{j \in \{i_1, \ldots, i_n\}} \tau_j \right) \right) \right) \ldots \right) \frac{\partial^n \sigma}{\partial t^n} \]

\[ = E_0 \varepsilon \]

\[ + \sum_{n=1}^{N} \left( \sum_{i_1=1}^{N-n+1} \ldots \left( \sum_{i_a=i_{a-1}+1}^{N-(n-a)+1} \ldots \left( \sum_{i_n=i_{n-1}+1}^{N} \left( E_0 \right) \right) \right) \right) \ldots \frac{\partial^n \varepsilon}{\partial t^n} \]

\[ + \sum_{j \in \{i_1, \ldots, i_n\}} E_j \left( \prod_{\kappa \in \{i_1, \ldots, i_n\}} \tau_j \right) \ldots \frac{\partial^n \varepsilon}{\partial t^n} \]

(6.2)

In which \( \sigma \) is Cauchy stress, \( \varepsilon \) is strain, \( t \) is time, \( E_i \) \((i = 1, \ldots, n)\) is the spring moduli for each Prony term, and \( \tau_i \) \((i = 1, \ldots, n)\) is the constant relaxation time for each Prony term. \( n \) is the Prony term number.

In which \( \tau_i = \frac{\eta_i}{E_i} \) \((i = 1, 2, \ldots n)\)  

(6.3)
Thus, for a two-term Prony series model, we have:

\[
\sigma + (\tau_1 + \tau_2)\dot{\sigma} + (\tau_1 \tau_2)\dot{\sigma} = E_0\varepsilon + ((E_0 + E_1)\tau_1 + (E_0 + E_2)\tau_2)\dot{\varepsilon} + ((E_0 + E_1 + E_2)\tau_1 \tau_2)\ddot{\varepsilon}
\]

(6.4)

For this two-term model, the Prony series parameters needed for Abaqus input are \(g_i (i = 1, 2)\), the instantaneous moduli \(E_{ins}\) and \(\tau_i (i = 1, 2)\), in which

\[
g_i = \frac{E_i}{E_0 + E_1 + E_2} (i = 1, 2)
\]

(6.5)

\[
E_{ins} = E_0 + E_1 + E_2
\]

(6.6)

All the parameters were obtained based on the strain vs. time data collected by the creep
& relaxation test through non-linear regression fitting of data into equation (6.4) using Matlab. And the values were further adjusted and validated by Abaqus before used in cell-matrix interaction model.

6.2.9. Finite element modeling

Three dimensional finite element models of the cell-encapsulated hydrogels were developed using Abaqus. In order to simplify the model, we have made assumptions as followed:

1. Cells were homogenously distributed inside the hydrogels;
2. The hydrogel is a homogenous, isotropic, linear viscoelastic, incompressible material described by a Prony series;
3. Cells within the gel have the exact same surrounding environment, thus they generate the same magnitude of traction force to the matrix (hydrogel);
4. Cell-generated tractions were evenly distributed on the cell-matrix interface.

Based on the assumptions above, cells were represented as spherical holes with the diameter of 10 μm. The gel was modeled as a 3D block with $3 \times 3 \times 3$ units. For each unit, its dimensions were set according to the cell-cell distance collected from real sample geometry and cell numbers (from dsDNA test result). The gel body was meshed with 10-node quadratic tetrahedron 3D solid elements.

The gel was assumed to be incompressible ($\nu = 0.495$) and linear viscoelastic with Prony series parameters collected from creep & relaxation tests as described above. A fixed, zero displacement boundary condition in the sample height direction (Z direction)
was specified on the nodes of the bottom surface. Two nodes along X and Y edge of bottom surface were fixed in the direction of Y and X respectively to allow model deformation along the XY plane, meanwhile avoiding its rotation and translation. Other surfaces of the model were set as free surfaces (Scheme 6.6). Cell traction was simulated by applying a constant pressure at the cell-gel interface, directed toward the center of the cells (represented as negative pressure in the holes in the FE model). Pressures were loaded on gel for 1 h before unloading for another 1 h. The loading experienced a linear decay during the first 200s within the whole unloading history. Different magnitude of pressures were tested and validated by comparing the displacement (displacement difference before and 60 min after unloading) from simulation with experimental results (from DIC approach as described in section 6.2.9). Finally, cell-generated tractions were correlated with cell differentiation result to better understand their interaction (Scheme 6.7).
Scheme 6.6. Finite element model to calculate the cell-generated traction in hydrogel.

Scheme 6.7. Work flow to get the correlation between cellular traction and stem cell differentiation.
6.2.10. Statistical analysis

Data were reported as mean ± standard deviation. Multivariate repeated-measures ANOVA were performed to test the interactions between different test groups. A statistical significance was considered when p < 0.05.

6.3 Results

6.3.1. Hydrogel mechanical properties

The synthesized APLA hydrogel is soft and flexible with fracture strain above 300% (no fracture happens before reaching the dimensional limitation of the tester) in a 37°C aqueous environment. According to the uniaxial tensile test results (Table 6.3), the elastic modulus of the hydrogel increased from 7 kPa to 35 kPa as the hydrogel concentration increased from 5% to 20%. In addition, the strength of the gel was also upregulated from 5 kPa to 13 kPa as the increase of the gel concentration. The representative stress-strain curves in Figure 6.1 demonstrated consistent trend of matrix modulus as data showed in Table 6.3.

<table>
<thead>
<tr>
<th>Hydrogel Concentration</th>
<th>Young’s Modulus (kPa)</th>
<th>Strength (kPa)</th>
<th>Fracture Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>5%</td>
<td>7.4 ± 0.6</td>
<td>5.5 ± 0.9</td>
<td>&gt; 300%</td>
</tr>
<tr>
<td>10%</td>
<td>17.1 ± 3.4</td>
<td>12.3 ± 0.7</td>
<td>&gt; 300%</td>
</tr>
<tr>
<td>20%</td>
<td>34.2 ± 6.8</td>
<td>13.3 ± 1.7</td>
<td>&gt; 300%</td>
</tr>
</tbody>
</table>
Figure 6.1. Representative stress-strain curves of hydrogels in a uniaxial tensile test (up to 70% of strain).

As expected, the hydrogels demonstrated viscoelastic properties as shown by the creep & relaxation curves in Figure 6.2. Upon loading of a constant weight/force, a small amount of instantaneous deformation was observed, followed by dramatically increased material deformation. This increase of material deformation rate was due to the increase of true stress on the specimen when their cross-section areas decreased as elongated. When unloaded, elastic deformation was recovered instantaneously, and a certain amount of permanent deformation was observed.
6.3.2. Prony series parameters

The Prony series parameters were determined by fitting the creep & relaxation test data into equation (6.4) through a non-linear regression method using Matlab. The collected parameters were listed in Table 6.4. As indicated, hydrogels prepared with different concentrations demonstrated different instantaneous moduli, and its trend was consistent with the gel tensile Young’s moduli as expected. The parameters were further validated in Abaqus by a model mimicking the experimental procedure. As Figure 6.3 demonstrated, the collected Prony series parameters can reproduce the experimental results well, thus they were proper to be used in the cell-matrix interaction models for cellular tractions calculation.
Table 6.4. Prony series parameters collected from creep & relaxation results.

<table>
<thead>
<tr>
<th>Hydrogel</th>
<th>$E_{\text{ins}}$ (kPa)</th>
<th>$g_1$</th>
<th>$g_2$</th>
<th>$\tau_1$ (s)</th>
<th>$\tau_2$ (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>APLA-5%</td>
<td>51.9975</td>
<td>0.9199</td>
<td>0.08</td>
<td>10</td>
<td>103.48</td>
</tr>
<tr>
<td>APLA-10%</td>
<td>72.0108</td>
<td>0.9699</td>
<td>0.03</td>
<td>4.03</td>
<td>103.48</td>
</tr>
<tr>
<td>APLA-20%</td>
<td>151.7512</td>
<td>0.9699</td>
<td>0.03</td>
<td>3.63</td>
<td>103.48</td>
</tr>
</tbody>
</table>

Figure 6.3. Experimental and Abaqus reproduced data for APLA-5%, APLA-10% and APLA-20%.
6.3.3. MSC survival and proliferation in hydrogels

MSCs were encapsulated in APLA hydrogel with different concentrations. They had the same chemical structure and composition, but largely different elastic modulus ranging from 7kPa to 35kPa, thus can be used to study the effect of matrix modulus on cell-generated tractions and cellular activities including cell survival, proliferation and differentiation. As illustrated in Figure 6.4, MSCs survived and proliferated in all three conditions (APLA-5%, APLA-10% and APLA-20%) during the 1-week culture period. The most significant cell growth was observed in the hydrogel with the largest elastic modulus (35 kPa).

![Figure 6.4](image)

Figure 6.4. dsDNA concentration of cell-encapsulated hydrogels after 7 days in vitro culture under normal culture conditions (*p<0.05).
6.3.4. MSC myogenic differentiation in hydrogels

The myogenic differentiation of seeded MSCs in APLA gel was evaluated at the gene level by real-time RT-PCR. Myogenic markers MHC, Desmin, Myogenin, MyoD1, MEF2 and β-actin as endogenous control were used. MSCs cultured on TCPs were used to calculate the fold increase. As the result in Figure 6.5 showed, when encapsulated in APLA-10% with Young’s modulus of 17kPa, MSCs showed significantly higher gene expressions up to more than 115, 13140, 89650, 4 times for Desmin, Myogenin, MyoD1 and MHC respectively, compared with those seeded in APLA-5% (elastic modulus of 7 kPa) or APLA-20% (elastic modulus of 35 kPa). These results demonstrated that by controlling the matrix elasticity, myogenic differentiation was successfully induced in MSCs.
Figure 6.5. Myogenic gene expressions of MSCs encapsulated in gel after 7 days in vitro culture under normal culture conditions (*p<0.05, **p<0.01).
Speckle patterns were successfully produced on hydrogels using air-flush, filter method of silicon carbide and charcoal powders, as well as spray method as described above. Previous works have pointed out that above a pattern density of 25%, there was no significant reduction of signal noise [17]. As shown in Figure 6.6, all the approaches were capable of producing random shaped speckle patterns with the density above this.
threshold, ranging from 33% to 46%. Compared with the charcoal powder and spray method, the silicon carbide produced patterns have reflection and comparatively lower contrast. The spray-produced patterns had the advantage of being capable of adhering on the gel surface, thus samples can be immersed into the water during the trypsinization and imaging process. While for other patterns, great cautions had to been taken during the whole operation process to keep the sample surface above the buffer to avoid powder flow.

Figure 6.6. Speckle patterns produced by various powders and methods.

The accuracy of strain/displacement measurement based on these produced speckle patterns were checked by VIC-2D DIC software based on digital translation images using the same DIC parameters (subset size of 61 and step size of 3). As demonstrated in Table 6.5, the differences between DIC-detected displacement and digital translation distance
were below 1.6% for all the patterns, and errors due to the spatial range of the displacement noise were smaller than 0.2%. The low intensity and spatial distribution of displacement noise indicated that the produced speckle patterns provided a proper approach for gel deformation measurement.

Table 6.5. Displacement measurement accuracy of different speckle patterns *

<table>
<thead>
<tr>
<th>Speckle Pattern Type</th>
<th>Area Fraction</th>
<th>Error % in $U_{avg}$</th>
<th>Error % due to spatial range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silicon Carbide (Filter)</td>
<td>45.4%</td>
<td>0.1%</td>
<td>0.0013%</td>
</tr>
<tr>
<td>Silicon Carbide (Air flush)</td>
<td>44.1%</td>
<td>0.885%</td>
<td>0.0056%</td>
</tr>
<tr>
<td>Charcoal (Filter)</td>
<td>35.1%</td>
<td>1.216%</td>
<td>0.191%</td>
</tr>
<tr>
<td>Spray</td>
<td>33.2%</td>
<td>1.571%</td>
<td>0.00578%</td>
</tr>
</tbody>
</table>

*: all the displacement accuracy were calculated based on digital translated images using the same DIC parameters with the subset size of 61 and step size of 3.

6.3.6. Strain/displacement measurement accuracy of VIC-2D DIC software and experimental system

The accuracy of the VIC-2D DIC software and the experimental system was tested by comparing images before and after digital transformations (translation, uniform and non-uniform transformations) and experimental transformation (translation and uniaxial extension).
According to the results listed in Table 6.6, compared with the digital method, experimental transformations generally produced larger error of displacement/strain as well as larger spatial distribution of the noise. These increased errors may result from the possible flow/movement of powders, lens distortions, reflection of the powders or the buffer, error in the operation process and system set-up (lightening, imaging, vibration and so forth). On the whole, this experimental system gave a strain/displacement measurement error below 5% at the subset size of 61 and step size of 3.

Table 6.6. Deformation field accuracy/spatial resolution of VIC-2D DIC software.

<table>
<thead>
<tr>
<th>Transformation Type</th>
<th>Area fraction</th>
<th>$\text{Error % in } U_{\text{avg}}$ or $\varepsilon_{\text{avg}}$</th>
<th>Error % due to spatial range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digital translation*</td>
<td>35.1%</td>
<td>1.216%</td>
<td>0.191%</td>
</tr>
<tr>
<td>Digital uniform transformation*</td>
<td>35.1%</td>
<td>0.988%</td>
<td>0.0304%</td>
</tr>
<tr>
<td>Digital non-uniform transformation*</td>
<td>35.1%</td>
<td>0.855%</td>
<td>1.293%</td>
</tr>
<tr>
<td>Sample translation*</td>
<td>41.7%</td>
<td>4.768%</td>
<td>1.318%</td>
</tr>
<tr>
<td>Sample uniform extension *</td>
<td>33.2%</td>
<td>1.53%</td>
<td>3.58%</td>
</tr>
</tbody>
</table>

*: The analysis was conducted using a subset size of 61 and step size of 3 based on speckle patterns produced by charcoal powders.

6.3.7. Toxicity of produced speckle patterns

The toxicity of produced speckle patterns by charcoal powder and spray paint were tested
by MTT assay. Four different concentrations (0.001g, 0.005g, 0.01g and 0.02g per well) of powders/spray paint were used. As demonstrated in Figure 6.7, there was no significant difference of cell viability when cultured in medium with spray paint at all the tested concentrations compared with those cultured in normal culture medium, indicating that the speckle pattern produced by spray method had no significant toxicity to MSCs. However, the fluorescence intensity was significantly decreased for wells with MSCs cultured in medium supplied with charcoal powder. This decrease could due to the reducibility of charcoal powder to the fluorescence, or the potential toxicity of charcoal powder to MSCs. Thus spray method was chosen and used for all the following tests in cell-gel interaction samples to minimum the potential speckle pattern toxicity on the cell functions or traction generation capability.

Figure 6.7. Toxicity of produced speckle patterns by charcoal powder (filter) and spray methods at different concentrations (0.001, 0.005, 0.01 and 0.02 g/well).
6.3.8. Strain/displacement measurement accuracy affected by DIC parameters

In order to detect the VIC-2D DIC parameters effect on the strain/displacement measurement accuracy as well as choose optimal DIC parameters for the cell traction produced gel deformation measurement, different subset sizes and step sizes were used for displacement analysis of an image with speckle pattern produced by spray method. The image for this analysis had the speckle pattern density of 32%.

According to the results in Table 6.7, a smaller subset size and step size contributed to a better displacement field spatial resolution, but led to a larger noise intensity. Also the smallest subset size for analysis is limited by the speckle pattern spacing. If the subset size is similar to the distance between speckles, there would be an increased number of regions that could not be analyzed by DIC algorithm since they do not exhibit unique speckle patterns, as in the case of subset size 31 and step size 3. Careful comparison of data indicated that increasing subset size to reduce displacement noise intensity produces less loss of spatial resolution compared with increase of step size. Thus the optimal DIC parameters for strain/displacement field measurement should be a moderate subset size with a small step size. As a result, the optimal parameters were chosen as 61 pixels for subset size and 3 pixels for step size to minimize the noise intensity while maximum the deformation resolution.
Table 6.7. Deformation measurement accuracy and noise intensity as a function of selected VIC-2D parameters.

<table>
<thead>
<tr>
<th>Subset Size (pixels)</th>
<th>Step Size (pixels)</th>
<th>( U_{\text{mea}} \times 10^{-6}\text{mm} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>61</td>
<td>1</td>
<td>5.44405 ± 9.19535</td>
</tr>
<tr>
<td>61</td>
<td>3</td>
<td>5.34702 ± 9.20736</td>
</tr>
<tr>
<td>61</td>
<td>10</td>
<td>5.36071 ± 9.02143</td>
</tr>
<tr>
<td>61</td>
<td>20</td>
<td>4.72793 ± 9.61239</td>
</tr>
<tr>
<td>31</td>
<td>3</td>
<td>15.3774 ± 24.9033</td>
</tr>
<tr>
<td>91</td>
<td>3</td>
<td>9.03987 ± 9.98145</td>
</tr>
<tr>
<td>121</td>
<td>3</td>
<td>7.24191 ± 9.13471</td>
</tr>
<tr>
<td>151</td>
<td>3</td>
<td>6.01552 ± 8.49834</td>
</tr>
</tbody>
</table>

\( U_{\text{mea}} \): measured deformation/displacement by VIC-2D DIC software;

The same image (speckle pattern produced by spray paint with speckle density of 32%) was used for this analysis.
Figure 6.8. Spatial resolution and intensity of displacement noise decreased as the increase of subset size or step size. The subset size and step size were indicated at the top left of each displacement map.

6.3.9. Displacement caused by cellular traction force in hydrogels

Displacement of gels caused by cell-generated tractions was detected by DIC algorithm using VIC-2D software by comparing images before and after Trypsin-EDTA treatment. The trypsin-EDTA effect on gel deformation was detected by gel-only samples first and then these values were used to adjust the displacement caused by cell traction.

As demonstrated in Figure 6.9, obvious shrinkages (negative $\epsilon_{xx}$ and $\epsilon_{yy}$) of gels were observed after 60 min of trypsin treatment. The shrinkages were homogenous, indicating the homogeneity of gel properties. Among the tested gels (APLA-5%, 10% and 20%) with different matrix moduli, the soft gel APLA-5% had the most significant shrinkage.
(average strain= -0.00946) compared to the stiffer gels. In addition, the shrinkage (strain in the radial direction in a polar coordinate system) of gel was decreased almost linearly as the gel concentration or matrix elasticity increased. This may due to the potential trypsin-EDTA effect on dragging the polymer long chains closer. With lower gel concentration, APLA-5% was very likely to have larger spacing between polymer chains. Thus there was more spacing for getting polymer chains closer, which may cause larger shrinkage.

Figure 6.9. Gel deformation caused by trypsin treatment.
Despite the large shrinkage in APLA-5% gel, the cellular traction force generated deformation of gel was most significant in APLA-10% gel, in which most significant myogenic differentiation of MSCs was also detected. The gel shrinkages caused by trypsinization was simplified as linear, and used to adjust the gel deformation produced by cell tractions. As Figure 6.10 showed, 60 min after trypsin treatment, gels expanded due to the release of tractions and APLA-10% demonstrated a larger average strain of around 3.8%, while smaller strains were found for softer gels (1.1% and 0.85% for APLA-5% and APLA-20% gels, respectively). Interestingly, the APLA-10% also showed a much more uniform deformation (constant strain over the whole tested surface) compared with the other gels. Both the larger and uniform deformation of APLA-10% gel caused by the cell-generated traction force may help explain the better myogenic differentiation of MSCs within it.

Continued

Figure 6.10. Deformation of cell seeded hydrogel samples (after 7 days in vitro culture) 60 min after releasing the cellular tractions by trypsin treatment.
Figure 6.10 continued
6.3.10. Finite element analysis of cellular traction in hydrogels with different matrix modulus

Finite element model based on real time sample geometries and cell densities (Table 6.8) were built up using ABAQUS. Here in this model, cell-matrix interactions were simplified by applying a negative pressure within the spherical holes (represent cells) on the interface.

Table 6.8. Dimensional parameters of cell-seeded hydrogels.

<table>
<thead>
<tr>
<th>Hydrogel</th>
<th>Thickness* (μm)</th>
<th>Surface area* (μm²)</th>
<th>Cell density# (million/mL)</th>
<th>Cell-cell spacing (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>APLA-5%</td>
<td>940</td>
<td>8154400</td>
<td>180</td>
<td>18</td>
</tr>
<tr>
<td>APLA-10%</td>
<td>1717</td>
<td>11137251</td>
<td>78.4</td>
<td>23</td>
</tr>
<tr>
<td>APLA-20%</td>
<td>2627</td>
<td>7089358</td>
<td>65.8</td>
<td>25</td>
</tr>
</tbody>
</table>

*: Sample thickness and surface area were collected after 7 days in vitro culture by imaging using optical microscopy and ImageJ;

#: Cell density was calculated based on dsDNA result of samples after 7 days in vitro culture, and volume of hydrogel after gelation at a cell encapsulation efficacy of 94% [18].

As the simulation results demonstrated in Figure 6.11, a higher cellular traction produced a larger gel deformation for all the tested gels (APLA-5%, APLA-10% and APLA-20%) as expected. Within the tested traction range, APLA-5% gel demonstrated larger deformation when applied with the same magnitude of traction due to its lower matrix elasticity (APLA-20% had the smallest deformation). By comparing these simulation
results with the gel deformation collected from DIC, we found that the average cell-generated traction in APLA-10%, in which MSCs exhibited the most significant myogenic differentiation, was larger (above 5 kPa) than that in other gels (3 kPa for APLA-5% and 4.5 kPa for APLA-20%). This may indicate that cells have a limited capacity to achieve a unique range of tractions given a certain differentiation-inducing environment.

Figure 6.11. Average strain vs applied cellular traction collected from finite element modeling using ABAQUS.

6.4 Discussions

Recent studies showed that cell-generated tractions had potential effect on physiological and pathological processes, both at the cell and tissue levels. Biologists have found that these cellular tractions may influence many cell and tissue activities through the
clustering of proteins and activation of certain signaling cascades as well as nuclei deformation/movement through actin-myosin contracting. Thus it’s of great importance to understand how these cell-generated tractions play a role in cell biology, which may contribute to the development of biomaterials, tissue engineering, and even disease treatments. In this chapter, we introduced a novel technique capable of quantitatively calculating cell tractions within a 3D matrix by combing digital image correlation with finite element modeling.

6.4.1. Application of digital image correlation in biomaterials

Digital image correlation method is widely used to investigate the full-field displacement/strain of materials with various loading conditions, especially for stiff materials such as metals and ceramics based on the tracking of unique details on the specimen surface. Its application on biological soft tissues or material is usually limited on the difficulty of creating a suitable speckle pattern on them. The speckle patterns should not require sample dehydration, not cause change of the specimen’s chemical or mechanical properties as well as have no toxicity to the cells. In earlier works, a natural texture (brain tissues, for examples) or nuclei staining were usually used. Here we demonstrated three potential methods (air flush, filter of silicon carbide or charcoal powders, and spray method) to produce speckle patterns on biological soft materials such as hydrogels and checked the strain/displacement measurement accuracy of the produced speckle patterns by VIC-2D DIC software. As indicated in Figure 6.2 and Table 6.5, speckle pattern with area fraction above the minimum fraction (25%) was successfully produced and all the patterns produced a comparatively low intensity of noise in the
displacement field as well as small spatial distribution of the displacement data. Compared with other speckle pattern production approaches such as nuclei staining, these methods have the advantages of high contrast, controllable speckle spacing, tunable area fraction, random speckle pattern shape, and low influence on the mechanical properties of materials, thus served as promising strategies for DIC method application in biology.

In addition, the patterning and DIC parameters effect on the strain/displacement measurement accuracy was also checked. As summarized in Table 6.7, increase of the subset size or step size contributed to a decrease in the intensity of displacement field noise and an increase of spatial distribution of the noise. These results are consistent with works from other groups [17]. This could be explained by the founding of Walley et al. that a non-random spatial distribution of elliptical spots, which have their long axis perpendicular to the displacement/strain measurement, was observed during the VIC-2D calculation [19]. Increase of the subset size or step size could result to an increase of the size of these spots as well as a decrease of their periodicity. As a result, data was smoothed and higher data resolution was obtained.

6.4.2. Cellular traction correlation with stem cell differentiation

The interaction between cell generated tractions and various biochemical processes has been thought to play important roles on regulating cell fates such as cell migration, proliferation and differentiation [1, 2, 4-6, 8, 20]. Traction force measurement can provide quantitative information about this cell-matrix interaction. In this chapter, we introduced a novel method using FEM with DIC to calculate the average cellular
tractions. Our results indicated that cells generated tractions of around 3-6 kPa when seeded in hydrogels with matrix moduli ranging from 7 to 35 kPa. Compared with the values collected from other works (Table 6.9), our calculated values were slightly higher. This could result from various factors including cell phenotype, the mechanical, chemical or morphological characteristics of materials as well as different cellular processes.

Table 6.9. Summary of some works about cell-generated tractions and cell fates.

<table>
<thead>
<tr>
<th>Cellular Traction or Matrix displacement</th>
<th>Matrix</th>
<th>Cell Type</th>
<th>Method</th>
<th>Other important conclusions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-3kPa</td>
<td>3D PEG hydrogel (E ~ 1kPa)</td>
<td>Fibroblasts</td>
<td>3D Traction Force Microscopy</td>
<td></td>
<td>[20]</td>
</tr>
<tr>
<td>0-1 μm</td>
<td>3D covalently crosslinked HA hydrogel (E ~ 4kPa)</td>
<td>hMSCs</td>
<td>3D Traction Force Microscopy</td>
<td>hMSCs differentiation is directed by the generation of degradation-mediated cellular traction.</td>
<td>[21]</td>
</tr>
<tr>
<td>0-3 μm</td>
<td>3D fibrin gel (E ~ 100Pa)</td>
<td>3T3 fibroblasts</td>
<td>3D Traction Force Microscopy</td>
<td>Cell traction forces direct the orientation of cell division axis.</td>
<td>[2]</td>
</tr>
<tr>
<td>0-1.5kPa</td>
<td>2D PAM gel (E ~ 40kPa)</td>
<td>SMCs</td>
<td>Particle Image Velocimetry and FEM</td>
<td></td>
<td>[22]</td>
</tr>
</tbody>
</table>
In addition, we found that cellular tractions were correlated with cell differentiation condition, in which cells in gels that induced higher cell differentiation exhibited larger tractions to their surrounding matrix. This trend may be explained by cell traction’s influence on stimulating integrin clustering, activating integrin-associated signal cascades or on nuclei deformation/movement. When considering purified FA components talin and vinculin, for example, del Rio et al. found that forced extension of talin contributed to the exposure of cryptic sequences, which then helped binding of vinculin. Processes like this can thereby regulate FA growth and later cytoskeleton remodeling to affect cellular activities [23]. However, due to the complexity within these mechano-sensitive responses, it’s yet unclear if successful differentiation requires a unique range of cellular tractions.

6.5 Conclusions

In this chapter, a novel approach combining DIC algorithm and FEM were introduced to calculate cell-generated tractions to the surrounding matrix. Random speckle patterns on hydrogels with area fractions between 30-40% and high contrast were produced by the seeding of carbide silicon or charcoal powders or the spray method. And these patterns produced low intensity and low spatial distribution of noise in displacement data, indicating their potential application in DIC method for biological soft materials. Gel deformation was observed after releasing the cellular traction by trypsinization. Among all the tested gels, APLA-10% hydrogel with matrix tensile modulus of around 17kPa demonstrated the most significant deformation compared with the other gels. In addition,
MSCs myogenic differentiation was also found to be higher in APLA-10% gel according to the real-time RT-PCR test result. According to the FE simulation result, it seems that MSCs with higher myogenic differentiation exhibited higher average traction to their surround gel. As far as we know, this is the first study to correlate the average cellular tractions with stem cell differentiation.
6.6 References


Chapter 7: Conclusions and Future Directions

Stem cell based therapy is a promising approach for cardiac and skeletal muscle regeneration for the treatment of diseases such as myocardial infarction (MI) or critical limb ischemia (CLI). Successful treatment requires a high survival and differentiation rate of transplanted cells into desired cell types (cardiomyocytes for cardiac regeneration, skeletal muscle cells for myogenic regeneration and endothelial cells for fast vascularization of ischemic tissues). In order to reach these treatment goals, biomaterial-based scaffolds were used to serve as: 1). a protection for the transplanted cells from the harsh (low oxygen and low nutrient) local environment; 2). a support for the cells to adhere, grow, and differentiate; 3). a delivery vehicle for biomolecules, DNAs or RNAs for the improvement of tissue regeneration and 4) a proper microenvironment to better control the cellular processes including stem cell differentiation. In Chapter 2, the mechanical and morphological properties of the 3D electrospun scaffolds based on hydrogel and polyurethane were controlled to induce the cardiac differentiation of CDCs. Results indicated that a smaller macro modulus and a comparatively lower fiber alignment were preferred. In order to understand how the matrix modulus along affect stem cell differentiation, hydrogels with different oligoester side groups and lengths were synthesized to decouple the matrix modulus from other properties such as the chemical structure, composition and water content (Chapter 3). A matrix tensile modulus of
around 20kPa, which matches the modulus of native skeletal muscles, was found to better induce the myogenic differentiation of MSCs.

In the first part of this work, cells were cultured under normal culture condition in vitro for cardiac/myogenic differentiation. However, after the happen of MI or CLI, the transplanted cells are suffered from a harsh low oxygen and low nutrient environment instead of a normal healthy one. Thus it’s critical to assess this cell delivery system under an ischemic environment as well. The second part of this work mainly focuses on its application in vitro under ischemic conditions and in vivo using a mice hindlimb ischemia model. To overcome the low nutrient issue, angiogenesis growth factor bFGF was loaded into the hydrogel for the fast formation of blood vessels in ischemic limbs (Chapter 4). To conquer the low oxygen condition, oxygen release microspheres were fabricated via electrospray approach and delivered with the cells to improve their survival and differentiation (Chapter 5). Results indicated that by delivering MSCs in hydrogel with bFGF or ORM, significant up-regulation of MSC survival and myogenic differentiation was observed both in vitro and in vivo under ischemic conditions. In addition, the new skeletal muscle regeneration, blood flow recovery as well as the mechanical and functional properties in the ischemic limbs were dramatically improved 4 weeks after injury/treatment with the delivery of MSCs and ORM (or bFGF) in hydrogel.

In order to better understand how the matrix modulus affect stem cell differentiation. A novel approach using DIC and FEM to calculate the average cell-generated tractions to the surrounding matrix was introduced in Chapter 6. By correlating stem cell
differentiation condition (from real time RT-PCR) with the average cellular tractions, we found that those MSCs with higher myogenic expressions exerted higher tractions to their surrounding matrix. Despite of the significant progress achieved in this dissertation, there are still some issues remain answered. In the hydrogel system, the optimal matrix tensile modulus to stimulate the myogenic differentiation of MSCs was found to be around 20kPa. In the electrospun fibrous system, the optimal scaffold modulus for cardiac differentiation of CDCs was found to be around 30kPa. These values were obtained under a certain combination with other properties such as chemical structure, composition and various micro-scale morphological characteristics. As a result, in order to better understand if or how each of these factors influences cellular activities, hydrogels or scaffolds with better control over the various properties may need to be produced to decouple these factors from each other.

Secondly, although significant expressions of cardiac/myogenic markers were demonstrated both at the gene and protein levels in this work, the functional condition of these differentiated cells was still unknown. Further experiments on the electrical conductivity, calcium flux, beating property, myotube morphology of the delivered cells need to be conducted to better assess the effect of materials’ physical properties on stem cell differentiation.

Thirdly, this cell delivery system using hydrogel with native-tissue-matched matrix modulus was assessed and proved to be capable of fully regenerating damaged skeletal muscles caused by ischemia. This significant improvement was found to be possible from
the contributions of 1). improved cell survival and differentiation (protective effect of bFGF or released oxygen from ORM); 2) paracrine effect of transplanted stem cells; 3) fast angiogenesis (from bFGF and stem cell secreted growth factors). For its future application in other ischemic tissues or different injury models, how and when the delivered stem cells or biomolecules play a role in the tissue regeneration must be further explored and understood at the tissue, cellular and bio-molecular levels.

Finally, Chapter 6 demonstrated a novel approach to calculate cell-generated traction within a 3D matrix. This system can be further improved in the areas include but not limited to:

1). Cell shape has been found to have potential effect on stem cell fate. Here in our model, we simplified cells as spheres. More complex geometry based on real-time imaging results could be used in the future work;

2). When cells were encapsulated in matrices, cellular tractions were applied through focal adhesion sites at/near the cell-matrix interface. Instead of assuming a uniform distribution of focal adhesion sites, a more accurate model can be developed given adhesion sites number and distribution;

3). The APLA hydrogel used in this work has a comparatively slow degradation rate so that the degradation effect was neglected during the calculation in this work. However, many widely used biomaterials for tissue engineering were biodegradable and some of them can degrade fast especially in vivo. Along with the degradation of materials in vitro or in vivo, materials properties including matrix modulus, chemical structure and water
content may also change. Thus it would be interesting to apply this system into other biomaterials, especially those with comparatively fast degradation rate to detect the degradation effect on cell traction generation;

4). Here in this work, a single time point (7th day) was chosen to study the correlation between cell-generated tractions with stem cell differentiation. However, many cellular activities depend more on the history of certain properties than their condition at a certain time point. Thus different time points can be further studied to better understand the traction history during various cellular activities and physiological processes.
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