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PCL-PDMS-PCL Copolymer-Based Microspheres Mediate Cardiovascular Differentiation from Embryonic Stem Cells

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PCL-PDMS-PCL COPOLYMER-BASED MICROSPHERES MEDIATE
CARDIOVASCULAR DIFFERENTIATION FROM EMBRYONIC STEM CELLS

By
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ABSTRACT

Poly- ϵ -caprolactone (PCL) based copolymers have received much attention as drug or growth factor delivery carriers and tissue engineering scaffolds due to their biocompatibility, biodegradability, and tunable biophysical properties. Copolymers of PCL and polydimethylsiloxane (PDMS) also have shape memory behaviors and can be made into thermoresponsive shape memory polymers for various biomedical applications such as smart sutures and vascular stents. However, the influence of biophysical properties of PCL-PDMS-PCL copolymers on stem cell lineage commitment is not well understood. In this study, PDMS was used as soft segments of varying length to tailor the biophysical properties of PCL-based copolymers. While low elastic modulus (<10 kPa) of the tri-block copolymer PCL-PDMS-PCL affected cardiovascular differentiation of embryonic stem cells, the range of 60-100 MPa PCL-PDMS-PCL showed little influence on the differentiation. Then different size (30-140 μm) of microspheres were fabricated from PCL-PDMS-PCL copolymers and incorporated within embryoid bodies (EBs). Mesoderm differentiation was induced using bone morphogenetic protein (BMP)-4 for cardiovascular differentiation. Differential expressions of mesoderm progenitor marker KDR and vascular markers CD31 and VE-cadherin were observed for the cells differentiated from EBs incorporated with microspheres of different size, while little difference was observed for cardiac marker α -actinin expression. Small size of microspheres (30 μm) resulted in higher expression of KDR while medium size of microspheres (94 μm) resulted in higher CD31 and VE-cadherin expression. This study indicated that the biophysical properties of PCL-based copolymers impacted stem cell lineage commitment, which should be considered for drug delivery and tissue engineering applications.

Key words: Poly- ϵ -caprolactone, copolymers, embryonic stem cells, cardiovascular differentiation, microspheres

CHAPTER 1

INTRODUCTION

Cardiovascular disease is the leading cause of death in the world and accounts for more than one-third of all deaths in United States each year [1]. The total direct and indirect cost of cardiovascular disease and stroke is estimated to be \$315 billion in 2010 in the United States [1]. The treatment of cardiovascular disease requires the available cells, tissues, and models for vascular prosthetics, development of new drugs, or establishing the disease-relevant models to understand the pathological disease development. Pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced PSCs (iPSCs), are ideal cell sources to generate unlimited number of cardiovascular cells or to construct cardiovascular tissues for cell therapy, drug screening and disease modeling [2-5].

Efficient scalable differentiation of cardiovascular cells from PSCs usually relies on the microspheres to support cell adhesion or to deliver the lineage-inducing growth factors in suspension [6-8]. Microspheres of different materials such as gelatin, agarose and poly lactic-co-glycolic acid (PLGA) were shown to induce differential expression of genes and proteins for mesoderm and endoderm lineage commitment from PSCs [9]. In addition, the biophysical properties (e.g., microsphere size and the elastic modulus) of microspheres also have profound effects on stem cell lineage commitment. For example, the comparison of different microsphere/microparticle size showed the accelerated and more complete cystic embryoid bodies when delivering retinoic acid using smaller size particles (1 μm) [10]. Optimized size of PLGA microparticles was also studied in the range of 100-240 μm during chondrogenic differentiation of mesenchymal stem cells (MSCs), where the use of particles of 175 μm resulted in better differentiation [11]. PLGA microspheres were incorporated into the collagen gels to increase the

stiffness of extracellular microenvironment and promote osteogenic differentiation of MSCs by providing the stiff microenvironment [12]. All these studies indicate that biophysical properties of microspheres or microparticles influence stem cell differentiation.

Poly- ϵ -caprolactone (PCL) based microspheres have wide applications in drug delivery and tissue engineering applications [13,14]. PCL-based microspheres (about 30-210 μm) can be fabricated by various methods such as emulsion solvent evaporation, spray drying, solution enhanced dispersion etc. [13,14]. PCL is biocompatible with a variety of human tissues and its long-term biodegradability allows the drug release up to several months [15]. PCL-based materials have flexible mechanical properties (Young's modulus, tensile strength, etc.) [14]. PCL also allows the modification of its physical, chemical, and mechanical properties through the formation of copolymers, e.g., gelatin, chitosan, polydimethylsiloxane (PDMS), poly(ethylene glycol) (PEG), poly(vinyl alcohol) (PVA), and PLGA, or forming composites by blending with other polymers [14].

In particular, the co-polymers of PCL-PDMS-PCL can be used to synthesize and fabricate thermo-responsive shape memory polymer (SMP) based materials or scaffolds [16,17]. SMPs have been used to fabricate biomedical devices, such as smart sutures, vascular stents, and tissue engineering scaffolds [18]. For example, the cell alignment (i.e., cytoskeleton orientation and nuclear alignment) of MSCs was shown to be modulated by the shape-memory-actuated fiber alignment of the scaffolds [19]. In addition, PDMS serves as soft segments of varying length to tailor the mechanical properties of PCL (serving as hard segments)-based polymers. Varying the segment lengths of PCL and PDMS can tune the mechanical properties of the resulting copolymers to fabricate the co-polymers with desired stiffness [16].

The objective of the present study is to fabricate PCL-PDMS-PCL microspheres and evaluate the influence of biophysical properties (i.e., microsphere size and elastic modulus) of PCL-PDMS-PCL copolymers on cardiovascular differentiation of ESCs. This study indicates the importance of biophysical properties of microspheres on cardiovascular lineage commitment and has the significance in biomaterials design for stem cell-based tissue engineering, cellular differentiation, and growth factor delivery.

CHAPTER 2

BACKGROUND

2.1. The role of biomaterials in stem cell-based tissue engineering

Both natural biomaterials (e.g., Matrigel, collagen) and synthetic biomaterials (e.g., poly lactic-co-glycolic acid (PLGA), poly(ϵ -caprolactone) (PCL)) have been extensively used in tissue engineering and drug delivery [20,21]. The properties of biomaterials in cellular microenvironment have been recently found as critical factors in regulating stem cell self-renewal, proliferation, and lineage commitment [22]. Different types of tissue engineering scaffolds mainly include 2-D based substrates, foam-like scaffolds, fibrous scaffolds, and microparticles/microspheres or nanoparticles (**Figure 2.1**). Compared to 2-D surface, 3-D scaffolds provide structural supports and porous environment, which more mimic extracellular matrix (ECM) environment in native tissues.

Figure 2.1 **Illustration of the use of biomaterials in tissue engineering.** 2D substrate, micro/nano particles, nanofibers, and foams can be fabricated from natural and synthetic biomaterials. After that, the cells are seeded into scaffolds with growth factors.

PCL is one of the extensively studied synthetic biomaterials in tissue engineering and drug delivery (**Figure 2.2**) [23]. Some commercialized PCL-based scaffolds include Osteoplug™ (Psteopore), Artelon sportmesh™ (SBI), and 3D Biotek 3D Insert™ (Sigma). Common PCL tissue

engineering scaffold fabrication methods include gas foaming (e.g., use supercritical CO₂), electrospinning, particulate leaching, extrusion etc. For example, electrospinning can generate the aligned, or random oriented fibrous scaffolds that guide tissue development [21]. In general, the physical properties of the scaffolds include structural properties, geometrical properties, and mechanical properties.

Structural properties such as pore interconnectivity, porosity (75-95% in general) and pore size (100-400 μm in general) are critical parameters to regulate tissue cell distribution, organization and the subsequent proliferation and differentiation [24]. Appropriate pore structure depends on the tissue type and the type of scaffold materials. The basic principle is to allow the efficient nutrient diffusion and waste removal in the meanwhile supporting tissue development.

Figure 2.2 **Different modifications of poly(ϵ -caprolactone) (PCL) polymer.** (A) graft copolymer; (B) (a) diblock copolymer; (b,c) triblock copolymer; (d) tricomponent triblock copolymer; (C) star shaped copolymer; (D) hyperbranched copolymer; (E) molecular brush or comb shaped copolymer; (F) targeted block copolymer. Dash et al, 2012, *Mol Pharm* 9:2365-79. Copyright © 2012, American Chemical Society.

Geometrical properties: Micro/nanopatterned surfaces have been found to influence stem cell fate decision. For example, nanofibrous scaffolds with aligned fiber orientation were recently found to affect the epigenetic state of the cells and cell reprogramming efficiency [25]. The microtopography can promote mesenchymal-to-epithelial transition and significantly improve reprogramming efficiency to generate induced pluripotent stem cells (iPSCs). The microtopography can induce the changes in histone H3 acetylation and methylation that favor the activation of reprogramming genes.

Mechanical properties: Mechanical properties of the scaffolds are affected by the scaffold materials, structure, and degradability. For example, increased porosity may compromise the mechanical strength of the scaffolds due to large void volume. Sufficient mechanical strength of the scaffold is required to maintain tissue integrity. The elasticity of the biomaterials can affect cell morphology, cytoskeleton organization, and the subsequent cellular differentiation [26]. Decoupling the effects of different scaffold properties (e.g., porosity, elasticity) is critical for scaffold and biomimetic materials design. The basic aim of the biomimetic scaffold design is to enhance *in vivo* tissue-like extracellular matrix microenvironment to promote tissue function.

In this study, the PCL-based microparticles were fabricated for the purpose of tissue engineering and drug delivery. The targeted application is cardiovascular tissue engineering based on stem cells, given the importance of stem cell-derived tissue specific cells in the applications of cardiovascular disease.

2.2. Cardiovascular differentiation from pluripotent stem cells

Cardiovascular disease is the leading cause of death in the worlds and accounts for more than one-third of all deaths in United States each year [1]. The total direct and indirect cost of

cardiovascular disease and stroke is estimated to be \$315 billion in 2010 in the United States [1]. The total number of inpatients of cardiovascular operations and procedures increased 28% from the year of 2000 to 2010. The treatment of cardiovascular disease requires the available cells, tissues, and models for vascular prosthetics, development of new drugs, or establishing the disease-relevant models to understand the pathological disease development.

Figure 2.3. **The diagram of potential applications of human induced pluripotent stem cells (iPSCs) in treating human disease.** Human iPSCs can be used into two ways as shown in the diagram. One is to repair the mutated DNA sequences, followed by in vitro differentiation. Then the healthy cells are transplanted into the body of patient (right). The other is to perform the in vitro differentiation of the patient cells to screen the disease-specific drugs. Robinton et al, 2012. *Nature* 481:295-305. Copyright © 2012, Rights Managed by Nature Publishing Group.

Pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and iPSCs, are ideal cell source to generate unlimited number of cardiovascular cells or the construction of cardiovascular tissues for cell therapy, drug screening and disease modeling (**Figure 2.3**) [27,28].

PSCs are capable of differentiating into cells from three-germ layers- ectoderm, mesoderm, and endoderm. Currently, the strategies of efficient differentiation of cardiovascular cells from PSCs following mesoderm induction are being actively studied. The studies target on gaining the understanding of biochemical factors and biophysical factors in directing PSC lineage specification especially toward mesoderm induction.

2.2.1. PSC differentiation into mesoderm cells

Human PSCs (hPSCs) can be induced to simultaneously differentiate into cardiac cells and vascular cells due to the common origin of their early mesoderm progenitor cells (**Figure 2.4**). For examples, mesoderm differentiation of PSCs through the treatment of activin A, bone morphogenetic protein (BMP)-4, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) was initiated from epithelial-to-mesenchymal transition (EMT) with the up-regulation of NCAM (CD56) and the down-regulation of EpCAM (CD326) [29]. The CD326-CD56+ cells at day 3.5 showed the upregulation of Snail-1, Snail-2, Twist 1 and ECM proteins and the down regulation of E-cadherin. The decreased expression of Nanog, Sox-2, Oct-4, CD9 and SSEA-4 demonstrated the loss of pluripotency. The gene expression analysis indicated the up-regulation of mesodermal genes, such as Brachyury, Tbx-6, Snail-1, and Mesp-1 and -2, but not for endodermal and ectodermal genes. The day 3.5 CD326-CD56+ progenitor cells were able to give rise to hematopoietic cells, endothelial cells, mesenchymal cells (bone, cartilage and fat), cardiomyocytes and smooth muscle cells.

The generation of cardiovascular progenitors ($\text{KDR}^{\text{low}}/\text{C-KIT}^+$) was observed at day 6 after mesoderm induction of PSCs. The cells can further differentiate into endothelial cells with the treatment of basic FGF (bFGF) or cardiomyocytes with the inhibition of Wnt signaling using Dickkopf-related protein (Dkk)-1 (**Figure 2.5**) [2]. Three populations are generated using

fluorescence-activated cell sorting (FACS) based on the co-expression of two markers KDR and C-KIT: $KDR^{high}/C-KIT^{+}$, $KDR^{low}/C-KIT^{-}$ and $KDR^{neg}/C-KIT^{+}$. Vascular markers, CD31, VE-cadherin, and SMA were highly expressed by the population of $KDR^{high}/C-KIT^{+}$. $KDR^{low}/C-KIT^{-}$ cells expressed high levels of cardiac markers, Nkx2.5, Isl1, and Tbs5. $KDR^{neg}/C-KIT^{+}$ population can be used to detect the residue undifferentiated cells. While hPSCs have demonstrated the capability to differentiate into cardiovascular progenitor cells, the purity and function of the derived cells are still yet to be fully characterized.

Figure 2.4. The surface markers expression during the first stages of mesodermal specification of human embryonic stem cells (hESCs). The initial stage was marked by the generation of KDR^{+} in the presence of activin A, BMP-4, VEGF, and FGF2. And the KDR^{+} cell population showed the abilities to generate three mesodermal lineages, blood cells, endothelial cells, and cardiomyocyte by observing the differential expression of surface markers, CD31, VE-cadherin, Nkx2.5, and α -actinin. The KDR^{-} cell population showed the ability to generate smooth muscle cells.

Figure 2.5. **A model for generating mesodermal progenitors that can differentiate into multiple cell types.** Yang et al, 2008. *Nature* 453:524-8. Copyright © 2008, Rights Managed by Nature Publishing Group.

2.2.2. Mesoderm differentiation into cardiac cells

Temporal modulation of Wnt signaling can promote cardiac differentiation of from mesoderm progenitor cells. Efficient cardiac differentiation from PSCs required early activation of canonical Wnt signaling followed by the inhibition of Wnt activity [30]. For example, high purity (>90%) of cardiomyocytes can be derived from hESCs with the early treatment of CHIR99021 (for 1 day) to activate Wnt signaling and then the inhibition of Wnt signaling by IWP4 (day 3-5) using the medium without insulin. The adaptation of this protocol in suspension toward the mass production of hPSC-derived cardiac cells was also recently demonstrated [31]. The differentiation was sensitive to the CHIR99021 and IWP4 concentrations, treatment duration, and treatment timing. Alternative cardiac differentiation from PSCs can be achieved using Activin A induction followed by BMP-4 treatment [5,32].

2.2.3. Mesoderm differentiation into vascular cells

Differentiation of vascular cells from mouse iPSCs and human iPSCs has been demonstrated recently (**Table 2.1**) [3,4]. From Flk1⁺VE-cadherin⁺ endothelial progenitor cells

(EPCs) derived from mouse iPSCs, more mature vascular cells can be generated by the treatment with VEGF on collagen IV surface. These vascular cells expressed markers of CD34, CD31, and VE-cadherin (VE-Cad). Plating the day 5 Flk⁺ and VE-cadherin⁺ EPCs on Matrigel-coated dishes, the cells were also able to generate vascular networks (branching point structures) in the presence of VEGF [4]. By activation of Wnt signaling, the mesoderm progenitors via CHIR99021 (Wnt activator) treatment have been shown to generate a population enriched in endothelial progenitors (CD34⁺CD31⁺ cells) when the endogenous canonical Wnt signaling was permitted (i.e., in the absence of Wnt inhibitor IWP4 treatment, IWP4 treatment would lead to the formation of cardiomyocytes) [33].

High purity of CD146⁺CD105⁺ cells (>90%), identified as the early vascular cells (EVCs), have been derived from multiple lines of hiPSCs (using VEGF and SB431542, an inhibitor of transforming growth factor- β pathway) and can give rise to both endothelial cells and the supporting pericytes [3]. Maturation of EVCs to endothelial cells was performed from the sorted VE-Cad⁺ cells. The matured cells expressed VE-Cad and CD31 on cell membrane and endothelial nitric oxide synthase (eNOS) and Von Willebrand factor (vWF) in cytoplasm. The cells also formed vascular networks on Matrigel. Maturation of EVCs to pericytes enriched cells expressing markers of CD73, NG2, CD44 and platelet-derived growth factor receptor (PDGFR)- β [3]. The pericytes exhibited filamentous calponin expression and have the mesenchymal differentiation potential. Encapsulation of EVCs in hyaluronic acid (HA) hydrogels showed the sprouting and open lumen morphology and bi-cellular vascular networks.

Vascular tissue models based on hiPSC-derived endothelial cells (ECs) were characterized [34]. More than 90% of the hiPSC-derived endothelial cells co-expressed CD31⁺, CD105⁺, and

CD144⁺ population. The proliferation properties of hiPSC-ECs are VEGF concentration-dependent and can be reduced by the addition of VEGF inhibitor SU1498 (**Figure 2.6**).

Figure 2.6. Vascular tissue models derived from human pluripotent stem cells (PSCs). (A) Schematic of cell spheroid formation in round-bottom low adhesion plates using Matrigel as a substrate to enable iPSC-EC sprouting. (B) Quantification of total sprout length of iPSC-ECs cultured in a VEGF-only Starvation Medium or Growth Medium (containing VEGF, FGF-2, insulin-like growth factor-1, and epidermal growth factor) along with a library of pharmacological inhibitors. (C) Fluorescent micrographs demonstrating sprouting after 6 full days in culture with Matrigel. Belair et al, 2015. *Stem Cell Rev.* 11: 511-525. Copyright © 2014, Springer Science+Business Media New York.

Table 2.1. Current protocols for vascular differentiation of pluripotent stem cells (PSCs).

Cell population	Culture parameters	Effect on differentiation	Reference
mESC-derived EBs	Mechanical strain (5%, 10%, 20%)	Mechanical strain increased CD31 ⁺ cells (2-fold) and the beating cardiomyocytes (2-3 fold) through the ROS generation	Schmelter et al, 2006 [35]
mESC-derived EBs	Spontaneous EBs, different surface stiffness (0.2, 6 and 40 kPa) when replating	α -actinin ⁺ cells were highest at 6 kPa, CD31 ⁺ cells were higher at lower stiffness (0.2-6 kPa). Maximal vascularized cardiac cells were found at stiffness similar to cardiac muscle (6 kPa).	Shkumatov et al, 2014 [36]
mESCs and miPSCs	Collagen IV surface, BMP4, bFGF, and VEGF	Characterization of Flk1 ⁺ VE-Cadherin ⁺ cells derived from iPSCs. Can form well-developed vascular structures.	Kohler et al, 2013 [4]
miPSCs	Spontaneous differentiation sorted by FACS on Flk1 ⁺ cells; then use Collagen IV surface and VEGF	Characterization of Flk1 ⁺ cells to generate vascular cells and cardiomyocytes.	Narazaki et al, 2008 [37]
hESCs	Encapsulation in dextran-based hydrogel	Encapsulation culture enhanced vascular differentiation (KDR ⁺ , CD34 ⁺).	Ferreira et al, 2007 [38]
hPSCs (hESCs H9 and H13, hiPSCs MR31, MMW2, BC1)	Collagen IV surface, VEGF, and SB431542	The derived early vascular cells can mature to endothelial cells and pericytes in hyaluronic acid (HA) gels.	Kusuma et al, 2013 [3]
hiPSCs	BMP4 and FGF2 for 4 days, followed by VEGF treatment during day 5-15	Generate cell sheet of cardiovascular cells for transplantation study. Cardiac function was improved.	Masumoto et al, 2014 [39]
hiPSCs (3 lines) and hESCs (H1, H13, H14)	CHIR99021 treatment to activate Wnt signaling	Generate high purity (55%) of CD34 ⁺ CD31 ⁺ endothelial progenitors by activating Wnt signaling.	Lian et al, 2014 [33]

The endothelial nature of hiPSC-ECs was characterized by testing the cell barrier properties that were related to wound healing process and inflammatory stimuli. The stimulated expression of cell adhesion molecules CD54 and CD146 was observed in response to the addition of tumor necrosis factor (TNF)- α . The sprouting of hiPSC-ECs was shown as VEGF concentration-dependent but did not depend on the regulated function of microtubules. HiPSC-

ECs sprouting were promoted by VEGF/VEGF2 signaling by influencing the single cell migration process. At the same time, the increased continuous sprout length in the growth medium containing FGF demonstrated FGF/FGFR signaling also promoted cell sprouting and cell integrity. HiPSC-ECs sprouting inhibition in the presence of mitogen-activated protein kinases (MAPK)/extracellular signal-regulated kinase (ERK) inhibitor demonstrated the positive role of ERK signaling in the iPSC-ECs sprouting process.

2.3. Importance of ECMs in cardiovascular culture

The role of ECMs in stem cell differentiation is bi-phasic: (1) the exogenously presented ECMs (e.g., fibronectin, laminin, collagens, vitronectin, heparin sulfate proteoglycans) affect cell adhesion, sequester and release the bound growth factors, and provide structural supports; (2) during the tissue development, the cells endogenously secrete ECM proteins that can be remodeled and affect the subsequent cellular events. The scaffolds for hPSC culture (also known as synthetic ECMs) usually require the surface coating with ECM proteins for initial cell adhesion and organization. Then endogenously secreted ECMs would play the dominant role in regulating cellular proliferation and differentiation. Here, the representative examples of ECM effects on cardiovascular culture are discussed.

2.3.1 The role of ECM proteins

In cardiac cell culture, ECM proteins have been found to promote cardiac differentiation from hPSCs. For example, the use of Matrigel sandwich culture, an ECM mixture with the dominant component of laminin (e.g., bind integrin $\alpha 6 \beta 1$), in hPSC mesoderm induction (induced by Activin A, BMP-4, FGF-2) was found to promote EMT transition and the generation of pre-cardiac mesoderm [40]. The subsequent differentiation resulted in high purity of cardiac cells. The evaluation of combinatorial fibronectin (FN) and laminin (LN) indicated that 70:30 FN:LN

reproducibly generated the greatest numbers of cardiac cells derived from hPSCs [41]. It was found that the gene expression of integrin $\beta 4$ (LN receptor), integrin $\beta 5$ (FN receptor), phosphorylated focal adhesion kinase, and phosphorylated extracellular signal regulated kinases were up-regulated on 70:30 FN:LN compared to gelatin [41].

In vascular cell culture, ECMs can drive the capillary morphogenesis through the bound growth factors and ECM receptors (integrins) [42]. ECMs also regulate the cytoskeleton reorganization and the cell shape change to control endothelial cell proliferation, survival, and the generation of vascular networks. Different ECM proteins have played a distinct role in vascularization process. For example, collagens and fibronectin regulate the tubular morphogenesis through the integrins $\alpha 2\beta 1$, $\alpha 5\beta 1$, $\alpha v\beta 3$ etc. In contrast, laminin regulates the vascular tube stabilization possibly through the integrins $\alpha 6\beta 1$ or $\alpha 3\beta 1$. Vascular cells derived from hPSCs were found to increase ECM secretion (fibronectin, laminin, and collagen IV) compared to undifferentiated cells, indicating the greater ability of vascular cells to deposit ECM proteins [43].

2.3.2 The role of ECM remodeling and degradation

MMPs control the ECM remodeling, degradation, and the dynamic modification of ECM microenvironment. Recently, both ECM secretion and ECM degradation are recognized as a critical process for stem cell lineage commitment [44]. ECM degradation can regulate cellular traction independent of cell morphology and matrix mechanics. Hyaluronic acid hydrogels that allows the ECM degradation (with the treatment of MMP-2) supported high cell spreading and contraction that favored osteogenic differentiation of human MSCs [45]. In contrast, reducing the hydrogel degradation (with cross-linking) inhibited cellular contraction and favored adipogenic differentiation. It was further demonstrated that the effects of non-degradable hydrogels were

similar to the effects of inhibition of myosin activity using the small molecules such as blebbistatin [45]. ECM degradation products have also been shown to possess chemotactic and mitogenic activities for multipotent progenitor cells but inhibit the proliferation of endothelial cells, indicating the influence on the cell recruitment during ECM remodeling [46]. MMP-9 (activated expression via Wnt/beta-catenin signaling) was shown to increase the migration and proliferation of neural stem cells under hypoxia and the presence of MMP-9 inhibitor reduced cell migration [47]. For vascular cells, MMPs allow the cell invasion into the 3-D collagen or fibrin gels and MMP-9 can accelerate the release of VEGF from the ECM sites [42]. So the dynamic interaction of cells with ECMs plays a critical role in cellular differentiation.

2.4. Biophysical properties of the substrates/scaffolds regulate cardiovascular differentiation

To regulate the specific lineage commitment of cardiovascular progenitors, there are two types of inducing factors: (1) biochemical factors such as the growth factors; (2) biophysical factors such as surface stiffness and biomechanical stress. Recently, the role of biophysical factors has drawn lots of attention in order to modulate the cellular microenvironment. In most cases, biophysical factors and biochemical factors work synergistically to direct the lineage-specific differentiation.

Biophysical microenvironment of stem cells has been recognized as an important factor in cell fate decision recently [26,48]. Biophysical properties of the substrates and scaffolds include the mechanical properties such as elastic modulus and the geometrical properties such as the dimension (or size) of the substrates and scaffolds. Here, the effects of elastic modulus of the substrates/scaffolds on cardiovascular differentiation are discussed.

2.4.1. Effects of elastic modulus of the substrates on cardiac differentiation

Elastic modulus of the scaffolds differentially promoted mesoderm (stiff), endoderm (intermediate), or ectoderm (soft) differentiation as well as cardiac cell functions [49-51]. The effects of elasticity of the substrates on cardiac differentiation depend on the differentiation stage: early stage differentiation from PSCs, late stage of differentiation from cardiac progenitor cells, or maturation of cardiomyocytes (**Table 2.2**). For late stage differentiation or maturation of cardiomyocytes, substrates with stiffness that was similar to native myocardium (around 18 kPa) were found to significantly enhance ventricular myocyte maturation and beating function [50,52,53]. HPSC-derived cardiomyocytes also showed stiffness-dependent contraction with the higher modulus (4.4-76 kPa) leading to the higher contraction stress [49]. However, the early commitment of hPSCs to cardiac lineage was found to be promoted on surfaces with intermediate stiffness (50 kPa) or rigid substrates such as tissue culture polystyrene (TCPS) (1.2 MPa to 3 GPa) (**Figure 2.7**) [54,55]. While cardiomyocytes differentiation in TCPS had the comparable purity to the surface of 50 kPa, it was postulated that different materials chemistry and ECM deposition for TCPS and hydrogel of 50 kPa may also contribute to the high differentiation efficiency. Similarly, when increasing the stiffness from 78 kPa to 1.2 MPa, mesoderm differentiation of hESCs was favored compared to endoderm differentiation [56]. It was found out the effect of substrate stiffness on cardiac differentiation of hESCs was restricted at the early stage of mesodermal induction because the use of Nkx2.5⁺/Isl1⁺ progenitor cells did not show the difference in cardiac cell purity for hydrogels with different stiffness [55]. Therefore, the exposure of hPSCs to stiffer surface at mesodermal induction stage can enhance the cardiac differentiation efficiency.

Table 2.2. Summary of current study for cardiac differentiation on surface with different stiffness

Cell population	Substrate Stiffness	Effect on differentiation	Reference
Effects on cardiac cell function			
Neonatal rat ventricular myocytes	1-50 kPa	10 kPa (near the stiffness of native myocardium) leads to maximal force generation	Jacot et al, 2008 [52]
Neonatal rat ventricular myocytes	2.3-5.4 kPa	Rigidity of 4 kPa (matching the cell stiffness) promoted myocardial contraction	Horning et al, 2012 [50]
Embryonic chicken cardiac myocytes	4-100 kPa, and 3 GPa	Cells on 18 kPa (comparable to native myocardium) surface had the highest beating rate	Bajaj et al, 2010 [57]
hESC-derived cardiac cells	4-100 kPa	Contraction stress increased with stiffness	Hazeltine et al, 2012 [49]
Human adult cardiac progenitor cells	0.5-40 kPa	Soft matrix (<1 kPa) promoted vascular differentiation while the harder matrix (>10 kPa) promoted cardiac differentiation.	Mosqueira et al, 2014 [17]
Effects on early stage cardiac differentiation of PSCs			
hESCs	50 kPa or 3 GPa (TCPS)	50 kPa and 3 GPa had the highest cardiac differentiation, but stiffness did not impact the differentiation of Nkx2.5 ⁺ /Isl1 ⁺ progenitor cells	Hazeltine et al, [55]
mESCs and hESCs	12 kPa, 1.2 MPa, 3 GPa (TCPS)	TCPS had the highest cardiac differentiation and beating rate	Arshi et al, 2013 [54]
hESCs	78 kPa to 1.2 MPa	Stiffer surface increased the mesoderm differentiation of hESCs	Eroshenko et al, 2013 [56]
Human EBs	0.05-7 MPa	Low modulus (<0.1 MPa) promoted ectoderm, intermediate modulus (0.1-1 MPa) promoted endoderm, and high modulus (1.5-6 MPa) promoted mesoderm	Zoldan et al, 2011 [51]

Figure 2.7. **Temporal impact of hydrogels with different modulus on cardiac differentiation from human ESCs.** (A) The human ESCs were seeded on different hydrogels and tissue culture polystyrene plastics (TCPS) and fixed on day 15 for cTNT expression. (B) cTNT⁺ cell expression at day 15 after replating the day 6 human ESC-derived cardiac progenitor cells (CPCs). Hazeltine et al, 2014. *Acta Biomater* 10:604-612. Copyright © 2013 Acta Materialia Inc. Published by Elsevier Ltd. All rights reserved.

2.4.2. Effects of elastic modulus of the substrates on vascular differentiation

Effects of elastic modulus of the substrates on vascular differentiation from PSCs have been evaluated based on embryoid body (EB)-induced differentiation. By changing the substrate stiffness, the percentage of CD31⁺ cells was found higher at lower stiffness (0.2-6 kPa) compared to the higher stiffness (40 kPa), and maximal vascularized cardiac cells were found at the stiffness similar to cardiac muscle (6 kPa) [36]. The effect of mechanical strain was also evaluated by applying the strain on the day 4 EBs [35]. Under the mechanical strain, both CD31⁺ cells and the beating cardiomyocytes increased at least 2-fold compared to the cells under no strain. It was demonstrated that the increased generation of reactive oxygen species (ROS) under the mechanical strain was responsible for the enhanced cardiovascular differentiation. In the range of stiffness 10 Pa to 650 Pa, the rigid surface (650 Pa) promoted the production of matrix metalloproteinase (MMP)-1 and MMP-2, the enzymes responsible for endothelial cell migration and vascularization,

compared to the soft surface (10 Pa) (**Figure 2.8**) [58]. However, in the *in vitro* tube morphogenesis, the vascular tube length increased (from 130 μm to 200 μm) with the decreasing modulus [58]. Different geometry of the substrates created by micropatterned surface showed the influence of substrate geometry and size on vascular differentiation of hiPSCs [59]. Small islands (e.g., 80 μm) that limited cell spreading (similar to soft substrates) supported higher vascular differentiation efficiency with 70% of endothelial cells while large islands (e.g., 225 μm) resulted in lower endothelial cell yield and was more similar to non-patterned surface.

Figure 2.8. Combination effects of vascular endothelial growth factor (VEGF) and substrate mechanics on vascular differentiation process. VEGF activated MMP to digest ECM proteins to initiate the cell migration. 10 Pa substrate activated the signaling pathway to initiate the membrane regulator, MMP-1 to accelerate cell elongation.

2.4.3. Signal transduction in the differentiation regulated by biophysical microenvironment

Signal transduction in stiffness-dependent cardiac differentiation has not been well studied to date. Both Rho GTPase signaling and Hippo/Yes-associated protein (YAP) signaling have been observed as the mechano-transduction mechanism for stem cells [60,61]. The role of Rho GTPase signaling was recognized initially through the modulation of cytoskeleton distribution [61]. Cells grown on hard substrate spread better and display the stress fibers while the cells grown on soft

surface tend to have round shape and redistribute the actin around the cell membrane. Inhibition of actin tension and polymerization (e.g., adding Cytochalasin D or ROCK inhibitor Y27632) was observed to abolish the effects of stiff surface on the cells. Apparently, cellular contraction regulated through RhoA signaling and actomyosin-based forces transduces the signals that affect the nuclear transcription [62]. Recently, Hippo/YAP signaling was also found in regulating cardiovascular differentiation on the substrates with different elasticity. For example, YAP silencing (similar effect to the soft substrate) correlated to the down-regulation of cardiomyocyte genes and the up-regulation of endothelial-specific genes as well as matrix metalloproteinase genes [17]. Consistently, the soft matrix leads to the cytoplasmic expression of YAP and the ability of the cells to form branching endothelial morphology. Rho GTPase signaling and Hippo/YAP signaling are related to each other because Rho and actin cytoskeleton (stress fibers) are required for the nuclear localization of YAP/TAZ. It was found that stress fibers reduced YAP phosphorylation and promoted nuclear YAP accumulation, thus disruption of stress fibers using cytochalasin D reduced nuclear YAP [63].

Other mechano-transduction mechanisms have also been investigated. For example, the role of ROS as the transducer of the mechanical strain in cardiovascular differentiation was revealed [35]. Higher level of ROS was observed when the cells were under the strain created by the elongated membrane, along with the increased capillary structures and the increased beating foci. The up-regulation of NADPH subunits was also detected. After the treatment with free radical scavengers, such as vitamin E or NMPG, the percentage of contractile area or area of capillary-like structures were reduced.

2.5. Microparticle/microsphere-mediated stem cell differentiation and tissue engineering

Microparticles/microspheres have been used in stem cell culture and tissue engineering as the vehicles to deliver the growth factors or as the scaffolds to regulate cellular differentiation (**Table 2.3**). For the purpose of growth factor or drug delivery, the particles are usually in the range of 5-30 μm to better distribute the delivered molecules. For the purpose of tissue engineering scaffolds, large size particles have been used in the range of 100-200 μm to support stem cell growth and differentiation. The biophysical properties of microparticles such as stiffness and size are the important parameters to regulate stem cell fate decisions in addition to the surface chemistry.

2.5.1. Effect of materials chemistry of microparticle/microsphere

The microparticles can be fabricated from different materials such as gelatin, agarose, and PLGA [9]. The effects of surface chemistry of gelatin, agarose, and PLGA microparticles (4-5 μm) have been studied during the incorporation of microparticles with embryoid bodies for spontaneous differentiation [9]. The presence of microparticles induced differential expression of genes and proteins for mesoderm and endoderm lineage commitment, e.g. AFP and MLC-2v. Gelatin particles induced higher expression of AFP and MLC-2v, followed by agarose and PLGA particles (**Figure 2.9**). The spatial distribution of mesoderm and endoderm markers was altered for the EBs incorporating different particles. For example, alpha-sarcomeric actin was not observed in EBs with PLGA particles at day 14, but was found in the interior of EBs incorporating gelatin (1:4 particle to cell ratio) particles. All the particles have been reported to maintain good cell viability. This study indicates that incorporating of biomaterials in EBs influence the mesoderm and endoderm lineage commitment.

Figure 2.9. **Differential gene expression analysis of PSC aggregates incorporating with different microparticles.** Pax-6 gene expression was not significantly different. However, the expression of AFP and MLC-2V was altered for different groups. Bratt-Leal et al, 2011. *Biomaterials* 32:48-56. Copyright © 2010 Elsevier Ltd.

2.5.2. Effect of elastic property of microparticles/microspheres

The elastic and mechanical properties of microparticles can impact stem cell differentiation. PLGA microparticles were incorporated into the collagen gel to increase the stiffness of the ECM-enriched extracellular microenvironment which increased osteogenic differentiation of MSCs by providing the stiff microenvironment [12]. Similarly, incorporation of gelatin microparticles in the MSC aggregates increased the stiffness of the spheroids (3-4 fold higher than the spheroids without microparticles) while maintaining the cell viability and aggregate structure [64]. No differences in cytoskeleton organization, cell distribution and aggregation properties were observed for the aggregates with or without microparticles. The mechanical properties of multi-block copolymer (20LP10L20-LLA40) microspheres were studied [65]. While at dry state the elastic modulus of microsphere was in the range of 56-95 MPa, at hydrated state the elastic modulus of multi-block copolymer microsphere decreased to 226-343 kPa, which was in the similar range of cartilage tissue.

2.5.3. Effect of microparticle/microsphere size

The microparticle/microsphere size has been found to be a critical factor in growth factor delivery and cellular differentiation. The comparison of different particle size showed the accelerated and more complete cystic EBs when delivering RA using small size particle (1 μm) [10]. The PLGA particle size less than 1 μm (e.g., 0.24 μm) was found to be easily taken up by the cells through endocytosis, while microparticles with the size of 6 μm were more highly dispersed in the EBs compared to 25 μm microparticles (**Figure 2.10**) [7]. Loading the microparticles of 6 μm with VEGF increased the expression of vascular markers KDR, CD31, CD34, and VE-cadherin in the EBs compared to the EBs with unloaded particles [7]. Optimize PLGA particle size was also observed in the range of 100-240 μm during chondrogenic differentiation of MSCs derived from umbilical cord, where 175 μm resulted in better differentiation [11].

Figure 2.10. **Schematic illustration of embryoid body (EB) formation in the presence of microparticles of different size.** Ferreira et al, 2008. *Adv Mater* 20:2285-2291. Copyright © 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

2.5.4. Effect of microparticles/microspheres loaded with biomolecules

The incorporation of biomolecules including RA, BMP-4, noggin, and BMP-2 with microparticles has been demonstrated in microparticle-mediated growth factor delivery to regulate site-specific PSC lineage commitment. The presentation of these inducing factors can be controlled in a spatiotemporal manner. For example, PLGA microparticles have been loaded with

RA and the RA was released inside the EBs [66]. It was observed that the EBs incorporating RA-loaded PLGA microparticles resembled the phenotype and structure of early streak mouse embryo (E6.75), while the EBs with unloaded microparticles displayed more mature phenotype and markers of epiblast and primitive streak [66] .

Table 2.3. Summary of the effects of microspheres/microparticles on stem cell differentiation

Materials	Particle Size	Effect on differentiation	Reference
PLGA particles	0.24, 6, 25 μm	Vascular differentiation of human EBs. 6 μm particles were loaded with VEGF, PIGF, and FGF-2. Incorporation of microparticles enhanced vascular differentiation (higher VE-Cad, CD31).	Ferreira et al, 2008 [7]
PCL microspheres	107 μm , 284 μm	For protein delivery and release, with modulus of 15-19 MPa; pore dimension, interconnectivity, and mechanical properties can be pre-defined.	Luciani et al, 2008 [68]
PLGA microspheres	1-11 μm	EB formation, sustained release of retinoic acid; smaller size induced accelerated and more complete cystic EBs.	Carpenedo et al, 2010 [10]
PLGA microparticles	20 μm 20-30 MPa	Increased the rigidity of collagen gel, enhanced osteogenic differentiation of MSCs.	DeVolder et al, 2012 [12]
Gelatin microparticles	3.6 μm	Use human MSCs, no effect on viability, no effect on cytoskeleton, increase aggregate modulus and stiffen the spheroids.	Baraniak et al, 2012, [64]
Gelatin microparticles	4-5 μm	Use to deliver BMP4 and noggin for mesoderm and ectoderm differentiation of ESCs. 12-fold less growth factor used to induce similar gene expression compared to soluble factors. Local control of mesoderm induction.	Bratt-Leal et al, 2013, [6]
PLGA microsphere-based scaffolds	100-240 μm (optimal 175 μm)	Primary chondrocytes; human umbilical cord MSC; support viability, support chondrocyte differentiation	Singh et al, 2010, [11]
Multi-block copolymer (20LP10L20-LLA40) microsphere	5-30 μm	Dry and hydrated states of microspheres have different mechanical properties (Dry: 56-95 MPa; Hydrated: 226-343 kPa)	Moshtagh et al, 2014 [65]
PLGA, agarose and gelatin microparticles	4-5 μm	The presence of microparticles within EBs differentially modulated the gene and protein expression patterns of several differentiation markers (e.g., AFP, MLC-2v).	Bratt-Leal et al, 2011, [9]
Heparin methacrylamide microparticles	2-10 μm	Heparin particles were used to deliver BMP-2, which stimulated comparable alkaline phosphatase activity of skeletal myoblasts to soluble BMP-2.	Hettiaratchi et al, 2014, [67]

Another example is the delivery of BMP-4 or noggin through gelatin microparticles [6]. Efficient gene expression of mesoderm and ectoderm lineages was observed using the BMP-4 loaded particles, which used 12-fold less growth factor compared to soluble factor delivery. In particular, spatial control of differentiation in EBs was demonstrated with half of the sphere expressing Brachyury due to the localized BMP delivery while the other untreated half showing the minimal expression of mesoderm marker Brachyury [6]. Heparin microparticles allowed the delivery of heparin-binding growth factors BMP-2, VEGF and FGF-2 [67]. BMP-2 loaded microparticles were observed to stimulate the alkaline phosphatase (ALP) activity of skeletal myoblasts and also increased the DNA content mediated by cell-particle contact. All these studies showed the active biological activity of microsphere or microparticle-mediated growth factor delivery during cellular differentiation of stem cells.

2.6. The application of PCL-based microparticles and microspheres

PCL is biocompatible with various drugs and enables the uniform drug distribution. Its long-term biodegradability allows the drug release up to several months. As tissue engineering scaffolds, PCL is biocompatible with a variety of human tissues [15]. It has flexible mechanical properties (Young's modulus, tensile strength, etc.) and is suitable for paramedical applications [14]. PCL has low glass transition temperature (-60°C). Degradation of PCL is slow compared to polyglycolic acid (PGA) and other polymers. So it is suitable for long-term delivery of over one year. PCL has high permeability to small drug molecules and does not generate acidic environment during degradation.

PCL also allows the modification of its physical, chemical, and mechanical properties by the formation of co-polymer (e.g. gelatin, chitosan, PDMS, PEG, PVA, PLGA, and PU) or forming composites by blending with many other polymers. For examples, PCL is compatible with many

natural polymers such as gelatin, chitosan and starch and synthetic polymers such as PEG, polyurethane (PU), and PVA [14].

PCL microparticles or microspheres (about 30-210 μm) can be fabricated by various methods such as emulsion solvent evaporation (w/o, w/o/w, w/o/o etc., w: water; o: organic solvent), spray drying, solution enhanced dispersion etc. [13,14]. During the fabrication, the drugs can be entrapped or encapsulated into the spheres. The disadvantage of emulsion solvent evaporation method is the presence of residual organic solvents in the particles.

Various drugs (e.g., nitrofurantoin, insulin, and heparin) have been incorporated with PCL microparticles for the controlled delivery. For examples, PCL microparticles containing cyclosporine have been prepared which were stable for 12 months and can be used for cyclosporine administration through different routes [13]. Nerve growth factor (NGF) was loaded in microspheres of a blend PCL/PLGA copolymer in which PLGA was used to tailor degradation rate. A bi-phasic release profile was observed which showed the first phase resulted from the NGF release from the surface and the second phase due to polymer degradation[13].

2.7. The proposal of using PCL-PDMS-PCL co-polymer-based microparticles

2.7.1. Why use PCL-PDMS-PCL co-polymer?

1. PCL can be made into a variety of drug delivery carriers, tissue engineering scaffolds and thermo-responsive shape memory polymer (SMP).

PCL has received much attention as drug delivery carriers and tissue engineering scaffolds due to their biocompatibility, biodegradability, and elasticity. PCL-based scaffolds include microparticles, nanoparticles, foams, films, hydrogels and micelles, composites, and nanofibers etc. Another example is that PCL can be made into thermo-responsive SMPs. The shape of SMPs

is modulated by heat based on their thermal transition temperature (T_{trans} , can be T_g or T_m). A temporary shape can be formed when $T > T_{\text{trans}}$, fixed by cooling to $T < T_{\text{trans}}$, and then recovered when $T > T_{\text{trans}}$ [69]. SMPs can be used to fabricate biomedical devices, such as smart sutures and vascular stents, and tissue engineering scaffolds [18]. The biocompatibility of PCL-based SMP network has been evaluated for MSCs [19,70]. The osteogenic and adipogenic differentiation of MSCs were supported by SMP and the shape memory effect activation had minimal effect of the cells [70]. The cell alignment (i.e., cytoskeleton orientation and nuclear alignment) of MSCs was shown to be modulated by the shape-memory-actuated fiber alignment of the scaffolds [19].

2. PDMS can regulate the physical property of the particles (e.g., elastic modulus).

Polydimethylsiloxane (PDMS) can serve as soft segments of varying length to tailor the mechanical properties of PCL-based polymers. PCL serves as hard segments to balance the effects of PDMS. Varying the segment lengths of PCL and PDMS can tune the mechanical properties of the resulting co-polymers (T_{trans} from 50-53°C) (**Figure 2.11**) [16]. The compressive modulus of PCL-PDMS-PCL based foams (porosity 70-90%) at room temperature was shown to be tailored from 0.04 MPa to 4.3 MPa [16]. An increase in PDMS segment length (from 20 to 130) was shown to decrease the tensile modulus of PCL-PDMS-PCL solid co-polymers from 75 MPa to 3.4 MPa [69]. PCL-PDMS-PCL co-polymer can be further polymerized with other materials. For example, triblock PCL-PDMS-PCL has been co-polymerized with DL-lactic acid to control the degradation rate and is shown to support fibroblast growth [71].

Figure 2.11. **Schematic illustration of geometrical structure of PCL-PDMS-PCL based copolymers.** Both hard segment (PCL) and soft segment (PDMS) are included to form SMP materials.

2.7.2. Why use 1,6-hexamethylene diisocyanate in the synthesis?

1,6-hexamethylene diisocyanate (HDI), is a coupling agent that can increase the molecular weight of PCL-PDMS-PCL co-polymer and modifies the polymer physical properties. The use of HDI in the synthesis of alternating block based on PCL and PEG co-polymer was demonstrated through the selective coupling reaction of PCL-diol and diisocyanate end-capped PEG (**Figure 2.12**) [15,72].

Figure 2.12. **Schematic illustration of 1,6-hexamethylene diisocyanate (HDI) coupling effect on the co-polymerization of PCL-based polymer.** Niu et al, 2014. *Biomaterials* 35:4266-77. Copyright © 2013 Wiley Periodicals, Inc.

The alternating block polymers showed higher mechanical properties and higher crystal degree compared to their random counterpart. In addition, better cell compatibility of fibroblasts and rat glial cells was observed on the alternating block polymers.

2.7.3. The influence of PCL-PDMS-PCL copolymer on cardiovascular differentiation from pluripotent stem cells is unknown

With the wide applications of PCL-based copolymers, the influence of biophysical properties of PCL-PDMS-PCL microparticle/microsphere on stem cell lineage commitment is unclear. This study will focus on two aspects of the biophysical properties of PCL-PDMS-PCL copolymers: the elastic modulus and the size of the microspheres (30-150 μm).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

ϵ -caprolactone monomer, poly (dimethylsiloxane)-bis(3-aminopropyl) terminated ($M_n \sim 2500$ g/mol), hexamethylene diisocyanate (HDI), stannous 2-ethylhexanoate and solvents were purchased from Sigma Aldrich (St. Louis, MI). Aminopropyl terminated polydimethylsiloxane DMS A12 ($M_n \sim 1,000$ g/mol), DMS A15 ($M_n \sim 3,000$ g/mol), DMS A21 ($M_n \sim 5,000$ g/mol), DMS A31 ($M_n \sim 25,000$ g/mol) were purchased from Gelest, Inc (Morrisville, PA).

3.2. Synthesis of PCL-PDMS-PCL co-polymers

Macromers of PCL_n -block- $PDMS_m$ -block- PCL_n were synthesized by ring-opening polymerization of ϵ -caprolactone in the presence of $NH_2 - PDMS_m - NH_2$ and tin-catalyst (**Figure 3.1A**) [16,69]. The repeat units for PCL segment length were varied at $n=20, 40$, and 50 . The PDMS segment lengths were varied at $m=10, 34, 58$, and 296 . Specifically, various amounts of ϵ -caprolactone ($37.75, 75.49$, or 94.36 mmol), aminopropyl terminated polydimethylsiloxane DMS A12, A15, A21, or A31 (2.20 mmol), and stannous 2-ethylhexanoate (0.123 mmol) were added into a 250 mL three-neck flask at 145°C and stirred for 24 hours when the co-polymers were synthesized. Co-polymers with $PDMS_{28}$ were synthesized with poly (dimethylsiloxane)-bis(3-aminopropyl) terminated similarly.

For the chain propagation between oligomers by adding HDI as coupling agent (**Figure 3.1B**), the synthesized oligomers were transferred into a 1000 mL flask, and cooled down from 145°C to 100°C . Synthesized oligomers were dissolved in 1, 2- dichloromethane and water. The 1, 2-dichloroethane was removed under 100°C for about 60 min until no bubbles exist in the

solution. When the flask was cooled down from 100°C to 50°C, two drops of stannous octanoate and 0.002 mole HDI were added into the system sequentially and reacted for 5 hours.

Figure 3.1. **Mechanism of PCL-PDMS-PCL (w/o HDI or w HDI) co-polymer synthesis.** (A) Structure of PCL-PDMS-PCL macromers. Macromers of PCL_n-block-PDMS_m-block-PCL_n were synthesized by ring-opening polymerization of ϵ -caprolactone in the presence of NH₂-PDMS_m-NH₂ and tin-catalyst; (B) Structure of coupling agent HDI and the synthesis in the presence of HDI.

3.3. Dynamic mechanical analysis (DMA)

DMA analysis was used to measure the Young's modulus of different co-polymers. The samples were characterized using dynamic mechanical analyzer (Q800, TA Instruments). For tensile modulus, the rectangular samples were exerted under strain at a strain rate of 0.1 min^{-1} and 30°C. Compressive modulus was measured for certain samples (low elastic modulus) that cannot be measured for tensile modulus. In a compression model, the cylindrical samples were

compressed with a 15 mm compression clamp at a strain rate of 0.1 min⁻¹ and 30°C. The Young's modulus was calculated based on the initial slope of the stress-strain curve.

3.4. Differential scanning calorimetry (DSC) analysis and ¹H NMR

For DSC analysis, the samples were run on the differential scanning calorimeter (Q100, TA Instruments) from 30°C to 100°C at heating rate of 10°C/min. The samples were then cooled down from 100°C to 30°C at cooling rate of 10°C/min. The melting point T_m was calculated from the heat flow-temperature curve. The enthalpy change was determined from the endothermic melting peak. The % crystallinity was calculated using the equation:

$$\% \text{ crystallinity} = \frac{\Delta H_m}{\Delta H_m^0} \times 100$$

Where ΔH_m is normalized based on the % mass of PCL segments in the PDMS-PCL macromere. ΔH_m⁰ is 139.5 J/g for 100% crystalline PCL [73].

¹H NMR spectra of the co-polymers were obtained on a Bruker 500M spectrometer at 200 MHz. The spectrum was taken in deuterated chloroform at 20°C. The composition of copolymers was calculated from the ratios of absorbance at 0.07 and 4.05 ppm. Chemical shift for the protons in the PCL repeating unit, -CH₂CH₂CH₂CH₂ CH₂OH, are assigned at δ=4.05, 2.30, 1.64 and 1.37ppm, which were labeled with different characters, *a*, *d*, *b+b'* and *c*. The peak represented the PDMS repeating units, -Si(CH₃)₂-, which had the chemical shift at δ=0.04-0.11ppm, was labeled with *e*. The ratio of PCL and PDMS can be determined by the integrations of methylene group (δ=4.05ppm, *a*) from PCL repeating units and methyl group (δ=0.04-0.11ppm, *e*) from PDMS repeating units in the ¹H-NMR spectrum. Average molecular weight (M_n) was calculated according to the following equation:

$$M_n = M_{PCL} \times 40 + M_{PDMS} \times \left(\frac{40}{3}\right) \left(\frac{M}{N}\right) + M_{PCL} \times 40$$

In this equation, it was assumed that the synthesized copolymers had constant number of PCL repeating units, $n=40$ or 20 , and the integration for methylene group in the $^1\text{H-NMR}$ spectrum from PCL repeating units is M and for methyl group in PDMS repeating units is N .

3.5. Fabrication of PCL-PDMS-PCL co-polymer based microspheres

PCL-PDMS-PCL microspheres were fabricated using a reversed phase precipitation method. Briefly, after the co-polymer synthesis reaction, the copolymer solution in 1, 2-dichloromethane was slowly poured into a 1000 mL big beaker containing 500 mL methanol. Microspheres were formed and precipitated at the bottom of beaker when stirring the solution at 100 rpm for two hours. Then the precipitate was filtrated to remove the mixture of 1, 2-dichloromethane and methanol. In order to remove the remaining oligomers and tin (II) 2-ethylhexanoate, the filtrate was washed three times with methanol. The final products were dried under vacuum for more than 24 hours before used for characterization or cell culture.

3.6. Scanning electron microscopy (SEM)

The morphologies of microparticle samples were investigated using field emission scanning electron microscope (SEM) (JEOL 7401F). Samples were sputter-coated with a thin layer of gold before observation. The SEM images were analyzed using ImageJ software for the size distribution of microspheres.

3.7. Undifferentiated ESC cultures

Murine ES-D3 line (American Type Culture Collection, Manassas, VA) was maintained on 6-well culture plates coated with 0.1% gelatin (Millipore, Temecula, CA) in a standard 5% CO_2

incubator [74,75]. The culture medium is composed of Dulbecco's Modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% ESC-screened fetal bovine serum (FBS, Hyclone, Logan, UT), 1 mM sodium pyruvate, 0.1 mM β -mercaptoethanol, penicillin (100 U/mL), streptomycin (100 μ g/mL) (all from Invitrogen), and 1000 U/mL leukemia inhibitory factor (LIF, Millipore). The cells were seeded at $2\text{--}4 \times 10^4$ cells/cm² and sub-cultured every 2-3 days with 0.05% trypsin.

3.8. Cell seeding with microspheres and cardiovascular differentiation

For cell culture, PCL-PDMS-PCL microspheres were sterilized with 70% ethanol and then washed three times with phosphate buffer saline (PBS). Different types of microspheres (0.2 mg/mL) were mixed with the cells and seeded in the wells of low attachment 24-well plates at 2×10^5 cells in 1 mL (**Figure 3.2**). The differentiation medium was composed of DMEM, 10% FBS, 1 mM β -mercaptoethanol, penicillin (100 U/mL), and streptomycin (100 μ g/mL).

Figure 3.2. **Schematic illustration of EB-induced differentiation in the presence of microspheres.**

The medium was supplemented with 10 ng/mL bone morphogenetic protein (BMP)-4 (R&D Systems) for the first 4 days. At day 4, the formed embryoid bodies containing microspheres were replated onto the 0.1% gelatin-coated tissue culture treated 24-well plates. The

cells were fed with the differentiation medium without growth factors. At day 2, 5, 11, and 14, the cells were characterized for cardiovascular markers. For culture medium experiments, the replated cells were put in either differentiation medium (DMEM + 10% FBS) or RPMI-B27 serum-free medium (Invitrogen). The cells were then characterized for cardiovascular markers at day 1 and 5 when in different media.

To illustrate the influence of elastic modulus only, the PCL-PDMS-PCL copolymers (9.3 kPa, 58 MPa, and 103 MPa) were processed into 2-D disks (diameter: 10 mm, thickness: 1 mm). The day 4 EBs without particles were replated onto different 2-D disks coated with 0.1% gelatin. The cells were grown for 11 days before the analysis of cell proliferation and cardiovascular marker expression.

3.9. Biochemical assays

Live/Dead[®] staining kit (Molecular Probes) was used to assess cell viability of the cells with microspheres [76]. After a 5-day culture, the cells with microspheres were incubated in DMEM containing 1 μ M calcein AM (green) and 2 μ M ethidium homodimer I (red) for 30 min. The samples were imaged under a fluorescent microscope. Cell numbers of microsphere cultures at day 1, 3 and 5 were determined using a hemocytometer after cell trypsinization. The cell aggregates with microspheres were also incubated with 5 mg/mL 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) solution at day 1, 3 and 5 to evaluate metabolic activity. The absorbance of the samples was measured at 500 nm using a microplate reader (Biorad, Richmond, CA).

3.10. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from ESC samples using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol followed by the treatment of DNA-Free RNA Kit (Zymo, Irvine, CA). Reverse transcription was carried out using 2 mg of total RNA, anchored oligo-dT primers (Operon, Huntsville, AL), and Superscript III (Invitrogen, Carlsbad, CA) (according to the protocol of the manufacturer). Primers specific for target genes (**Table 3.1**) were designed using the software Oligo Explorer 1.2 (Genelink, Hawthorne, NY). The gene beta-actin was used as an endogenous control for normalization of expression levels. Real-time RT-PCR reactions were performed on an ABI7500 instrument (Applied Biosystems, Foster City, CA), using SYBR1 Green PCR Master Mix (Applied Biosystems). The amplification reactions were performed as follows: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 95°C for 15 sec and 55°C for 30 sec, and 68°C for 30 sec. Fold variation in gene expression was quantified by means of the comparative Ct method: $2^{-(C_{t\ treatment} - C_{t\ control})}$, which is based on the comparison of expression of the target gene (normalized to the endogenous control beta-actin) between day 4 EB samples and the day 0 undifferentiated cells.

Table 3.1. Primer sequence for target genes.

Gene	Forward primer 5' to 3'	Reverse primer 5' to 3'
Oct-4	CAGCAGATCAGCCACATCGCC	TGAGAAAGGAGACCCAGCAGCC
Nanog	CCTGTGATTTGTGGGCCTG	GACAGTCTCCGTGTGAGGCAT
Beta-actin	GTACTCCGTGTGGATCGGCG	AAGCATTGTGCGGTGGACGATGG

3.11. Immunocytochemistry

Briefly, the cells were fixed with 4% paraformaldehyde (PFA) and permeabilized with 0.2-0.5% Triton X-100 (for intracellular markers only). The samples were then blocked and incubated

with mouse or goat primary antibody against: KDR (Millipore), CD31 (PECAM-1) (Santa Cruz Biotechnology Inc., Dallas, Texas), VE-cadherin (Santa Cruz), and α -actinin (Sigma). After washing, the cells were incubated with the corresponding secondary antibody (Molecular Probes): Alexa Fluor® 594 donkey anti-goat IgG (for CD31 and VE-cadherin), or Alexa Fluor® 488 goat anti-mouse IgG1 (for KDR and α -actinin). For F-actin staining, the cells were incubated with Alexa Fluor® 594 Phalloidin (Molecular Probes). For YAP staining, the cells were incubated with rabbit primary antibody (Santa Cruz), followed by Alexa Fluor® 594 goat anti-rabbit IgG. The samples were stained with Hoechst 33342 (to reveal the cell nuclei) and visualized under a fluorescent microscope.

3.12. Flow cytometry

To quantify the cardiovascular marker expression, the cells were harvested by trypsinization and analyzed by flow cytometry. Briefly, 1×10^6 cells per sample were fixed with 4% PFA and washed with staining buffer (2% FBS in PBS). The cells were permeabilized with 100% cold methanol (for intracellular markers only), blocked, and then incubated with primary antibodies against KDR, CD31 (PECAM-1), VE-cadherin, Nkx2.5 (Santa Cruz) and α -actinin followed by the corresponding secondary antibody: Alexa Fluor® 594 donkey anti-goat IgG (for CD31 and VE-cadherin), Alexa Fluor® 594 goat anti-rabbit IgG (for Nkx2.5), or Alexa Fluor® 488 goat anti-mouse IgG1 (for KDR and α -actinin). The cells were acquired with BD FACSCanto™ II flow cytometer (Becton Dickinson) and analyzed against isotype controls using FlowJo software.

3.13. Vascular structure assay

Briefly, 24-well plates were coated with 200 μ L/well 1:1 diluted Geltrex (B&D Biosciences) at room temperature for more than 30 min. The cells were plated at 5×10^5 cells on

Geltrex-coated plates in 500 μ L EGM-2 medium (Lonza, for endothelial cells) and incubated at 37°C in 5% CO₂. The morphology of the cells was photographed by a phase-contrast microscope daily for 5 days. The cells were then fixed and assessed for marker expression of CD31, VE-cadherin, and F-actin.

3.14. Statistical analysis

Each experiment was carried out at least three times. The representative experiments were presented and the results were expressed as [mean $\pm$ standard deviation]. To assess the statistical significance, one-way ANOVA followed by Fisher's LSD post hoc tests were performed. A *p*-value <0.05 was considered statistically significant.

CHAPTER 4

RESULTS

4.1. Fabrication of PCL-PDMS-PCL copolymers and characterization

Macromers of $\text{PCL}_n\text{-block-PDMS}_m\text{-block-PCL}_n$ ($n=20, 40, \text{ and } 50$; $m=10, 34, 58, \text{ and } 296$) were synthesized by ring-opening polymerization of ϵ -caprolactone in the presence of $\text{NH}_2\text{-PDMS}_m\text{-NH}_2$ and tin-catalyst as shown in **Figure 3.1**. Composition, molecular weight, and thermal properties of different PCL-PDMS-PCL copolymers were characterized (**Table 4.1**). ^1H NMR analysis confirmed the feed ratio of the two polymers (**Figure 4.1A and Table 4.1**). T_m of PCL-PDMS-PCL copolymer ($54\text{-}57^\circ\text{C}$) was slightly lower than the PCL (60°C). Decreasing the length of PCL segments showed the decrease in % of crystallinity (**Table 4.1**). Young's modulus of copolymers was measured by DMA and the representative stress-strain curve was shown in **Figure 4.1B**. By varying the segments of PCL and PDMS, the copolymers displayed different elastic modulus (0.8 kPa to 160 MPa) (**Figure 4.1C**). HDI is used as coupling agent, which can increase the chain length of copolymer. Higher modulus was observed (from 80 MPa to 160 MPa) when the ratio of HDI was increased (**Figure 4.1D**). These results demonstrate the successful synthesis of PCL-PDMS-PCL copolymers and the elastic modulus of the copolymers can be tailored by varying hard segment (PCL) and soft segment (PDMS).

Figure 4.1. **Characterization of PCL-PDMS-PCL co-polymers.** (A) representative ^1H NMR analysis of co-polymer; (B) representative stress-strain curve of co-polymer; (C) The Young's modulus of PCL-PDMS-PCL co-polymers with different PCL and PDMS length; (D) The Young's modulus of PCL-PDMS-PCL co-polymers with different HDI ratio.

Table 4.1. Composition, molecular weight, and thermal properties of different PCL-PDMS-PCL copolymers.

Copolymers	Polymer 1	Polymer 2	Polymer 3	Polymer 4
PCL(n)	40	40	40	20
PDMS(m)	28	34	58	58
Molecular Weight	11,588	12,100	13,586	9,100
M_n /g.mol ⁻¹				
Feed ratio	80/28	80/34	80/58	40/58
^1H NMR ratio	80/31	80/37	80/57	40/50
Tm/°C	57.4	45.7	57.3	54.0
Cryst.(%)	45	39	35	23

4.2. Effect of elastic modulus of PCL-PDMS-PCL copolymers

Prior to the evaluation of the copolymers in cardiovascular differentiation of ESCs, the differentiation media was compared. The differentiation was initiated with BMP-4 induction to obtain the KDR⁺ progenitors which can further differentiate into cardiac cells and vascular cells (**Figure 4.2A**). The day 4 EBs were replated onto the gelatin-coated surface in DMEM-FBS or RPMI-B27 media. At early time point (day 3), KDR was expressed at a higher level for cells grown in DMEM/FBS compared to RPMI/B27 media (20.1% vs. 8.8%), but decreased quickly at day 7 (10.4% vs. 39.0%) (**Figure 4.2B**). Compared to RPMI/B27 culture, CD31 expression was higher at both day 3 (27.3% vs. 5.9%) and day 7 (40.9% vs. 16.3%) for DMEM/FBS culture. For cardiac markers, DMEM/FBS and RPMI/B27 cultures had comparable levels of NKX2.5 and α -actinin. Based on these results, DMEM/FBS media was used in further study.

2-D disks of three types of copolymer (PCL20-PDMS58-PCL20: 9.3 kPa, PCL40-PDMS28-PCL40: 58 MPa, and PCL20-PDMS34-PCL20+ HDI: 103 MPa) were evaluated for cardiovascular differentiation (**Figure 4.3**). Highest expression of vascular markers CD31 (43%) and VE-cadherin (56%) was observed for the 2-D disk of 9.3 kPa. For 58 MPa and 103 MPa PCL-PDMS-PCL copolymer, slightly higher expression was observed for 103 MPa disk. Since vascular differentiation was shown to be promoted at the lower elastic modulus [17,36,58], these data indicate little effect of modulus range of 58-103 MPa on the vascular differentiation from EBs replated on the 2-D disks. The expression level of α -actinin was similar (8-11%) for all three conditions.

Figure 4.2. **Cardiovascular differentiation from embryonic stem cells.** (A) Schematic illustration of cardiac differentiation and vascular differentiation from embryonic stem cells; (B) Representative flow cytometry histograms of cardiovascular marker expression in different media: DMEM plus 10% FBS and RPMI plus 2% B27 serum replacement.

Figure 4.3. **Effect of elastic modulus of PCL-PDMS-PCL co-polymers.** (A) Representative flow cytometry histograms of cardiovascular marker expression for PCL-PDMS-PCL co-polymers with different elastic modulus. (B) Fold increase in cell number for cells grown on PCL-PDMS-PCL co-polymers with different elastic modulus.

Figure 4.4. **Characterization of PCL-PDMS-PCL co-polymer based microspheres.** (A) Scanning electron microscopy (SEM) images of PCL-PDMS-PCL microspheres; (B) Size distribution of the three types of PCL-PDMS-PCL microspheres.

4.3. Biocompatibility of PCL-PDMS-PCL microspheres

PCL-PDMS-PCL microspheres (with modulus of 60-100 MPa) were then fabricated with three different sizes (**Figure 4.4**). The morphology of the microspheres was shown in the SEM images and the size distribution of the microspheres was characterized. The average diameter of the three types of microspheres was 32 μm (S), 94 μm (M), and 140 μm (L).

Figure 4.5. **Incorporation of PCL-PDMS-PCL microspheres with embryoid bodies.** (A) Phase contrast images of EBs with microspheres and cell viability determined by LIVE/DEAD assay; Scale bar: 200 μm . (B) MTT activity; (C) Cell number kinetics.

The microspheres were mixed with ESCs at the same concentration (0.2 mg/mL) for the EB formation. The microspheres were incorporated into the EBs as shown in the phase contrast images (**Figure 4.5A**). About all the EBs (100%) contained the S-spheres, while about 40% of

EBs contained M-spheres and about 25% EBs contained L-spheres. The EBs incorporating the microspheres were viable as shown in the images of LIVE/DEAD assay. The dead cells were only observed for the single cells that were not able to form EBs. MTT activity showed the increased reading with culture time for all three conditions, further confirmed the viability and the proliferative activity of the EBs (**Figure 4.5B**). M-sphere condition showed the highest level of MTT activity. About 4-fold increase in cell number was observed over 5-day culture (**Figure 4.5C**). All these data demonstrate the biocompatibility of PCL-PDMS-PCL microspheres when incorporating with the EBs.

4.4. Cardiovascular differentiation of ESCs grown with microspheres

The EBs incorporating the microspheres were evaluated for the gene expression of Oct-4 and Nanog (**Table 4.2**). The decrease of Oct-4 and Nanog indicated the loss of pluripotency of the differentiated cells. The cells were further replated and grown for 11-15 days toward cardiovascular differentiation. At the end of differentiation, the cells expressed CD31 and VE-cadherin but less α -actinin (**Figure 4.6**). The quantification of progenitor marker showed the high expression of KDR (70% vs. 30%) at day 3 for S-sphere condition. However, the M-sphere condition showed the highest expression of CD31 (65% vs. 30%) and VE-cadherin (65% vs. 20-30%) at day 11 after replating. The BrdU expression of the replated cells was evaluated (**Supplementary Figure S4.1**). About 42% BrdU+ cells for S-sphere condition, 31% for M-sphere, and 35% for L-sphere were observed. The expression of F-actin and YAP was examined for the replated cells of the three groups (**Supplementary Figure S4.2**). The cells expressed the actin stress fibers and nuclear pattern of YAP for all three groups.

Table 4.2. The expression of Oct-4 and Nanog in day 4 embryoid bodies (EBs) incorporating microspheres.

Culture conditions	Oct-4	Nanog
S-sphere	0.66±0.04	0.95±0.05
M-sphere	0.60±0.07	0.17±0.17 ^a
L-sphere	0.75±0.06	0.37±0.15 ^a

^a*p* value<0.05

Figure 4.6. Cardiovascular differentiation from embryoid bodies containing PCL-PDMS-PCL microspheres. (A) Immunocytochemistry analysis of cardiovascular markers CD31, VE-cadherin, and α -actinin expression in the cells differentiated from EBs incorporated with microspheres; Scale bar: 50 μ m for CD31 and VE-cadherin; Scale bar: 100 μ m for α -actinin. (B) Flow cytometry analysis of KDR, CD31, and VE-cadherin expression by cells differentiated from EBs incorporated with microspheres.

The differentiated cells were also harvested and replated onto Geltrex-coated well in the presence of endothelial cell growth medium and growth factors. Under endothelial cell growth condition, CD31 and VE-cadherin positive populations were enriched (**Figure 4.7**). Elongated endothelial cell-like morphology was observed and the M-sphere condition showed a few branching points.

Figure 4.7. Vascular network assay for the cells derived from EBs incorporating different PCL-PDMS-PCL microspheres. (A) Morphology of vascular cells derived from EBs grown in the endothelial cell medium and the enrichment of vascular marker expression; (B) Vascular cells grown in the endothelial cell medium for the cells from EBs incorporated with different microspheres. Scale bar: 100 μ m.

CHAPTER 5

DISCUSSION

Complex and interactive niche signals including the mechanical and topographical cues have played a critical role in stem cell lineage commitment. The microstructures and elastic properties of micro-scale spheres, particles, ribbons, or scaffolds have shown the influences on stem cell fate decisions and draw attention in biomaterials design and characterizations [12,77]. In this study, PCL-PDMS-PCL co-polymers were synthesized, characterized and used for regulating cardiovascular differentiation from ESCs. In particular, biophysical properties including microsphere size and elastic modulus of PCL-PDMS-PCL co-polymers were studied.

PCL-PDMS-PCL co-polymers are suitable for fabricating thermoresponsive shape memory substrates/scaffolds for various biomedical applications [78]. The use of PCL and PDMS also enable the tuning of elastic properties of the materials by varying the ratio of PCL segments and PDMS segments. This study successfully synthesized PCL-PDMS-PCL co-polymers with different PCL and PDMS segments, which were characterized by ¹H NMR, DSC and DMA. Increasing PDMS segment length decreased the elastic modulus while increasing PCL segment length increased the elastic modulus. The elastic properties of PCL-PDMS-PCL co-polymers can be further tuned using HDI coupling agent. Potentially, PCL-PDMS-PCL co-polymers can be modified with other materials depending on the purposes. For example, triblock PCL-PDMS-PCL was co-polymerized with DL-lactic acid in order to control the degradation rate [71].

The influence of PCL-PDMS-PCL co-polymers on cardiovascular differentiation at 2-D level was evaluated using 2-D disks. The effect was mainly observed for vascular differentiation rather than cardiac differentiation. Consistent with the report from the literature that evaluated

other materials [17,36], soft PCL-PDMS-PCL copolymers (<10 kPa) promoted vascular differentiation compared to the hard copolymers (>50 MPa). These results demonstrated the feasibility of using PCL-PDMS-PCL co-polymers in a biological system to regulate stem cell differentiation.

The influence of PCL-PDMS-PCL co-polymers on cardiovascular differentiation at 3-D level was evaluated using microspheres. While PCL-PDMS-PCL with tunable physical and thermal properties has been recently studied [16,69], the use of such copolymers in stem cell culture has not been well reported. The PCL-PDMS-PCL microspheres were readily incorporated with the EBs and showed the biocompatibility during cardiovascular differentiation of ESCs. The EBs incorporating PCL-PDMS-PCL microspheres displayed high viability, normal MTT activity, and the proliferation activities.

In this study, differential expression of vascular markers was observed for the microspheres with different sizes. Higher KDR expression was found for the S-sphere group, but higher CD31 and VE-cadherin was observed for the M-sphere group. It is thought that the stiffening of EBs with microspheres contributed to the differential expression of vascular markers. Incorporation of gelatin microparticles with stem cell aggregates has shown to increase the stiffness of the aggregates by 3-4 fold than those without microparticles [64]. Stiff substrates or scaffolds were reported to favor the early stage mesoderm commitment from PSCs. For example, elastic modulus of the scaffolds differentially promoted mesoderm (6 MPa), endoderm (1 MPa), or ectoderm (0.1 MPa) differentiation from human ESCs [51]. Similarly, when increasing the stiffness from 78 kPa to 1.2 MPa, mesoderm differentiation of human ESCs was favored compared to endoderm differentiation [56]. The EBs with S-spheres had the highest frequency of microsphere incorporation and almost all the aggregates were stiffened with the microspheres, which may help

the mesoderm commitment. M-sphere condition had less frequency of microsphere incorporation followed by the L-sphere condition. The higher CD31 and VE-cadherin expression for M-sphere condition may suggest the balanced mesoderm commitment and the further differentiation into vascular lineage.

The effect of microsphere/microparticle size has been observed in the range of 0.24 μm to 25 μm for PLGA. The particle size less than 1 μm (e.g., 0.24 μm) was found to be easily taken up by the cells through endocytosis, while microparticles with the size of 6 μm were more highly dispersed in the EBs compared to 25 μm microparticles [7]. The comparison of different particle size (1-11 μm) also showed the accelerated and more complete cystic EBs when delivering RA using smaller size particle (1 μm) [10]. This study showed the influence of PCL-PDMS-PCL microspheres in the size range of 30-140 μm .

Both Rho GTPase signaling and Hippo/Yes-associated protein (YAP) signaling have been investigated as the mechano-transduction mechanism for stem cells [60,61]. The soft matrix leads to the cytoplasmic expression of YAP and the ability of the cells to form branching endothelial morphology [17]. It was found that stress fibers reduced YAP phosphorylation and promoted nuclear YAP accumulation, thus disruption of stress fibers using cytochalasin D reduced nuclear YAP [63]. However, in this study, the replated cells with different microspheres all had nuclear expression of YAP, which suggested that the difference in differentiation should result from the suspension stage (e.g., EB culture) of differentiation, where EBs were incorporated with microspheres with different frequency.

The microspheres are usually used as the carriers to deliver the drugs or growth factors, or as scaffolds/microcarriers to influence stem cell differentiation. Delivery of BMP-4 through

gelatin micropsheres/microparticles showed the increased gene expression of mesoderm and ectoderm lineages, while the BMP-4 loaded microparticles used 12-fold less growth factor compared to soluble BMP-4 delivery [6]. Heparin microparticles allowed the delivery of heparin-bounding growth factors BMP-2, VEGF and FGF-2 [67], which can stimulate the alkaline phosphatase (ALP) activity of skeletal myoblasts and increase the DNA content mediated by cell-particle contact. The PCL-PDMS-PCL copolymer-based microspheres can be used as the drug carriers and tissue engineering scaffolds as shown for PCL-based microspheres [13,68]. The shape memory property of PCL-PDMS-PCL copolymers needs to be further investigated to design smart materials for regulating stem cell fate decision. As the initial step to apply smart materials in stem cell culture, the present study highlights the importance of biophysical properties of the PCL-PDMS-PCL microspheres during stem cell lineage commitment.

CHAPTER 6

CONCLUSIONS

This study synthesized and characterized the biodegradable copolymers that comprised PCL hard segments and PDMS soft segments of variable lengths. The PCL-PDMS-PCL copolymers were evaluated as the substrates and the microspheres that supported cardiovascular differentiation of embryonic stem cells. The main conclusions are as follows:

1. The effects of biophysical properties of PCL-based copolymers are investigated in the present study. PDMS acted as soft segment to tailor the copolymer with controllable mechanical properties. The elastic modulus in the range lower than 10 kPa can promote vascular differentiation from ESCs. However, the modulus in the range of 50-120 MPa showed little effect on cardiovascular differentiation.

2. The PCL-PDMS copolymers with modulus in the range of 50-120 MPa are fabricated into microspheres to investigate the effect of microspheres on the cardiovascular differentiation from EBs. PCL-PDMS-PCL copolymers displayed the biocompatibility in embryonic stem cell differentiation. Differential expression of mesoderm progenitor marker KDR and vascular marker CD31 and VE-cadherin were observed. Little difference was observed for cardiac marker α -actinin expression.

3. Specifically, smaller size of microspheres (30 μm) resulted in higher expression of KDR while medium size of microsphere (94 μm) showed higher expression of CD31 and VE-cadherin.

4. This study demonstrated the influence of biophysical properties of the PCL-PDMS-PCL copolymers on stem cell lineage commitment at the interface of biomaterials and stem cells.

Therefore, the influence of biophysical properties of the substrates/microspheres should be considered for drug delivery and tissue engineering applications.

APPENDIX A

SUPPLEMENTARY MATERIALS

Cardiovascular differentiation from embryoid bodies without PCL-PDMS-PCL microspheres. (A) Immunocytochemistry analysis of cardiovascular markers CD31, VE-cadherin, and α -actinin expression in the cells differentiated from EBs without microspheres; Scale bar: 100 μ m. (B) Quantification of cardiovascular marker expression.

Figure A.1. **Cardiovascular markers, CD31, VE-CAD, and α -actinin expressions for day 7 embryonic bodies.** (A) Immunocytochemistry staining for embryonic bodies without microspheres treated. Scale bar: 100 μ m. (B) Quantification of cardiovascular marker expression.

EBs with different types of microspheres were replated and assessed using BrdU staining [74]. Briefly, the cells were incubated in growth medium containing 10 μ M BrdU (Sigma) for 90 minutes. The cells were then fixed with 70% cold ethanol, followed by a denaturation step using 2N HCl/0.5% Triton X-100 for 30 minutes in the dark. The samples were reduced with 1 mg/mL sodium borohydride for 5 minutes and incubated with mouse anti-BrdU (1:100, Life Technologies) in blocking buffer (0.5% Tween 20/1% bovine serum albumin in PBS), followed by the incubation

with Alexa Fluor® 488 goat anti-Mouse IgG₁ (1:100, Life Technologies). The cells were counterstained with Hoechst 33342 and visualized using a fluorescent microscope (Olympus IX70, Melville, NY).

Figure A.2. BrdU expression in the cells derived from embryoid bodies containing three types of PCL-PDMS-PCL microspheres. Scale bar: 100 μ m.

Figure A.3. Expression of F-actin and YAP in the cells derived from embryoid bodies containing PCL-PDMS-PCL microspheres. Scale bar: 100 μ m.

APPENDIX B

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PUBLICATIONS

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2. Yuanwei Yan, **Liqing Song**, Yan Li. Toward Bio-manufacturing of Pluripotent Stem Cell-derived Products: Scale Out and Scale Up. *Pharmaceutical Bioprocessing*. 2015, 3: 25-33.