A SPIN-COATED THERMORESPONSIVE SUBSTRATE FOR RAPID CELL SHEET DETACHMENT AND ITS APPLICATIONS IN CARDIAC TISSUE ENGINEERING

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A SPIN-COATED THERMORESPONSIVE SUBSTRATE FOR RAPID CELL SHEET DETACHMENT AND ITS APPLICATIONS IN CARDIAC TISSUE ENGINEERING

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ABSTRACT

A modular approach to create organized tissue that has gained much attention since the introduction of thermoresponsive surfaces is the assembly of cell sheets. With a change in temperature of grafted poly-N-isopropylacrylamide (pNIPAAm), a thermoresponsive polymer, cell sheets can be harvested with their deposited extracellular matrix (ECM) intact. PNIPAAm has been covalently grafted to cell culture substrates by two primary methods: electron beam irradiation and plasma polymerization. Most tissue engineering laboratories have difficulties using these approaches to custom-make their thermoresponsive surface for specific applications due to the complexities of associated procedures and limited access to required equipment (e.g. e-beam). The goal of this study was to develop a simple, cost-effective approach for the creation of thermoresponsive surfaces using commercially available pNIPAAm for cell sheet harvest. Methods to effectively manipulate viable cell sheets (e.g. transfer and stack) were developed. In addition, a study of cell sheet interaction on fibrin gels is presented and analyzed for future cardiac tissue engineering applications.
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CHAPTER I
INTRODUCTION AND BACKGROUND

1.1 Significance of Study

Myocardial infarction is the leading cause of death in the United States with ~450,000 deaths occurring annually due to cardiovascular disease [1]. Myocardial infarction is caused by the blockage of blood to the heart, resulting in death of heart tissue from lack of nutrients and oxygen. Patients with myocardial infarction and subsequent heart failure have limited life spans even with optimal medical treatment.

Current medical approaches to treat end stage heart failure are very limited. Heart transplantation is the last option that has optimal long term results, but the limited amount of donors and associated immunosuppressive complications [2] makes the treatment option unfeasible for most patients. Thus, it is necessary to develop new therapeutic strategies to treat cardiovascular disease and to better understand how cell therapy can be used to reverse the negative effects of heart remodeling after myocardial infarction.
1.2 Mesenchymal Stem Cell Therapy for Cardiac Repair

Mesenchymal stem cells (MSCs) are adherent adult progenitor cells that can be harvested from many tissues including bone marrow, muscle, skin, umbilical cord, and adipose tissue. These cells have the potential to differentiate into any cell type found in tissue of mesenchymal origin [3]. The predominant origin of MSC used in research comes from bone marrow due to the ease of cell isolation. These cells can be expanded numerous times in vitro while maintaining their differential potential [4]. An important advantage to using MSCs is that they are immune-privileged [5]. This is advantageous because allogenic cells can be used in cell based therapies rather than autologous cells, allowing for a possible off the shelf product. MSCs release pro-angiogenic factors such as vascular endothelial growth factor, fibroblast growth factor-2, insulin-like growth factor-1, and hepatocyte growth factor in response to hypoxia and ischemia-reperfusion injury [6]. These growth factors stimulate cell recruitment and vessel formation around secreted areas. Recently, MSCs have been shown to have the ability to differentiate into cardiomyocytes both in vitro and in vivo [7, 8]. Thus, these studies initiated extensive research into using MSCs for cardiac repair.

Using small and large animal models, researchers have injected MSCs into ischemic myocardium to find improved cardiac function while witnessing MSC engraftment and differentiation toward cardiomyocytes [7, 9-15]. MSCs reduce the infarct area [16], improve perfusion to the ischemic region [17], and increase vascular density [18]. Even with improved heart function, there remains no clear understanding of the exact mechanism of how MSCs act to treat the infarct region [5].
Challenges still remain in effective use of MSCs. Cell survival of injected MSCs is very low for prolonged periods of time [19]. Through fluorescent labeling and tracking, several research groups have shown MSC numbers rapidly decline and become barely detectable after several weeks post injection into ischemic myocardium [20-23]. Low cell survival can be attributed to the stressful nature of the region of injection. The area is ischemic and lacks nutrient supply. In addition, pumping motion of the heart may prevent cells from properly adhering to desired locations. Therefore, it is imperative to increase engraftment rates of MSCs to see further improvement in cardiac function [24].

1.3 Tissue Engineering Strategies for Myocardial Infarction Treatment

Tissue engineering approaches are designed to repair lost or damaged tissue through the use of biomaterial scaffolds. After myocardial infarction, there is an influx of macrophages, monocytes, and neutrophils that initiate the inflammatory response. The extracellular matrix (ECM) degrades in the infarct area resulting in wall thinning and ventricular dilation [25]. Death of cardiomyocytes results in negative left ventricular (LV) remodeling leading to increased wall stress in the surrounding healthy myocardium. The increased stress causes physiological and cellular responses that lead to LV dilation. This LV remodeling is shown to lead to progressive heart failure [26].

Injectable biomaterials that have the ability to gel in situ have been studied to treat post-infarct negative remodeling. The biomaterials can provide a temporary support to the LV wall to prevent negative remodeling by restricting LV dilation. Natural polymers such as collagen, alginate, and fibrin have been investigated for this purpose [25].
Collagen is the most abundant protein in the body and can gel under proper pH and temperature [27]. Being a naturally occurring protein, it has been investigated as an injectable biomaterial. For cardiac tissue applications, results vary when using collagen injected for MI treatment. One group found improved cardiac function without observing increased vascularization [28] while another group reported an increase in capillary density post injection of collagen [29]. Even with conflicting results, all have reported some form of cardiac improvement.

Alginate is a polysaccharide isolated from seaweed that gels in the presence of calcium ions [30]. It is approved for human use by the United States Food and Drug Administration (FDA) as a wound dressing material. Injection of alginate to ischemic heart tissue in swine has shown improved left ventricular (LV) function through reversal of LV remodeling after myocardial infarction [31]. Experiments comparing alginate gel to fibrin gel injection in rat models revealed increased angiogenesis with both gels, while infarct size was significantly reduced in the fibrin group while a trend to a smaller size was seen in the alginate group [32]. The improvement seen from fibrin gel injection to ischemic areas led to many studies on optimizing fibrin gels to treat myocardial infarction.

1.3.1 Fibrin Patch for Cardiac Repair

Fibrinogen is a plasma protein that has widespread use in tissue engineering. It has been extensively studied to form a fibrin gel with the addition of thrombin and has been used as a scaffold for the growth of many cell types including bone marrow stromal cells [33], osteoblasts [34], chondrocytes [35], nerve axons [36], and keratinocytes [37]. Fibrin networks are the first natural scaffolds that cells encounter in the body during its
roles in wound repair [38]. Thus, cells know how to interact with the network without adverse effects. Fibrin gels have many advantages including the option for direct cell entrapment during gel formation, the availability of sites for cell adhesion, and ability to bind to other matrix molecules and growth factors [39].

Fibrin gels form through a polycondensation reaction from fibrinogen when active thrombin removes the parts of fibrinogen polypeptides that prevent its spontaneous polymerization [40]. Different concentrations of gels can be formed by varying fibrinogen concentration. Increasing fibrinogen concentration increases the stiffness of gels, while increasing thrombin concentration has no effect on gel stiffness [41], but reduces gelation time. The fibrinolytic system of the body can systematically breakdown a fibrin gel similar to a blood clot. Degradation can be prolonged through the use of plasmin inhibitors, such as aprotinin or ε-aminocaproic acid [42, 43]. Slowing degradation of fibrin gels can be advantageous to gradually release adhered growth factors or other molecules. Rapid degradation can result in insufficient support for cells if the cells were not able to secrete sufficient ECM to replace digested regions. Excessive fibrinolytic inhibition may result in limited remodeling of the fibrin gel and integration into tissue and can result in construct failure [39]. Thus, a proper degradation scheme is needed for a cell-fibrin construct implant to achieve desired integration with host tissue.

There have been many recent studies exploring the use of cell based therapy for cardiac repair. MSCs delivered through transendocardial injection improved patient heart function and reduced scar formation [44]. One significant problem that is associated with injection of cells is the low retention rate in ischemic heart [45, 46]. Stem cells that do engraft to the injected region do not fully differentiate into host cardiac cell phenotypes
Therefore, stem cell therapy can be made more efficient through development of methods to increase engraftment rates and improve differentiation of cells into cardiac and vascular lineages.

Fibrin gels without cell entrapment improved infarct heart tissue [49-51]. The gels provide temporary physical support to infarct tissue to prevent negative remodeling to damaged ECM [52]. This in turn prevents LV dilation and improves the long term LV geometry and cardiac function. These gels are advantageous because they can be used to release growth factors to stimulate cells to form blood vessels and recruit other various cell types. In addition, fibrin degradation products stimulate collagen synthesis by cells [53] and have mitogenic effects on smooth muscle cells [54].

Fibrin gel scaffolds embedded with cells have also been investigated for cardiovascular applications [42, 55]. A fibrin gel was previously developed to aid in delivery of growth factors and to provide stem cells with a suitable environment for survival [56, 57]. The fibrin patch could induce entrapped hMSCs (human mesenchymal stem cells) to differentiate toward vascular lineages. Because the cells were entrapped within the fibrin gel as it was formed, cell engraftment was higher than direct cell injections. One key advantage to using fibrin gels to treat infarct cardiac tissue is that it is FDA approved for clinical applications.

Although cardiac function improves with fibrin gels embedded with cells, little cell integration into host tissue has been observed. Low cell retention and integration can be attributed to the harsh environment the cells encounter. The pumping of the heart washes cells away. The injured heart has type I collagen fibers replaced with type III fibers, which has worse cell adhesion properties [52]. The hostile environment created
from the injury can reduce cell survival due to the cytokines and inflammatory factors present in the damaged myocardium [58]. The hypoxic conditions also make it hard for cells to survive. Increasing cell engraftment provides superior functional benefits of cell therapy [59]. Even with low cell engraftment to host tissue, improvement in cardiac function suggests enhancement of long term cell engraftment and migration into host tissue must be pursued.
1.4 Background of Cell Sheet Technology

1.4.1 Introduction

Cell sheet technology enables a sheet of interconnected cells to be layered to form tissue-like and organ-like structures. The rapid progression of the technology has emerged as a novel approach for cell based therapy. The most profound advantage of cell sheet technology is that it could achieve high cell density as well as retain cell-cell junctions and deposited ECM. In a series of studies, Okano’s group proved the superiority of cell sheet transplantation compared with cell injection for cardiac repair [60-64]. Using thermo-responsive culture dishes, confluent cardiac cell sheets were harvested without any enzymatic treatment. The stacked cardiac cell sheets developed sheet-to-sheet communication via cell-cell junctions and could pulsate synchronously [63]. After cardiac cell sheet transplantation, increased cell survival rate compared with cell injection was observed and cardiac function was significantly improved. Importantly, successful engraftment of cell sheets to the host tissue was demonstrated [61]. In addition to cardiac applications, cell sheet technology has been used to treat many other diseases and has generated promising results. For patients with bilateral total limbal stem-cell deficiency, autologous epithelial cells were harvested from oral mucosa and grown into cell sheets for transplantation onto the ocular surface. The presence of ECM in the cell sheet provided sufficient adhesion to stabilize it to the host eye without sutures. Visual acuity was dramatically improved 2-8 weeks after the epithelial cell sheet transplantation [65]. Layered fibroblast cell sheets are a novel lung air leak sealant [66]. The feasibility of using cell sheet technology to treat bone fractures [67], periodontitis [68], esophageal cancer [69], diabetes [70] and liver disease [71] have all been explored. The results, both
experimental and clinical, have demonstrated the efficacy of cell sheet technology as an effective approach for regenerative medicine.

Technologies that enable fabrication of viable, transplantable cell sheets for various tissue engineering applications offer to significantly impact the field. Currently, the majority of cell sheet studies use thermo-responsive systems for cell sheet detachment. However, other responsive systems began showing their potentials for cell sheet harvest. This review provides an overview of the current techniques in creating cell sheets using different fabrication techniques. Their mechanism, approach and applications for cell detachment have been included.

1.4.2 Thermo-responsive Systems

The use of thermo-responsive systems has created a novel way to harvest cell sheets and greatly accelerated the progress of cell sheet engineering. The most studied thermo-responsive system is a poly(N-isopropylacrylamide) (pNIPAAm) modified surface. pNIPAAm is a well characterized thermo-responsive polymer that undergoes a sharp coil-globule transition at its lower critical solution temperature (LCST) of 32°C in aqueous solution, changing from a hydrophilic state to a hydrophobic state [72]. Therefore, a surface retaining insoluble pNIPAAm is hydrophobic, allowing cells to attach, when the temperature is higher than 32°C. The surface becomes hydrophilic to release cells once the temperature falls below 32°C. The critical temperature of pNIPAAm can be further altered by copolymerization using hydrophilic or hydrophobic monomers for broad biomedical applications [73].

Different methods have been developed to fabricate pNIPAAm modified thermo-responsive surfaces for cell sheet engineering (Table 1.1). Electron beam (EB)
polymerization is the most widely used method to graft N-isopropylacrylamide (NIPAAm) onto tissue culture polystyrene (TCPS) for cell sheet engineering [74]. Briefly, TCPS dishes are uniformly coated by NIPAAm monomer solution (in 2-propanol) and irradiated with 0.3 MGy EB. The non-grafted NIPAAm monomers are rinsed off with deionized (DI) water after irradiation and the resulting pNIPAAm-modified dishes are used to culture cells. Okano’s group, the pioneers of cell sheet engineering, has extensively studied the surface properties of the pNIPAAm-modified dishes fabricated by EB polymerization and reported that cell adhesion is greatly affected by the thickness of the grafted NIPAAm layer. A thin layer, ~15.5 ±7.2 nm, with a low density of pNIPAAm allows for cell attachment at 37°C and detachment from the surface at 20°C [75]. However, a thick layer, 29.3 ± 8.4 nm, with a high density of pNIPAAm was found to have no cell adhesion at 37°C although both graft thicknesses show a change in wettability between temperatures above and below LCST. According to their studies, an effective thickness of a grafted pNIPAAm layer should be between 15 nm and 20 nm to obtain optimal cell attachment and detachment in response to temperature changes. This electron beam polymerization of NIPAAm has been used in cell sheet technology for many different cell types including keratinocytes, corneal and oral mucosal epithelia cells, myocardial cells, hepatocytes, and skeletal myoblasts [75-79].
Table 1.1: Comparison of different types of pNIPAAm modified thermoresponsive surfaces for cell sheet engineering [80].

<table>
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<th>Fabrication Method</th>
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<th>Detachment Time</th>
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<td>Electron Beam Polymerization</td>
<td>Tissue Culture Polystyrene (TCPS)</td>
<td>Keratinocytes, Corneal and Oral mucosal epithelia cells, Myocardial cells, Hepatocytes, Skeletal myoblasts</td>
<td>1 h at 20°C</td>
</tr>
<tr>
<td>Plasma Polymerization</td>
<td>Silica Wafer, Glass, TCPS</td>
<td>Bovine artery carotid endothelial cells, Retinal cells</td>
<td>2 h at 20°C</td>
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<tr>
<td>UV irradiation</td>
<td>Polydimethylsiloxane, Poly (ethylene terephthalate)</td>
<td>Smooth muscle cells, Retinal pigment epithelial cells</td>
<td>30 min</td>
</tr>
<tr>
<td>Solvent Cast</td>
<td>Not Grafted; solid support underneath: TCPS, Glass</td>
<td>3T3 Fibroblasts, HUVEC</td>
<td>20 min at 4°C</td>
</tr>
<tr>
<td>Spin-Coated w/APTES</td>
<td>Si Wafer, Glass</td>
<td>Fibroblasts, MSCs</td>
<td>2 min at 20°C</td>
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Another tested method to graft pNIPAAm is through vapor phase plasma polymerization. A plasma glow discharge of NIPAAm monomer vapor was used to deposit pNIPAAm onto solid surfaces such as silicon, glass, and TCPS [81]. The deposition process includes an 80W methane plasma deposition followed by NIPAAm plasma deposition with stepwise decreasing powers from 80 to 1 W for 30 min. The pNIPAAm-grafted surfaces are then rinsed with DI water to remove non-cross-linked molecules [82]. The plasma glow discharge power is slowly reduced to form an adhesion promoting layer on the substrate and to deposit a functional coating at the outer surface [83]. The advantage of plasma polymerization is that it is a one step, solvent free vapor phase coating technique. A major concern over the use of this technique is the loss of chemical functional groups and material functionality in the coating due to possible monomer fragmentation. High power and high temperature conditions during the plasma
polymerization process yields the most stable films. However, high temperature also reduces the chemical functionality of the grafted monomer [84]. Pan et al. [81] were the first group to overcome the fragmentation problem by finding an optimal balance between power and temperature conditions during the polymerization of NIPAAm monomer. Contrary to EB polymerized pNIPAAm, the thickness of pNIPAAm film fabricated by plasma polymerization does not have an impact on cell attachment. Cell studies showed no difference between different batches of plasma polymerized films when the batches varied in thickness from one another [85, 86]. Thus, cell adhesion and proliferation are independent of plasma deposited film thickness. Grafting of pNIPAAm through plasma polymerization has been used to detach bovine artery carotid endothelial cells and retinal cells [85-88].

An alternative approach to graft pNIPAAm is through ultraviolet (UV) irradiation. Plasma-induced UV or heat polymerization of NIPAAm monomers has been successfully immobilized on polydimethylsiloxane (PDMS) surfaces and applied to smooth muscle cell (SMC) sheet detachment [89]. The PDMS surface is activated by Argon plasma to generate peroxides on the surface. These peroxides will decompose by UV light or heat to initiate the polymerization of NIPAAm solution. UV polymerization can also be used to graft pNIPAAm to TCPS surfaces as well. One such method involves the entrapment of copolymers NIPAAm and 4-(N-cinnamoylcarbamide)methylstyrene (CCMS) onto a poly(ethylene terephthalate) (PET) surface. The surface is irradiated with UV light to cross-link the copolymer through dimerization of the cinnamoyl groups [90]. Similar to plasma polymerized pNIPAAm, the thickness of the film does not affect cell attachment and proliferation.
Solvent casting methods have also been employed to create bulk pNIPAAm films. These films are not grafted to a substrate. Instead, the pNIPAAm films are conjugated with collagen and deposited on a solid support such as TCPS or glass. Here, cells attach due to the collagen in the film and detach because of the dissolution of pNIPAAm under cooling conditions [91]. Solvent casting methods have also been used to deposit thick pNIPAAm films which are coated with adhesion molecules such as collagen, fibronectin or laminin. The addition of the overlaying adhesion coating does not affect the detachment rates of cells, as evidenced by culture and detachment of 3T3 fibroblasts and human umbilical vein endothelial cells (HUVEC) [92, 93].

More recently, spin-coating techniques have been developed to deposit pNIPAAm thin films on substrates without the need for expensive equipment such as EB or plasma polymerization equipment. Reed et al. [94] diluted pNIPAAm in hydrochloric acid (HCl) and mixed it with tetraethyl orthosilicate (TEOS). They deposited this solution on glass coverslips and spun the substrate at 2000 rpm for 60 seconds to yield a thin pNIPAAm film on glass cover slips. Cells grew to confluence on the surfaces but detached in clumps instead of as whole cell sheets. Another disadvantage to the system is the dissolution of pNIPAAm, leading to possible cytotoxic effects on the cells. Nash et al. [95] spin-coated pNIPAAm/ethanol mixtures onto thin films on Thermanox disks. The resulting films exhibited a rapid cell sheet detachment for various cell types, ranging from detachment times of 5-10 min, and in some cases ~60 min when 4°C media was placed over the film. However, the authors could not provide a convincing explanation for the mechanism of their system since many others reported that bulk films with similar properties were incapable of attaching and growing cells due to the inability of pNIPAAm to provide
anchor points for cell attachment. The study, nevertheless, indicated the potential of spin-coating pNIPAAm films for cell attachment/growth and rapid cell sheet detachment without the use of expensive equipment.

Our laboratory has developed a novel approach to graft pNIPAAm films onto silica based surfaces [96]. Utilizing a spin-coating technique, thermo-responsive films were deposited on glass slides using pNIPAAm blended with 3-aminopropyltriethoxysilane (APTES). APTES was used to enhance the retention of pNIPAAm on the surface while providing the anchor points needed for cell attachment and proliferation. Additionally, changing the concentration of the adhesion promoting agent allowed for tunable detachment rates. When the surface was cooled, the pNIPAAm chains extended, pushing the cells away from the APTES anchor points, thereby releasing the cells. By changing the ratio of pNIPAAm to APTES, the detachment times of the cell sheets ranged from 2.5 minutes to 40 minutes (Figure 1.1). The spin coating technique used in our research provides a straightforward and economical approach for creating cell sheets in comparison to the traditional techniques used to create other thermo-responsive surfaces.
Electro-responsive systems, which enable cell attachment and release upon an electrical trigger, represent a second common platform for cell sheet engineering. One example of an electro-responsive system was developed by the Mrksich group \[97\]. Electroactive self-assembled monolayers (SAMs) on gold were utilized in their approach to immobilize ligands. The electroactive molecules tethered to the monolayer can be oxidized by applying electrical potential to the gold film releasing the immobilized...
ligands. By selecting the peptide ligand that mediates cell attachment, the system can be electrically switched to permit cell adhesion or detachment. In particular, the peptide Cys-Gly-Arg-Gly-Asp-Ser (CGRGDS) containing RGD as a cell adhesive ligand was tethered to monolayers of alkanethiolates via electroactive O-silyl hydroquinone. Fibroblast cells were cultured on the RGD-presenting monolayers. When an electrical potential of 550 mV was applied to the monolayers for 5 minutes, the O-silyl hydroquinone oxidized to benzoquinone and the silyl ether was hydrolyzed, releasing the RGD containing peptide from the monolayer with the attached cells. This approach has been used to control cell adhesion, pattern cells and activate cell migration [97, 98].

Fukuda et al. [99] exploited a similar electro-responsive system for efficient cell sheet detachment. The RGD-containing oligopeptide CCRGDWLC was designed to form a gold-thiolate bond on a gold-coated substrate. Fibroblasts were cultured on the substrate for 7 days and grown into a confluent cell sheet. The viable cell sheet was detached within 10 minutes after application of -1.0V electrical potential to the surface. The cell sheet detachment was caused by the peptide desorption from the gold substrate by electrical stimulus.

Another example of an electro-responsive system for controlling release of a cell sheet is polyelectrolyte-modified surfaces. Polyelectrolytes adsorb to oppositely charged surfaces due to electrostatic interaction and desorbs from the conducting substrates upon electrochemical polarization. Based on this mechanism, an electro-responsive system was developed for harvesting cell sheets. The Voros group had shown that a poly(L-lysine) (PLL) grafted poly(ethylene glycol) (PEG) monolayer on metal oxide can be desorbed by electrochemical polarization of the substrate [100]. By exploiting this concept, they were
able to desorb a PLL-g-PEG/PEG-RGD monolayer from indium tin oxide (ITO) by applying a short positive potential [101]. More specifically, the adsorbed PLL-g-PEG/PEG-RGD monolayer allows cells to attach to the metal oxide substrate. When the monolayer desorbs, the cells lose their attachment points and detach. The thickness of the polyelectrolyte layer has been shown to impact cell adhesiveness. Multiple layers lead to thicker, softer films with weaker adhesive properties. This can be overcome by increasing the pH at which polyelectrolyte films are assembled, leading to stiffer films and better adhesive properties. Several different cell types have been grown to confluence and detached as cell sheets from these surfaces including hepatocytes, fibroblasts, endothelial cells, and HeLa cells.

In order to create more complex cell sheets with multiple cell types, Voros et al. [102] used an electrochemically responsive platform for micro-patterning and release of cell sheets. Biointerfacial properties of the micro-patterned regions can be switched electrochemically by controlling the dissolution and adsorption of polyelectrolyte coatings. Micro-patterns were created by insulating SU-8 on transparent ITO in the desired formations through photolithography methods. A layer of a polyelectrolyte was placed over the platform while the polyelectrolyte was shielded from the electrochemical treatment by a photoresist stencil. Cells grown on the ITO detached upon electrochemical dissolution of the polyelectrolyte substrate. Cells grown on the neighboring weakly adhesive substrate detached through the contractile forces generated by the cells detaching from the ITO domains. This approach allows high precision in cell patterning for cell sheet formation. A drawback of using polyelectrolyte-modified surfaces to detach
cell sheets is that electrochemical dissolution of polyelectrolyte coatings could cause local pH change, which may be harmful to sensitive cells.

1.4.4 Photo-responsive Systems

Light is an ideal stimulus for responsive surfaces because it can be controlled with high spatial and temporal resolution. One strategy of developing photo-responsive surfaces is to use light to change the wettability of a surface. Metal oxides, mainly Zinc oxide (ZnO) and Titanium dioxide (TiO₂), are the most studied for this application since their wettability can be switched between hydrophilicity and hydrophobicity by light illumination. In a recent published article, a light-induced cell detachment approach has been investigated using TiO₂ due to its biocompatibility and wettability variation when illuminated at a cell safe wave length of 365 nm [103]. Mouse calvaria-derived, pre-osteoblastic MC3T3-E1 cells were seeded on TiO₂ nanodot films that were coated on a quartz substrate. The data demonstrated detachment of cell sheets after 20 minutes of UV365 illumination. The exact mechanism of cell detachment is still under investigation; however, it is probably caused by the release of adsorbed adhesive protein when the surface becomes more hydrophilic under illumination.

Photo-responsive molecules have been explored to control adhesion and detachment of cells. Spiropyran is a photosensitive molecule that isomerizes when exposed to UV light from a hydrophobic spiro conformation to the polar hydrophilic zwitterionic merocyanine conformation [104]. Higuchi et al. [104] have used this concept to graft spiropyran to poly(methyl methacrylate) (PMMA) and use the developed copolymer to realize UV-regulated detachment of fibrinogen, platelets, and mesenchymal stem cells.
Photo-responsive surfaces can also be fabricated by incorporating spiropyran into the side chains of pNIPAAm. Because irradiating spiropyran with UV light enhances cell adhesion, incorporating spiropyran into pNIPAAM can prevent cell detachment when the surface is cooled. The advantage to this system is the ability to spatially control detachment of cells for patterning. Edahiro et al. [105] used this concept by attaching cells at 37°C to a pNIPAAm-spiropyran surface. They UV-irradiated a region and then cooled and washed the entire surface. Cells remained adhered to the UV irradiated region while cells in the other region detached. By irradiating the unattached portion with visible light (400-440 nm), the spiropyran isomerizes back into the nonionic structure and the cells can be detached by cooling the surface. The researchers demonstrated the ability to spatially control cell attachment through the use of a photomask. This system can be used to control the location of a particular cell type in a co-culture and harvest the cell sheet with the intended arrangement.

Another photosensitive molecule being explored is azobenzene which isomerizes by UV light from trans to cis form. The isomerization is fully reversible and extremely fast. RGD peptides were coupled with azobenzene derivatives and coated on PMMA disks [106]. The azobenzene is responsible for controlling the spacing between RGD peptides and PMMA disks leading to either attachment or detachment of cells. Cell adhesion was achieved when the azobenzene derivative was in its trans form, while cells detached when azobenzene isomerized into its cis form after irradiating with 366 nm light. The light irradiation caused the azobenzene to shorten, moving RGD peptides closer to the surface, resulting in cell detachment. Although cell sheet detachment was
not shown, the photo-responsiveness of azobenzene can be utilized to control cell adhesion.

RGD peptides have also been used with a photocleavable linker to provide a photo-responsive surface for cell adhesion and release. Wirkner et al. [107] used a photolabile 4,5-dialkoxy 1-(2-nitrophenyl)-ethyl group to attach to the free amine groups of a surface. A tetraethyleneglycol (TEG) spacer was included in the structure enabling the photolabile group to also bind to RGD peptides. The RGD peptides provide a surface for cells to attach, while the photolabile group links the RGD to the substrate. Upon light exposure, the chromophore was photocleaved, removing the linker along with the RGD and the bound cells from the substrate. Using this method, about 85% of adhered HUVEC were detached from the surface, while the remaining cells displayed a rounded morphology. The inability of some cells to attach can be attributed to the incomplete cleavage of the photolinker. Although all the cells did not release, this technology has the ability to be applied to any substrate as long as the linker is properly designed.

A vastly different approach to releasing cells from surfaces using light was taken by the Mohwald group [108]. Fibroblasts were cultured on gold nanoparticle based surfaces. Gold nanoparticles have strong photoabsorption in the green spectral range. They irradiated the surface with a green laser (532 nm), causing a photochemical mechanism to produce reactive oxygen species (ROS). The ROS caused damage to cell membranes at the cell-surface interface, releasing the cells from the substrate. The cells did not immediately detach from the surface, but rather take up to 24 hours to fully detach. One advantage to the system is that the surface is able to recover, allowing the reattachment of cells in the irradiated areas within 72 hours. This property can be used to
spatially pattern cells, and create co-culture cell sheets by controlling the areas the green laser is irradiated and seeding different cell types once the surface has recovered to allow reattachment.

Another photo-responsive material explored for cell attachment and detachment is a methacrylate with a photocleavable unit, 4-[4-(1-methacryloyloxyethyl)-2-methoxy-5-nitrophenoxyl]butyric acid (PL) [109]. These PL groups were placed in the side chains of poly(2-methacryloyloxyethyl phosphorylcholine-co-n-butyl methacrylate) (PMB). PMB is a bioinert material preventing the adhesion of cells to its surface. The PL groups provide adhesion points for cell attachment. Under irradiation with 365 nm UV light, the 2-nitrobenzyl group is cleaved off, causing loss of the adhesion points on PL groups. Cells were seeded on the PMB-PL surface and allowed to attach for 4 hours. The surface was irradiated with UV light for 60 seconds, resulting in detachment of 50% of adhered cells. Cells seeded on previously irradiated regions showed no attachment to the surface, indicating the loss of the adhesion points on the PL groups. The photo-responsive surface used here shows the ability to rapidly detach cells although PL group density needs to be optimized to allow for complete detachment.

1.4.5 pH-responsive Systems

pH-responsive systems have been extensively studied as drug delivery carriers because of the varying pH in the human body that can be used to direct response. For example, tumor tissue has an extracellular pH of 6.5-7.2, which is lower than the normal pH of 7.4 [110]. Thus acid triggered pH-responsive systems can be used to specifically deliver anti-cancer drugs. The remarkable change in pH along the gastrointestinal tract (from acid in stomach to basic in the intestine) has also served as potent stimuli for pH-
responsive systems of oral drug delivery. Ionisable polymers containing either acidic or basic pendant groups in their structure that can accept or release protons in response to pH changes in the surrounding environment are candidates for pH-responsive systems. Examples of common polymers used in pH-responsive systems are: polyacrylamide (PAAm), poly(acrylic acid) (PAA), poly(methacrylic acid) (PMAA), poly(2-diethylaminoethyl methacrylate) (PDEAEMA) and poly(N,N-dimethylaminoethylmethacrylate) (PDMAEMA) [73, 111].

Only a few studies, however, have been performed using pH-responsive systems for cell-based applications due to the limited range of pH (6.8~7.4) for normal cell functions. Ehrbar et al. [112] showed the feasibility of controlling cell sheet detachment by either local or global pH lowering. The pH responsive substrates were formed by alternate layering of cationic poly(allylamine hydrochloride) (PAH) layers and anionic poly(styrene sulfonate) (PSS) layers on a conductive ITO surface. Placenta derived mesenchymal stem cells (PD-MSCs) easily adhered and proliferated to confluence on polyelectrolyte surfaces. Because this system is similar to an electro-responsive system, an electrical trigger with a current density of 30uA/cm2 was found to detach the cells with their ECM intact within 10-20 min. The Ehrbar group hypothesized that one of the mechanisms driving cell sheet detachment was the drop in local pH at the cell-substrate interface. Thus, instead of using an electrical trigger to detach cell sheets, they tried inducing the cells to detach by decreasing the bulk pH through change of culture media pH. A range of pH 5.0 to 7.4 resulted in no change in cell adhesion, while a pH of 4.0 resulted in complete cell sheet detachment within 2-3 min. After conducting viability assays and mesodermal plasticity experiments, the detached PD-MSCs retained their
viability and differential potential after exposure to acidic media. Thus, the pH responsive substrate used in this study provided another method to release cell sheets from a surface, although caution must be taken for cells sensitive to pH change.

Chen et al. [113] use chitosan, a natural polymer, to attach/detach cells with a change in pH. The primary amine of the glucosamine residue on chitosan makes the polymer a pH responsive cation [114]. When the cells are cultured on the chitosan in a medium with a pH of 7.2, the fibronectin excreted from the cells is adsorbed on the chitosan substrate. The adsorption allows the cells to attach to the chitosan surface. Chitosan has an isoelectric point at pH 7.4 [115]. At a pH of 7.2, the chitosan surface becomes positively charged, allowing for the adsorption of the negatively charged fibronectin. When the pH of the medium is raised to 7.65, the chitosan surface deprotonates and exhibits a positive charge. The fibronectin desorbs from the surface resulting in the detachment of cells. Although Chen et al. [113] did not demonstrate the detachment of an intact cell sheet, they have shown promise for creating cell sheet modules using pH responsive chitosan at physiological conditions.

1.4.6 Magnetic Systems

The use of magnetic force to aid in attachment and release of cells has been studied through ferrous based nanoparticle labeling of cells. Magnetite (Fe₃O₄) nanoparticles were mixed with N-(a-trimethylammonioacetyl)-didodecyl-D-glutamate chloride (TMAG) to create magnetite cationic liposomes (MCLs). The electrostatic interaction between positively charged MCL and negatively charged cell membrane resulted in a high adsorption rate of magnetite nanoparticles in target cells [116]. Cells labeled with magnetite nanoparticles are easily influenced by an external magnetic field,
resulting in an accumulation of cells toward the source magnet. Ito et al. [117] exploited this property to attach and release keratinocytes using magnetic force. Magnetite labeled keratinocytes were cultured on ultra low attachment plates that had a covalently bound hydrophilic and neutral hydrogel on its surface. Upon placement of a neodymium magnet under the plate, cells evenly spread on the surface, while no cell spreading was observed in the absence of a magnet. In order to detach the cell sheet, the magnet was removed from underneath the plate and a polyvinylidene fluoride (PVDF) membrane was placed on the surface of the magnet. The magnet was moved on top of the cells and the magnetic force resulted in the keratinocyte sheet sticking to the PVDF membrane on the magnet surface. With the use of the magnet, the cells could be transferred to a new location, and the PVDF membrane could subsequently be removed from the magnet to detach the cells from the magnet. No cell toxicity has been reported by the use of the magnetite nanoparticle technology and this technology has been applied to create and transfer cell sheets of many different cell types including keratinocytes, cardiomyocytes, hepatocytes, endothelial cells, mesenchymal stem cells, and retinal pigment epithelial cells [117-121]. Although the system is effective in releasing and transferring cells, the detached sheets are not cell monolayers, but aggregates of the detached cells clumped together forming several layer thick sheets.

1.4.7 Key Challenges and Future Directions

Responsive systems triggered by different stimuli have been developed to enable the detachment of confluent cell sheets, which is one of the most critical steps in cell tissue engineering. While mounting evidence has demonstrated the potential of these systems [62, 64, 67, 71, 92, 118, 122-125], more cell studies are needed for further
optimization. Current commercially available responsive surfaces for cell sheet detachment are very expensive especially in consideration for the number of cell sheets required to layer them into 3D tissue constructs. In addition, most of the approaches involve non-easily accessible facilities and complicated procedures, which will hinder tissue culture laboratories in custom designing their own surfaces for specific downstream applications. Therefore, one of the major challenges to overcome in the future is developing simple and economical methods for responsive surface fabrication, which will greatly encourage researchers to exploit cell sheet engineering. Another challenge is to accelerate the cell sheet detachment time. It takes upwards of 40 minutes for a single cell sheet to detach using the most popular commercial available thermo-responsive surface (UpCell®) [126]. The exposure to prolonged cooling will affect cell function and dramatically increase the time needed for cell sheet stacking. There are several aforementioned technologies discussed in this chapter that strive to address improving cell detachment times. But, these technologies are yet to be fully adapted in the field. With further improvement in techniques and incorporation with other microfabrication methods (e.g. micropatterning), cell sheet engineering will bring tissue engineering constructs one step closer to clinical applications.

1.5 Dissertation Outline

A new process for rapid cell sheet detachment must be developed for ease of cell sheet manipulation and better cell function. Current cell sheet detachment techniques require lengthy periods of time to adequately harvest cell sheets, often taking upwards of 40 minutes. Prolonged exposure to cool temperatures during the cell detachment process can negatively affect cell function by inhibiting certain metabolic processes. From a
research or clinical perspective, it would be more economical to obtain cell sheets in a short period of time. Production of cell sheets can only be achieved once properly functioning thermoresponsive substrates can consistently be made. In this research, a novel approach to create thermoresponsive surfaces for rapid cell sheet detachment through spin coating techniques was developed (Chapter II). Physical properties of the novel thermoresponsive substrate were characterized. The work described in Chapter II was previously published in the journal of Acta Biomaterialia [96].

Once cell sheets could consistently be created, a method to facilitate single cell sheet manipulation and multilayer stacking was explored (Chapter III). Consistent layering of cell sheets was achieved after a standard protocol was set for using a 1mL micropipette for manipulation. Low cell engraftment rates in current myocardial infarction therapeutic strategies led to the study of cell sheets in hopes of improving engraftment and integration. Chapter III explores the interaction between cell sheets and fibrin gels. Information obtained about cell sheet interactions with fibrin gels help give an insight toward future cardiovascular applications.
CHAPTER II

DEVELOPMENT OF A NOVEL THERMORESPONSIVE SYSTEM FOR CELL SHEET ENGINEERING

2.1 Introduction

The use of thermo-responsive polymers (TRPs), especially poly (N-isopropylacrylamide) (pNIPAAm) and its co-polymers, has attracted considerable attention in the area of cell sheet engineering [83, 127, 128]. For cell sheets formed on TRPs, a simple change of temperature allows cells to spontaneously detach from these surfaces. Compared to proteolytic enzymes (e.g. trypsin) or mechanical agitations, using TRPs to harvest confluent cell sheets minimize damage to cells and their excreted extracellular matrix (ECM), thus preserving their biological functions [93]. Tissue engineering constructs based on cell sheets allow increased cell-cell interactions and eliminate the risk of immunogenic materials present in scaffolds. Furthermore, cell sheets harvested from TRPs can be patterned and assembled together to mimic the microarchitecture of native tissue, which is crucial for functional tissue regeneration.

TRPs have the ability to respond to a change in temperature and can be classified into two main types: TRPs possessing a lower critical solution temperature (LCST) and TRPs possessing an upper critical solution temperature (UCST). pNIPAAm is one of the most commonly used TPRs that presents an LCST. Other polymers with thermoresponsive properties include poly(N,N-diethylacrylamide) (PDEAAm), poly(N-
vinlycaprolactam) (PVCL), poly[2-(dimethylamino)ethyl methacrylate] (PDMAEMA) and poly(ethylene oxide) (PEO) [129]. Among all the TRPs, pNIPAAm is of special interest in bioengineering applications because of the phase change that it undergoes in a physiologically relevant temperature range. It has a lower critical solution temperature (LCST) of 32°C in water. The polymer chains reside in a collapsed hydrophobic state above the LCST and in an extended hydrophilic state below LCST [130]. In most studies, a grafted pNIPAAm thin layer (< 30 nm in thickness) or a combination of adhesive proteins with bulk pNIPAAm allows cell attachment above LCST and detachment when cooled below LCST.

Two primary approaches have been adopted to covalently graft pNIPAAm chains onto tissue culture polystyrene dishes. One method uses electron beam irradiation [78] and the other uses plasma polymerization [131-133]. Although the covalently grafted pNIPAAm surface is a major achievement in cell sheet engineering, grafting pNIPAAm chains to form an optimal layer for cell seeding involves non-easily accessible (e.g. e-beam) facilities and complex fabrication methods. The use of commercially available thermo-responsive dishes is impractical for long term tissue culture and large scale investigation because of the price tag of those dishes (over $20 for one 35mm dish at the time of writing [95]). Therefore, a more practical approach to fabricate thermo-responsive surfaces for cell sheet harvesting is desired.

Recent publications have focused on attempts to simplify the production of pNIPAAm films utilizing commercially available pNIPAAm for cell culture studies [95, 122, 134, 135]. Bulk pNIPAAm films are generally not favorable for cell attachment and growth when not grafted on to the substrate [134, 136]. A recent study [95] has indicated
that pNIPAAm films spin-coated at a high enough speed (≥ 1000 RPM) could serve as thermo-responsive substrates for cell culture and cell sheet harvesting. Nonetheless, the underlying mechanism on why these spin-coated pNIPAAm films work while other bulk pNIPAAm films do not is unclear. Adhesive promoting agents, such as ECM proteins, are normally applied to enhance cell attachment and growth for bulk pNIPAAm films [93, 122, 132]. However, applying animal ECM proteins is expensive and in many cases could introduce the risk of disease transfer [95].

In this study we explored the possibility of using a common adhesion promoter, 3-aminopropyltriethoxysilane (APTES), to enhance the retention of spin-coated pNIPAAm films onto the surface of a glass substrate, thereby preventing it from dissolving in cool aqueous medium. The pNIPAAm/APTES film would function as a thermo-responsive substrate for cell culture and cell sheet detachment. APTES has been shown to enhance cell attachment and proliferation [137] due to its amine functionality. APTES also improves retention of polymer thin films on a substrate surface due to the formation of a complex 3-D network [138]. We hypothesized that curing a blend of APTES and pNIPAAm would cause the pNIPAAm chains to be interlocked in the APTES network as the network is being formed during the curing process. The amino groups of the APTES molecules residing at the surface of the pNIPAAm/APTES network would be exposed to the surrounding environment, especially when the pNIPAAm chains collapse at a temperature greater than its critical temperature. Exposed amino groups would promote cell attachment and proliferation. Upon cells growing to confluence and forming a sheet, lowering the temperature of the cell culture medium, or replacing it with fresh cold medium, would hydrate the pNIPAAm chains and change their conformation to the
extended form. This phase change would bury the APTES surface amino groups and push the cell sheet away from the surface, allowing the detachment of the confluent sheet of cells (Illustration 2.1). Furthermore, manipulating the amount of APTES blended in pNIPAAm/APTES films would control the cell sheet detachment rate, which could play a significant role in future tissue engineering applications.

Illustration 2.1: Sketch of Thermoresponsive Substrate. The sketch representations of the polymer/air (or polymer/water) and the polymer/substrate interfacial regions of the pNIPAAm/APTES films are shown. (A) the as spin-coated film, (B) the film cured at high temperature (≥ 160 ºC) for 3 days and submerged in a cell culture medium at 37ºC, and (C) the cured film with cells placed in a culture medium at room temperature (~ 25ºC). The solid, dash and dotted lines represent APTES molecules, the backbone of the pNIPAAm and the isopropyl acrylamide side chains, respectively. At room temperature, water molecules form hydrogen bonds with the isopropyl acrylamide side chains and the APTES molecules. The dotted box in each of the sketches indicates the omitted information of the complexity of the bulk pNIPAAm/APTES blend. Sketch is not drawn to scale.
2.2 Materials and Methods

2.2.1 Materials

pNIPAAm, MW 20-25 kg/mol, and 3-aminopropyltriethoxysilane (APTES) were purchased from Sigma Aldrich (St. Louis, MO, USA). Glass slides and P(100) test type silicon-wafers were purchased from Fisher Scientific (Waltham, MA, USA) and Silicon Quest International (Santa Clara, CA), respectively. All other chemical reagents were purchased from Sigma Aldrich unless otherwise indicated.

2.2.2 Preparation of glass and silicon wafer (Si-wafer) substrates

Glass slides and Si-wafers were cut into squares with a surface area of ~ 1cm². Slides and wafers were immersed in freshly prepared piranha solution (70 vol.% of concentrated H₂SO₄ and 30 vol.% of 30% H₂O₂) for 1 hour at 100°C to remove organic contaminants. After decanting the piranha solution, the slides were thoroughly rinsed with deionized (DI) water and dried with nitrogen (N₂) gas. Afterwards, the slides and/or wafers were oxidized in a UV/Ozone Cleaner (Jelight Company Inc, Irvine, CA) for 10 minutes for further cleaning.

2.2.3 Preparation of pNIPAAm/APTES solutions

A 3% wt. pNIPAAm solution and a 10% wt. APTES solution in ethanol (Pharmco-AAPER, Inc., Shelbyville, KT) were prepared separately. Solutions having pNIPAAm to APTES ratios (by mass) of 90:10, 80:20, 60:40, and 40:60 were prepared by mixing the proper ratios of the two above solutions and a small amount of ethanol to make a final solution containing ~ 3 wt.% of total solute. The solutions were subsequently filtered to remove particulates through an Acrodisc® CR 13mm Syringe filter with a 0.45μm PTFE membrane (Pall Life Sciences, Co., Ann Arbor, MI).
2.2.4 Preparation of pNIPAAm/APTES films

Polymer blend films were produced by spin coating the pNIPAAm/APTES mixed solution onto pre-cleaned substrates for 30 seconds at 2000rpm (p-6000 Spin Coater, Specialty Coating Systems, Inc, Indianapolis, IN). The spin-coated samples were cured inside a vacuum (< 100 mTorr) oven (VWR International, Radnor, PA) for 3 days at the following temperatures: 115°C, 145°C, 160°C, 175°C, and 205°C.

2.2.5 Water contact angles of pNIPAAm/APTES thin films

The sessile drop method was utilized to measure the static water contact angles on the pNIPAAm/APTES films. Contact angles were recorded using a goniometer (Ramé-Hart Instrument Co, Netcong, NJ) with a modified stage, which was heated to ~ 45°C and then placed in a petri dish. The sample was positioned on top of the heated stage, and a drop of DI water was placed on the sample. The temperature of the stage was decreased continuously to ~ 25°C by adding chilled water in the petri dish. As the temperature decreased, the sessile drop images of DI water on the sample were recorded using a Diamond VC500 one-touch video capture system (Diamond Multimedia, Chatsworth, CA) while the associated elapsed time was manually recorded. Still images were extracted out of the video clip and the contact angle was measured using ImageJ software (National Institutes of Health, Bethesda, MD).

2.2.6 Thickness of pNIPAAm/APTES films

A manual photoelectric ellipsometer (Rudolph Instruments, Inc., Fairfield, NJ) was used to measure the thicknesses of different pNIPAAm/APTES films on Si-wafers at a 632nm wavelength. A refractive index of 1.48 for pNIPAAm, measured by Tu et al. [139], was used for thickness calculations for the blended films, which might have a
slightly different refractive index compared to a pure pNIPAAm film. Thickness measurements were taken of films made with different pNIPAAm/APTES ratios and/or cured at different temperatures. Samples were measured before and after immersion in cold DI water (immersed samples were dried by a stream of nitrogen before measurement). Two thickness measurements for each sample, and multiple samples (n=3) treated under the same condition were measured to provide the statistical values.

2.2.7 Diffuse reflectance infrared Fourier transform (DRIFT) of pNIPAAm/APTES films

DRIFT spectra were obtained from films spin-coated on Si-wafers and cured under identical conditions as films on glass slides. A small sample, ~ 6 mm x 6 mm in size, was placed at the center of the sample holder after ensuring the IR beam was properly focused on the sample. A small hemi-spherical dome (with a base diameter of ~ 2 cm) covered the sample. After securing the dome by fastening the screws, argon (Ar) gas was allowed to flow into the dome for ~ 5 minutes to replace the air inside and minimize water content. Then, a single beam spectrum was recorded using a Nicolet Magna 560 at 2 cm\(^{-1}\) resolution using the Harrick Praying Mantis diffuse reflectance accessory at room temperature. Absorbance was obtained by Abs. = - log(I/I\(_o\)), where I and I\(_o\) are the normalized intensities of the sample and of an oxidized Si-wafer (i.e. reference) respectively. Normalization was achieved by dividing the intensity at a particular wavelength by the maximum intensity of the spectrum. Maximum intensity for all the spectra obtained ranged from 1.60 to 1.79, depending on how the IR beam was focused on the sample surface. Normalization was applied to minimize variation of the IR beam intensity used for scanning the samples under different scanning conditions and operated by different operators.
2.2.8 Cell attachment

Human mesenchymal stem cells (hMSCs) (Lonza, Walkersville, MD) were cultured in serum-containing MSCBM medium (Lonza) supplemented with MSCGM SingleQuots (Lonza) according to manufacturer’s specifications. To observe cell attachment, hMSCs (Passage 3) were seeded onto different substrates: a clean glass substrate, a pNIPAAm film, a 90:10 pNIPAAm/APTES film cured at 160°C, and a tissue culture polystyrene (TCPS) dish at a density of $1.5 \times 10^4$ cells/cm$^2$. Contrast phase pictures were taken 4 hours after cell seeding on each of the surfaces using an Axiovert 40 CFL (Carl Zeiss, Inc, Thornwood, NY) microscope equipped with an AxioCam MRm camera (Carl Zeiss, Inc). A MTT cell proliferation assay (Invitrogen, Carlsbad, CA) was performed to compare the proliferative potential of cells on different substrates as previously described [57]. The MTT solution absorbance was measured at a wavelength of 570nm using a Synergy H1 Hybrid microplate reader (BioTek, Winooski, VT). For each group, 3 samples were tested.

2.2.9 Cell sheet detachment

After the cells grew to confluence and formed a cell sheet on the pNIPAAm/APTES film, detachment was induced by replacing the warm cell culture medium with fresh cold medium (4°C). Cells grown on UpCell® dishes were used for comparisons. An Observer Z1Time lapse microscope (Carl Zeiss, Inc) was used to monitor and record cell detachment behavior. The cell images were taken every 10 seconds by an AxioCam MRm camera (Carl Zeiss, Inc) controlled by AxioVision (Carl Zeiss, Inc) software until the cell sheet was completely detached.
2.3 Results

2.3.1 pNIPAAm/APTES blend films support cell attachment and proliferation

hMSCs were seeded on different substrates and observed under a phase contrast microscope. After 4 hours of seeding, low hMSC attachment was observed on glass surfaces (Figure 2.1A). hMSCs exhibited a rounded morphology and poor attachment on pNIPAAm only films (Figure 2.1B) cured at 160°C for 3 days. Most of the hMSCs attached and spread on the 90:10 pNIPAAm/APTES films (Figure 2.1C), also cured at 160°C for 3 days, and on TCPS dishes (Figure 2.1D). Cell proliferation was measured using the MTT assay (Figure 2.1E). There was a significant increase in absorbance after 5 days for hMSCs on pNIPAAm/APTES films and the positive control (hMSCs on TCPS culture dish). The proliferation of hMSCs on the pNIPAAm/APTES films was comparable to proliferation on TCPS dishes after 5 days of seeding. A dip in absorbance was observed for the pNIPAAm only film, which may be due to the medium change performed at day 4. The pNIPAAm only films were not tightly bound to the underneath substrate and were easily rinsed away with the cells during medium change.
Figure 2.1: Cell Attachment and Proliferation on pNIPAAm-APTES Substrates. Human Mesenchymal Stem Cells seeded on (A) glass, (B) pNIPAAm only film on glass, (C) pNIPAAm/APTES blend film on glass, and (D) tissue culture polystyrene dish. Cell spreading occurs on (A), (C), and (D). Cell attachment is greater in (C) and (D) compared to (A), which has several unattached cells. Phase Contrast pictures were taken 4 hours after seeding with a density of $1.5 \times 10^4$ cells/cm$^2$. (E) MTT cell proliferation assay for hMSCs cultured on glass (dashed line), pNIPAAm film (dash-dotted line), 90:10 pNIPAAm/APTES film (solid line) and tissue culture dish polystyrene surfaces (TCPS, dotted-line). Error bars are standard deviations from 3 samples. Scale bar = 200 $\mu$m.
2.3.2 General properties of pNIPAAm/APTES films

Prepared solutions were all transparent, indicating complete dissolution of the pNIPAAm/APTES mixture in ethanol. Films spin-coated on silicon wafers appeared to have a uniform color throughout the sample except at the edges where a slightly thicker film might result. There was a slight color change of each sample before and after curing, mainly from purplish/golden to mostly golden. Films cured at ~ 115°C and 145°C appeared to completely dissolve when dipped into a bath of DI water at room temperature (~ 25°C < LCST). Films cured at higher temperatures (160°C to 205°C) underwent a color change, but were still retained on the substrate.

The ellipsometry measurements (Table 2.1) showed that upon dipping in a DI water bath at room temperature, a layer as thick as ~ 200 - 250 nm was removed from a ~ 300 – 350 nm pNIPAAm/APTES film cured at 160°C and above. Thickness of films after dipping in room temperature cell culture medium was also measured and was found to be not significantly different from that of films after dipping in DI water (data not shown). Repeated dipping of the films did not further reduce the film thickness. Prolonged (e.g. 3 days) immersion in room temperature water showed minimal reduction in thickness (~ 4%; data not shown). Films cured at temperatures of 145°C and below showed that only a very thin layer (< 10 nm) remained after dipping.

For pure pNIPAAm and pNIPAAm/APTES blended films, the infrared spectroscopy signature peaks of pNIPAAm (N-H at ~ 3300 cm\(^{-1}\), C=O at ~ 1640 cm\(^{-1}\), and the doublet for –HC(CH\(_3\))\(_2\) at ~ 1390 and ~ 1360 cm\(^{-1}\)) were observed (Figure 2.2). For blended films, the peaks of free APTES molecules (–OH stretching in =Si-OH at 3050 – 3700 cm\(^{-1}\), 2880 – 2980 cm\(^{-1}\) for –CH\(_2\) stretching, 1475 cm\(^{-1}\) for –CH\(_2\) scissoring)
were hard to distinguish since they overlapped with the –NH and –CH$_2$ peaks of pNIPAAm. The peak associated with cross-linked APTES molecules (=Si-O-Si= stretching at 1050 – 1150 cm$^{-1}$) [140-142] was observed for films cured for three days at a temperature of 160°C or higher, but the peak intensity was relatively weak, as shown in Figure 3C. For the high temperature cured films, the non-hydrogen bonded N-H stretching was also observed at 3442 cm$^{-1}$ [143]. After dipping in cool DI water, all peak intensities decreased while the signature peaks associated with pNIPAAm remained noticeable.

Table 2.1: Film thickness of pNIPAAm/APTES films prepared under different conditions and the cured films before and after dipping in DI water at room temperature. Error bars are standard deviations from 3 samples at each condition.

<table>
<thead>
<tr>
<th>pNIPAAm/APTES</th>
<th>Thickness (nm)</th>
<th>Temperature (°C)</th>
<th>Thickness (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
<td>Initial</td>
</tr>
<tr>
<td>90/10</td>
<td>353.1 ± 16.3</td>
<td>120.6 ± 3.8</td>
<td>396.1 ± 0.7</td>
</tr>
<tr>
<td>80/20</td>
<td>377.4 ± 8.5</td>
<td>128.7 ± 11.6</td>
<td>378.3 ± 1.1</td>
</tr>
<tr>
<td>70/30</td>
<td>389.3 ± 4.4</td>
<td>106.2 ± 3.3</td>
<td>353.1 ± 16.2</td>
</tr>
<tr>
<td>60/40</td>
<td>409.1 ± 1.2</td>
<td>116.8 ± 25.3</td>
<td>293.9 ± 0.6</td>
</tr>
<tr>
<td>50/50</td>
<td>343.1 ± 77.6</td>
<td>118.6 ± 0.7</td>
<td>322.6 ± 0.2</td>
</tr>
<tr>
<td>40/60</td>
<td>379.8 ± 1.9</td>
<td>113.0 ± 0.8</td>
<td></td>
</tr>
</tbody>
</table>
Water contact angles (Figure 2.3 and Table 2.2) confirmed pNIPAAm/APTES films were thermo-responsive. The water contact angle decreased with a decrease in temperature, dropping from an angle of ~70° at a temperature of ~ 40°C to about 30° at a temperature of ~ 26°C. In general, the contact angle change rapidly occurred in the
temperature range of 31-34°C for the pNIPAAm/APTES films cured at 160°C or above for 3 days.

![Graph showing contact angle vs. temperature](image)

**Figure 2.3**: Contact Angle vs. Temperature on a 90:10 pNIPAAm/APTES Substrate. Contact angles are taken above and below pNIPAAm’s lower critical solution temperature (LCST) of 32°C. Contact angles are significantly lower when the temperature is below 32°C than those above LCST. The flattening of the sessile drop at temperatures below 32°C indicates that the surface is more hydrophilic below LCST. An example of a contact angle vs. temperature curve for a 90:10 film cured at 160°C is shown. The contact angle rapidly starts to decrease in the temperature range of 30 and 35°C while slowing its decrease below 30°C. Error bars are standard deviations from 3 samples at each condition.

**Table 2.2**: Contact angles (°) for surfaces treated at different conditions. Error bars are standard deviations from 3 samples at each condition.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Surfaces cured at 160°C with different pNIPAAm/APTES ratios</th>
<th>Surfaces with the pNIPAAm/APTES ratio of 90/10 at different cure temperatures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>90/10</td>
<td>70/30</td>
</tr>
<tr>
<td>40</td>
<td>69.3 ± 7.5</td>
<td>54.2 ± 2.8</td>
</tr>
<tr>
<td>37</td>
<td>66.1 ± 9.3</td>
<td>48.8 ± 2.1</td>
</tr>
<tr>
<td>32</td>
<td>49.6 ± 2.4</td>
<td>42.3 ± 3.6</td>
</tr>
<tr>
<td>27</td>
<td>29.1 ± 0.2</td>
<td>34.8 ± 0.0</td>
</tr>
</tbody>
</table>
2.3.3 Cell detachment on pNIPAAm-APTES films

hMSCs were cultured on pNIPAAm/APTES films to assess individual cell and cell sheet detachment resulting from temperature change. Upon 80% confluence, cell detachment was achieved by replacing the cell culture medium with fresh cold medium (4°C). The cell detachment behaviors are shown in Figure 2.4A-C. Most cells returned to a round morphology as they lost their anchor points, while a few cells that had formed ECM connections remained bound together as shown in Figure 2.4C. All cells detached within 2.5 minutes after adding cold medium (see the video clip in the supplemental data section). Under the same conditions, it took nearly 3 hours for the cells to completely detach from the commercial thermo-responsive cell culture surface (UpCell®).

The cell sheet detachment was assessed the same way as the aforementioned individual cell detachment. After adding cold medium, the confluent cell sheet detached from one end and rolled up (Figure 2.4A). The entire detachment process took approximately 2.5 minutes from the point cold media was added to when all cells had detached from the 90:10 160°C cured pNIPAAm/APTES film (Figure 2.5). Cell viability before and after detachment was also examined using trypan blue staining. Figure 2.4C shows minimal cell death after detachment from our surface, revealing that the detachment process does not harm the cells. UpCell® surfaces were found to take ~11 hours for the cell sheet to completely detach when using the same protocols.

2.3.4 Cell detachment as a function of pNIPAAm-APTES ratios

The proposed mechanism in Illustration 2.1 describes cell detachment as being due to the extension of pNIPAAm chains burying the NH₂ groups of APTES, covering the anchor points needed for cell attachment. Increasing the amount of APTES in the
pNIPAAm/APTES blend would lead to an increased amount of anchor points for cells. This would lead to slower cell detachment upon cooling. A simple experiment was set up to test this hypothesis by creating films of different pNIPAAm/APTES ratios, all cured at a temperature of 160°C for 3 days. Cells were seeded on each of these films and cell detachment times for each surface were recorded. Figure 2.6 shows the different cell detachment times for the surfaces with various pNIPAAm/APTES ratios. By increasing APTES, cell detachment times increased from approximately 2.5 minutes for 90:10 films to ~ 40 minutes for 40:60 pNIPAAm/APTES films, while the 20:80 pNIPAAm/APTES films showed no detachment.

Figure 2.4: Cell Sheet Detachment and Viability. Human MSCs are seeded on 90:10 pNIPAAm/APTES films cured at 160°C until confluence is reached. (A) The “rollup” of the cell sheet as the cells detach. Upon confluence, cells are connected together because of their deposition of ECM. The “rollup” of the cells indicate that the ECM is undisturbed upon detachment and that the cells remain bound together as cell sheets. (B) Trypan blue staining of attached cells prior to cooling. (C) Detached cells from cooling are re-seeded on a tissue culture dish and stained with trypan blue after 4 hrs is allowed for attachment/spreading. Dark cells indicate the cell membrane has been damaged and the cell has died. Scale bar denotes 200 µm.

90:10 pNIPAAm/APTES film surfaces were tested for reusable functionality. After detaching cells, surfaces were rinsed with cold media, and then reseeded with hMSCs. Cells were able to reattach and reach confluence. Once again, cold media was added over the surfaces and the cells detached as a cell sheet within 2.5 minutes. When
surfaces were tested for reseeding/detachment for a third time, proper cell spreading was not observed. Instead, cell attachment was sporadic although patches of cells were still able to detach upon cooling.

Figure 2.5: Comparison of Cell Sheet Detachment Times. Prior to reaching full confluence, individual cells detach and exhibit a round morphology after cold medium is added. Early stages of cell sheet formation are observed with cells remaining attached to each other during detachment. Elapsed time is given after addition of cold medium over the surface. Cell detachment from (A-C) 90:10 pNIPAAm/APTES films cured at 160°C, and from (D-F) the commercially available thermoresponsive UpCell® Culture Dish. Compared to UpCell® culture dish, our surface allows a much quicker cell sheet detachment (< 2.5 minutes). Total cell detachment is observed 2hrs and 50 minutes after cold medium is added to the commercial UpCell® dish. Note: once cold medium is added, the medium and dish is left at room temperature until cell detachment is observed. The dish is never placed in a cooled environment. Scale bars = 200µm.
2.4 Discussion

2.4.1 Cellular behavior on pNIPAAm films

Thermo-responsive polymers, especially pNIPAAm, have been widely used as a substrate for engineering cell sheets. Over the years, many different methods have been investigated to graft pNIPAAm to surfaces. Each of these methods results in different
surface characteristics which influence cell attachment, proliferation and detachment. For example, previous works by Okano’s group have demonstrated that cell-adhesion properties are dependent on the thickness of the grafted pNIPAAm film. pNIPAAm layers of more than 30nm thick exhibited non cell-adhesive surfaces. In this study, we characterized the surface properties of pNIPAAm films produced by our method and analyzed the resulting cellular response to verify that these surfaces are biocompatible and support the maintenance of normal cellular functions. Our data suggested that our surfaces support cell attachment regardless of film thickness. Cells proliferated on our surface at a similar rate to regular cell culture dishes.

Different mechanisms have been proposed for cell detachment from pNIPAAm films. Different groups have hypothesized a passive step involving the hydration of pNIPAAm chains and an active step involving cell shape change. Our surface achieves rapid cell detachment with 4°C medium indicating that the passive hydration of pNIPAAm chains could be the main detachment mechanism as low temperature would limit the cytoskeletal action and metabolic processes to drive changes in cell morphology. We also tested different types of mammalian cells on our surface (data not shown) and found that cell detachment times are not affected by different cell types or by cell-cell interactions, since the detachment rates remained the same.

2.4.2 The need of rapid detachment of confluent cell sheets

A pressing issue associated with existing TRP technologies is that cell detachment times remain long when cooled below LCST, often 30-60 minutes depending on the detachment protocols used. Normally, the slow detachment process occurs gradually from the sheet periphery toward the center [144]. Rapid detachment is believed to be
essential for retaining the biological functions of detached cell sheets for constructing tissue-mimicking structures [126, 144], which often require the assembly of numerous cultured cell sheets. When adhered cells are kept at 20°C or below for a lengthy time, the cooler environment may damage cells and their functions. Several attempts have been carried out to accelerate cell sheet detachment and recovery from TRPs to prevent damage from cooler temperatures [126, 144-147]. Okano and co-workers [126, 144-146] have grafted pNIPAAm and co-grafted hydrophilic poly (ethylene glycol) (PEG) with pNIPAAm onto porous cell culture membranes, introduced hydrophilic poly (2-carboxylisopropyl-acrylamide) into pNIPAAm gels, and created comb-type grafted pNIPAAm gel modified surfaces to accelerate water penetration and hydration of the pNIPAAm layer upon cooling. Wang et al. [147] developed a semi-interpenetrating nanocomposite hydrogel containing polysaccharide alginate and pNIPAAm, which contains rough surface textures that allow faster water penetration, leading to quicker cell sheet detachment. Even with these different types of modifications, complete cell detachment still takes at least 15 to 20 minutes. Nash and co-workers [95] spin-coated pNIPAAm into thin films on Thermanox disks, and the resulting films exhibited a rapid (mostly 5 to 10 minutes, in some cases ~ 60 minutes, in a ~ 4°C medium) cell sheet detachment for various cell types. However, the authors were not sure why their spin-coated pNIPAAm films worked while many others reported bulk films with similar properties were incapable of attaching and growing cells. The study, nevertheless, indicated the potential of spin-coating pNIPAAm films for cell attachment/growth and rapid cell sheet detachment when proper post-treatments of the spin-coated films were administrated. In our study, we utilized the spin-coating process to deposit a mixture of
pNIPAAm and an adhesion promoting agent to retain pNIPAAm films on the surface, to enhance cell attachment/growth, and to allow rapid detachment of the confluent cell sheets. In addition, we symmetrically tuned the concentration of the adhesion promoter to control the detachment time.

2.4.3 APTES as a cell adhesion promoter and retention agent for bulk pNIPAAm films

There were two main reasons for blending a small amount (< 20 wt.%) of APTES with pNIPAAm to generate thermo-responsive films. One was to enhance cell attachment and growth on the pNIPAAm surface, and the other was to retain the pNIPAAm films on the substrate surface once the aqueous medium is cooled below the LCST (32°C) of pNIPAAm.

Earlier studies have indicated that cell attachment and growth on bulk pNIPAAm films generated by simple casting are unfavorable [134, 136]. The same results were observed in our study. When spin-coated pNIPAAm only films on glass microscope slides were placed in a vacuum oven at 115°C or higher for three days to remove solvent, the resulting films were unable to allow cells to attach and grow (Figure 2.1B). Since spin-coating could likely distort the conformation and alter the properties of adhesive proteins if they were blended with pNIPAAm, we decided to use a simpler adhesion promoter, aminopropyltriethoxysilane (APTES). APTES is a small molecule widely utilized for immobilizing peptides or proteins, such as RGD, for cell biological studies [137, 148-150]. APTES has also been shown to enhance cell attachment and proliferation [137, 151]. By blending APTES with pNIPAAm and spin-coating this mixture into thin films, some of the amino groups of the APTES molecules could reside on the surface of the pNIPAAm/APTES films. These amino groups would be more prominent at the
surface when the pNIPAAm chains collapse at a temperature greater than their LCST (Illustration 2.1B). The exposed amino groups would promote cell attachment and proliferation.

APTES also has a unique molecular feature, containing an amino (−NH₂) end group and three ethoxyl (−OCH₂CH₃) head groups that can quickly hydrolyze into hydroxyl (−OH) groups in the presence of water. Due to the presence of these hydrogen bonding moieties, both intra and inter molecular hydrogen bonding can result [151-154]. In most cases, multiple hydrogen-bonds can be formed for each APTES molecule under ambient temperature. In addition, the intermolecular van der Waals forces between the short alkyl chains (i.e. three −CH₂) of APTES are too weak to allow the APTES molecules to form an ordered monolayer [155]. As a result, under ambient conditions, APTES molecules normally form a loose three-dimensional multilayer network [156]. APTES molecules chemically graft to a surface or with each other by forming Si-O-Si bonds through a condensation reaction. This reaction can be greatly enhanced by increasing the reaction temperature. Hence, the loose APTES network can be tightened (i.e. further cross-linked) by a thermal annealing process. As the network is being tightened, segments of polymer chains could be locked in the network and allow retention of otherwise non-retained polymer chains on the substrate (Illustration 2.1A, 2.1B). The slight enhancement of peaks, although weak when compared to other peaks, associated with the non-hydrogen bonded N-H stretching (3442 cm⁻¹) and the Si-O-Si linkage (1040 – 1120 cm⁻¹) shown in the DRIFT spectra (Figure 2.2B, 2.2C) for films cured at higher temperature (≥ 160°C) suggests this possibility. With the presence of APTES molecules, which attract water molecules to form multiple hydrogen bonds, it is highly possible a
faster hydration of the top portion of the pNIPAAm/APTES network results upon cooling. The faster hydration leads to a quicker detachment of the confluent cells.

The above interpretation provides an explanation on cell attachment/detachment behaviors on our thermo-responsive pNIPAAm/APTES films when cells are seeded at ambient temperature. Another potential factor may play an equally important role when a warm medium above the LCST of pNIPAAm is used to seed cells on the pNIPAAm/APTES films. This factor involves the partial or complete dissolution of the pNIPAAm/APTES layer when the medium is cooled below LCST. We have noticed that all of the cured pNIPAAm/APTES films exhibited some layer loss when they were dipped into cold DI water. Under warm medium, films remained adhered to the substrate and allowed cells to attach and grow. When medium temperature was lowered below LCST, either the entire film or a top layer of 200 – 250 nm dissolved. The dissolution of this layer could also cause fast cell detachment through the loss of anchorage points.

A possible reason for the films dissolving at cool temperatures is the way the APTES network forms. At our annealing temperatures (generally ≥ 115°C), pNIPAAm is more hydrophobic (i.e. having a lower surface energy) than APTES. This could cause most of the APTES molecules, due to their higher surface energy, to settle to the bottom of the pNIPAAm/APTES layer and/or segregate to the polymer/substrate interface. As a result, a denser APTES network could form at the lower portion of the layer to retain those pNIPAAm chains, while a looser APTES network could form at the upper pNIPAAm/APTES layer. The looser APTES network might not be able to lock the pNIPAAm chains in place. Furthermore, a tighter APTES network only forms when the annealing temperatures are relatively high (e.g. ≥ 160°C in this case) and the annealing
time is sufficient (e.g. 3 days). Consequently, films cured at a temperature of 145°C or below form APTES networks that could be too loose to retain pNIPAAm chains.

We noticed films cured at high temperatures were reusable to an extent, even after the top layer was removed during previous cell culture. The detachment time and cell morphology for cells reseeded a second time were found to be the same as those of the previously unseeded films. When films were reseeded for a third time, cells were sparsely attached and spread, but still detached successfully. After two heating/cooling cycles, pNIPAAm chains may fail to return to the same conformation of cured films prior to cell seeding. The inability to return to the initial conformation could prevent the NH$_2$ groups of the APTES molecules from residing at or close to the surface. We speculate that when the pNIPAAm extended chains return to the collapsed state upon reheating, they could bury the APTES molecules, thus preventing cell attachment.

2.4.4 Cytotoxicity of APTES and pNIPAAm

The detached cell sheets, once harvested, will eventually be implanted in vivo making it imperative that any residual APTES and/or pNIPAAm will not harm a patient’s body. Acute toxicity tests have been conducted in which repeated dermal doses of 126mg of APTES/kg (or 126 ppm) body weight resulted in no systemic toxicity leading to its classification as a low risk chemical to human health [157]. Other studies also concluded APTES was nongenotoxic [157]. Cell attachment, spreading, and proliferation were achieved on the cured pNIPAAm/APTES film surfaces, indicating that the cured pNIPAAm/APTES films as well as any loose pNIPAAm and APTES molecules within the network express little or no toxicity toward the cells under our culture conditions. Previous studies have reported that surface grafted APTES monolayers have no
significant effect on cell proliferation [158]. We expect cross-linked (i.e. cured) APTES to possess similar properties to surface grafted APTES (i.e. posing no significant effect on cell proliferation). If 5% of the APTES molecules in a pNIPAAm/APTES blend (e.g. 80:20), which is a relatively high estimate, were not cross-linked and were all retained with the cell sheet (assuming a sheet thickness of 20 microns), these molecules would only result in a ~ 100 ppm of APTES to the cell mass. This is lower than the acute toxicity value (126 ppm) tested, which showed no systemic toxicity to humans. It follows that when the cell sheets are ready for implantation, the remaining APTES will be so minimal that it may be considered negligible. Nevertheless, further studies, both in vitro and in vivo, should be conducted to evaluate the toxicity of free APTES molecules prior to implantation.

Many other groups have evaluated pNIPAAm for cytotoxicity [91, 136, 159]. Concentrations of 10 mg/ml and below were well tolerated by cell cultures indicated by MTT and LDH tests [160, 161]. The maximum amount of pNIPAAm cells come in contact with our films is 20 – 30 µg/ml, which is far less than the concentration tolerated by cells. Moreover, pNIPAAm is trapped within the APTES network with little exposure to the surface where cells would anchor. With these findings, one can conclude pNIPAAm/APTES films have little to no detrimental effect on cell behavior.
CHAPTER III
STACKED STEM CELL SHEETS ENHANCE CELL-MATRIX INTERACTIONS

3.1 Introduction

Myocardial infarction (MI) is one of the leading causes of morbidity and mortality in industrialized nations. It affects more than 10 million people in the United States and hundreds of millions worldwide [162]. Various therapeutic strategies exist to treat MI [163, 164]. However, they do not restore normal heart function that has been lost as a result of dead myocardium. Cardiac cells have low proliferative potential and endogenous cells are unable to replenish dead heart cells. The cardiomyocyte deficit in patients with MI is in the order of one billion myocytes [165]. Therefore, true cardiac repair would require restoring approximately one billion cardiomyocytes and ensuring their synchronous contraction via electromechanical junctions with host myocardium. Stem cells, which have the unique capability to differentiate into multiple lineages, could potentially regenerate lost myocardium and restore cardiac function. However, low cell viability and poor engraftment after transplantation has limited the therapeutic effects of stem cell-based cardiac therapy. Currently, only approximately 1–10% of stem cells remain in the heart 1–2 h after injection into the beating heart. More than 90% of injected cells migrate to other organs, and less than 10% of the transplanted cells are identified in the heart 4 weeks after transplantation [46, 166]. In addition, injections of a large number of cells can cause cell clumping, microinfarction and arrhythmogenic foci formation.
The recently developed cell sheet engineering approach has enabled the production of confluent cell sheets stacked together for use as a cardiac patch to increase cell survival rate and engraftment after transplantation, providing a promising strategy for high density stem cell delivery [168, 169].

One key challenge in using cell sheet technology is the difficulty of cell sheet handling due to its weak mechanical properties. A single-layer cell sheet is generally very fragile and tends to break or clump during harvest. Effective transfer and stacking methods are needed to move cell sheet technology into widespread clinical applications. Currently, two main methods have been used to transfer cell sheets: using a sheet of PVDF membrane or a hydrogel-coated, plunger-like manipulator [170]. Most recently, a cell sheet transfer device has been developed, which has a scooping part to transfer cell sheets by a movable belt and a handling part to operate the device [171]. However, all of these methods require applying a direct force on the cell sheet, which may affect cell viability, activities, long-term functions and cause variation between users.

In the present study, we developed a simple and effective micropipette based method to aid cell sheet transfer and stacking. The cell viability after transfer was tested and multi-layer stem cell sheets were fabricated using the developed method. Furthermore, we examined the interactions between stacked stem cell sheets and fibrin matrix. Fibrin, as a nature biomaterial, has been widely used in tissue engineering applications [172-174]. When applied as a cardiac patch to treat MI, fibrin has been shown to provide temporary physical support of the infarct tissue and prevent negative left ventricular remodeling [57, 175]. The investigation on cell sheet-fibrin matrix
interactions would provide valuable information on the feasibility of combining cell sheets and fibrin as a composite patch for cardiac repair.

3.2 Methods

3.2.1 Materials

pNIPAAm, MW 20-25 kg/mol, 3-aminopropyltriethoxysilane (APTES), fibrinogen from human plasma, and thrombin from human plasma were purchased from Sigma Aldrich (St. Louis, MO, USA). Glass slides were purchased from Fisher Scientific (Waltham, MA, USA). All other chemical reagents were purchased from Sigma Aldrich unless otherwise indicated.

3.2.2 Preparation of glass substrates

Glass slides were cut into squares with a surface area of ~ 1cm². Slides were immersed in freshly prepared piranha solution (70 vol.% of concentrated H₂SO₄ and 30 vol.% of 30% H₂O₂) for 1 hour at 100°C to remove organic contaminants. After decanting the piranha solution, the slides were thoroughly rinsed with deionized (DI) water and dried with nitrogen (N₂) gas. Afterwards, the slides were oxidized in a UV/Ozone Cleaner (Jelight Company Inc, Irvine, CA) for 10 minutes for further cleaning.

3.2.3 Preparation of pNIPAAm/APTES solutions

A 3% wt. pNIPAAm solution and a 5% wt. APTES solution in ethanol (Pharmco-AAPER, Inc., Shelbyville, KT) were prepared separately. Solutions having pNIPAAm to APTES ratios (by mass) of 80:20 were prepared by mixing the proper ratios of the two above solutions and a small amount of ethanol to make a final solution containing ~ 3 wt.% of total solute. The solutions were subsequently filtered to remove particulates
through an Acrodisc® CR 13mm Syringe filter with a 0.45μm PTFE membrane (Pall Life Sciences, Co., Ann Arbor, MI).

3.2.4 Preparation of pNIPAAm/APTES films

Polymer blend films were produced by spin coating the pNIPAAm/APTES mixed solution onto pre-cleaned glass substrates for 30 seconds at 2000 rpm (p-6000 Spin Coater, Specialty Coating Systems, Inc, Indianapolis, IN). The spin-coated samples were cured inside a vacuum (≤ 100 mTorr) oven (VWR International, Radnor, PA) for 3 days at 160°C. The glass slides were sterilized by placing under UV light (100-280 nm) for 20 min.

3.2.5 Fibrin Matrix Preparation

Fibrinogen (Sigma Aldrich, MO) was dissolved in TBS at 37°C to make a final concentration of 40mg/ml. The solution was filtered through a Steriflip 0.2μm filter (Millipore, Billerica, MA). Using this solution and TBS, fibrinogen concentrations of 20 mg/ml and 10 mg/ml were also made. Thrombin was dissolved in sterile 40mM CaCl₂ to make a thrombin solution containing 25 units/ml.

Fibrin matrices were prepared by mixing 0.5ml of fibrinogen solution and 0.5ml of thrombin solution to make fibrin matrices with the final fibrinogen concentrations of 5 mg/ml, 10 mg/ml, and 20 mg/ml. These fibrin matrices were allowed to fully crosslink for 1 hour at room temperature. The matrices were washed twice with PBS to remove non-cross-linked solution from the matrix.

3.2.6 Cell Seeding, Detachment and Manipulation from Thermoresponsive Substrates

Human mesenchymal stem cells (hMSCs) (Lonza, Walkersville, MD) were cultured in serum-containing MSCBM medium (Lonza) supplemented with MSCGM
SingleQuots (Lonza) according to manufacturer’s specifications. hMSCs from passages 2-5 were used in the experiments. 1.0 x 10^5 cells were seeded on pre-warmed substrates for 24 hrs. After cells reached desired confluence and formed a cell monolayer on the pNIPAAm/APTES film, cell sheet detachment was induced by replacing warm cell culture medium with fresh cold medium (4°C).

The detached cell sheet was then transferred to the desired location using a modified 1ml micropipette as shown in Fig 1. The floating cell sheet was drawn into the pipette tip and transferred to the desired location.

3.2.7 Cell Proliferation

A PrestoBlue cell proliferation assay (Invitrogen, Carlsbad, CA) was performed to evaluate the efficiency of using the micropipette method to manipulate cell sheets by comparing 1 cell sheet comprised of 1.0 x 10^5 cells to 1.0 x 10^5 cells seeded on to a tissue culture plate. It was also used to compare the proliferative potential of cell sheets on fibrin matrices with fibrinogen concentrations of 5mg/ml, 10mg/ml, and 20mg/ml. The PrestoBlue solution fluorescence was measured at an excitation wavelength of 560nm and emission wavelength of 590nm using a Synergy H1 Hybrid microplate reader (BioTek, Winooski, VT). For each group, 3 samples were tested.

3.2.8 Visualization of Cell Sheets

hMSC sheets were prepared as previously described and transferred onto fibrin matrices with the final fibrinogen concentration of 10mg/ml using the developed micropipette method. After cultured for 1-7 days, the samples were fixed in 10% formalin. Fixed samples were dehydrated, paraffin embedded, sagittally sliced in 10µm sections using a microtome (Leica Microsystems,Buffalo Grove, IL) and placed on
microscope slides. These slides were stained with hematoxylin and eosin (H&E) (Leica). Pictures of the sectioned gels were taken using an Axiovert A1 (Carl Zeiss, Inc, Thornwood, NY) microscope equipped with an AxioCam MRc camera (Carl Zeiss, Inc).

Confocal microscopy was used to visualize the success of layering cell sheets on top of each other. hMSCs were labeled with CellTracker Red CMTPX (Invitrogen) prior to forming cell sheets using the thermoresponsive substrate. Non-labeled hMSCs were seeded on a microscope slide. A cell sheet labeled with CellTracker Red CMTPX was placed on top of the confluent cell layer on the microscope slide. After allowing 20 minutes of attachment, a 2nd cell sheet layer that was not labeled was placed on top of the labeled cell sheet. After allowing 2 hours of full attachment of the cell layers the slide was fixed using 4% formaldehyde. Cells were permeabilized using Triton-X100 (Alfa Aesar, Ward Hill, MA) and stained with Alexa Fluor 488 Phalloidin (Invitrogen). Cell images were taken using an inverted microscope equipped with a Zeiss 510 META laser scanning module (Carl Zeiss).

3.2.9 Statistical Analyses

Statistical analyses were performed using SAS 9.3. Data are reported as means ± standard deviations. All statistical comparisons were made by performing singly factor analysis of variance (ANOVA), followed by a Student-Newman-Keuls and Tukey’s comparisons test. P values less than 0.05 are considered statistically significant.

3.3 Results and Discussion

3.3.1 Transfer and Stacking of Cell Sheets Using a Micropipette Method

A simple and effective micropipette based method was developed to transfer and stack cell sheets. The mechanism of the developed method was illustrated in Fig 3.1.
Human mesenchymal stem cells (hMSCs) were cultured on a thermo-responsive surface fabricated using our newly developed spin coating technique [96]. The detachment of hMSC sheets was achieved by replacing cell culture medium with fresh cold medium (4°C). No residual cells were detected after cell sheet detachment. The detached floating cell sheet was collected by a modified micropipette tip (Fig. 3.1A) and transferred to other surfaces easily and rapidly. Cell viability testing was conducted and confirmed that no significant cell death was caused during the cell sheet transfer using the described micropipette method (Fig. 3.2C). Moreover, the developed micropipette method allowed stacking of multi-layer cell sheets within the modified micropipette tip and transferring of the stacked cell sheets without any clumps and breakages (Fig. 3.1C). Confocal microscopy was used to show the successful stacking of triple layer hMSC sheets (Fig. 3.3) using the developed micropipette method. Multiple layer cell sheets were precisely transferred onto a fibrin gel surface, suggesting the manipulability of the method (Fig. 3.2D).
Figure 3.1: Micropipette Transfer Method. (A) The modified micropipette tip is fabricated by cutting the 1ml micropipette tip by the depicted configuration. (B) A cell sheet detached from the thermo-responsive surface is drawn into the modified micropipette tip along with media and dispensed to the desired location. (C) Multiple cell sheets can be transferred and stacked within the tip by drawing them into the tip, allowing them to settle to bottom of tip, and then dispensing on desired substrate.
Figure 3.2: Cell sheet transfer and cell viability. (A) The morphology of confluent hMSC monolayer growing on the thermoresponsive substrate prior to cell sheet detachment. (B) hMSC sheet morphology after transfer and attachment to the new cell culture substrate using the developed micropipette method. (C) Cell viability of single layer cell sheet after manipulation with a micropipette compared with the same amount of dissociated cells. No significant difference ($p = 0.98$) is identified between the two groups. (D) H&E staining of multi-layer hMSC sheets on fibrin matrix. Scale bar is 200µm for (A) and (B) and 50µm for (D).
Figure 3.3: Representative confocal images of 3-layer hMSC sheets. (A) Gallery image of sectional view showing the 3 layers of hMSC sheets. Cells at the top and bottom layers are labeled with AlexaFluor Phalloidin Green and cells at the middle layer are labeled with CellTracker Red CMTPX. (B) Cross section view. Scale bar is 100 µm.
The developed micropipette method can aid cell sheet transfer and improve the efficiency of cell sheet stacking and transplantation. The modified tip used in our method was made from a universal 1ml micropipette tip and operated by a regular micropipette. They are available in most laboratories allowing the method to be readily adapted by many cell culture and tissue engineering researchers. Compared with other existing methods (e.g. using a PVDF membrane or a plunger-like manipulator), our method minimizes the force placed on the transferred cell sheet. A pipette-based method has been previously reported for transferring cell sheets, where a cell sheet was drawn into a 10-ml pipette and push-released onto another surface. The cell sheet tends to clump together in the pipette and needs to be flattened after transfer [170]. When a cell sheet was transferred using our method, the modified micropipette tip collected the floating cell sheet and kept it flat within the tip. No re-spaying was needed after the cell sheet was transplanted onto the intended surface. Furthermore, continuous collecting and stacking of cell sheets is enabled using our method and could significantly increase the efficiency of cell sheet transfer. The method has been designed to collect and stack small sizes of cell sheets. It could be further modified to work with different sizes of cell sheets by using customized tips and modified operating devices.

3.3.2 Interactions between Cell Sheets and Fibrin Matrix

A single layer of hMSC sheet (~1.0 × 10^5 cells) was detached and transferred onto the surface of fibrin matrix to examine the cell-matrix interactions. The transferred cell sheet immediately attached to the fibrin matrix upon contact and formed a uniform cell layer on the matrix without any floating cell pieces. Fibrin matrices with different fibrinogen concentrations (5mg/ml, 10mg/ml and 20mg/ml) were used to test their effects
on cell proliferation of the attached cell sheet. Our results indicated that cells within the
cell sheet grew at different rates and proliferated significantly faster (p < 0.05) on the
fibrin matrix with the final fibrinogen concentration of 10 mg/ml than on the other two
fibrinogen concentrations (Fig. 3.4A). Much to our surprise, the cell sheet degraded most
of the underneath fibrin matrix (10mg/ml) 24 hours after the initial seeding while no
noticeable fibrin degradation was observed with the same number of dissociated cells
under same culture conditions. We further tested the cell sheet and fibrin matrix
interactions using stacked multi-layer cell sheets and thicker fibrin matrix. Accelerated
fibrin degradation caused by the attached cell sheet was consistently observed (Fig 3.4C).
To the best of our knowledge, this is the first *in vitro* study demonstrating the enhanced
cell-matrix interactions between hMSC sheets and fibrin matrix.

One of the biggest advantages of using cell sheets detached by thermo-responsive
surfaces is that it preserves cell-cell contacts and cell secreted extracellular matrix (ECM)
during cell sheet transferring. Our results have shown that the preserved ECM associated
with the detached cell sheet greatly facilitates its adherence to fibrin matrix. While it
usually takes 2-6 hours for dissociated hMSCs to fully attach to fibrin matrix, the
attachment of cell sheet to fibrin matrix is instantaneous. When we stacked multiple
layers of cell sheets within the modified micropipette tip as previously described and
placed them on the fibrin matrix, effective bindings were found among the layers of cell
sheets as well as between the cell sheets and fibrin matrix. The interactions of cell sheet
and fibrin matrix are affected by the fibrinogen concentration of fibrin matrix. It has been
extensively reported that the substrate stiffness plays an important role on directing cell
behaviors [176, 177]. The stiffness of fibrin matrix is mostly determined by the
fibrinogen concentration. Our previous studies have also elucidated that dissociated hMSCs prefer to grow on fibrin matrix with 10 mg/ml fibrinogen [57, 178]. As expected, hMSC sheets also showed the greatest proliferation rate on fibrin matrix with 10 mg/ml fibrinogen, indicating that cell culture methods (e.g. as individual cells or cell sheet) may not influence the preference for the culture substrate. The most profound finding of this study is the accelerated fibrin degradation caused by attached cell sheets. Compared with the same amount of dissociated cells, a hMSC sheet degrades fibrin matrix significantly faster. The mechanisms behind the accelerated fibrin degradation caused by cell sheet are still unknown. In future studies, we plan to examine whether the enhanced interactions between cell sheets and fibrin matrix are induced by the improved cell-cell communication enabled by the cell sheet technology.
Figure 3.4: Cell sheet and fibrin matrix interactions. (A) The effect of fibrinogen concentration on cell proliferation. Cell proliferation was measured using a PrestoBlue cell proliferation assay on the monolayer hMSC sheet 24 hours after transferring to fibrin matrices with different fibrinogen concentrations. RFU is the fluorescent emission intensity of samples at 590nm when excited with 560nm light. Results are the means ± SD of 3 samples. Asterisk (*) indicates significant difference ($p < 0.05$). (B, C) H&E Staining of 4-layer hMSC sheets cultured on fibrin matrix for 1 day and 7 days, respectively. Scale bar is 50µm (B) and 200µm (C).
CHAPTER IV
CONCLUSIONS

4.1 Summary

4.1.1 Development of a Novel Thermoresponsive Substrate

The data demonstrate that thermoresponsive pNIPAAm/APTES films can be generated by simple blending, spin-coating and thermal curing. These films retain water soluble pNIPAAm on the glass surface in culture medium even at low temperature, exhibit enhanced cell adhesion and proliferation, and greatly accelerate detachment of confluent cell-sheets upon cooling. The pNIPAAm/APTES substrates can be used up to 3 times for cell sheet detachment and have the ability to control detachment times based on pNIPAAm and APTES ratios. Based on initial results, the spin-coating technique will be an economical step forward for the cell sheet engineering community. While current work has been focused on silica based substrates, the approach can be extended to other substrates containing sites that can covalently link to APTES and can withstand a high annealing temperature (e.g. ~ 160°C).

4.1.2 Cell Sheet Manipulation and Interactions with Fibrin Matrix

Thermoresponsive substrates were used to create cell sheets and a micropipette was found to be an efficient technique to transfer sheets to specific locations. Culturing hMSC sheets on fibrin gels revealed important information about their interaction with fibrin. Cell sheets bound to fibrin gels almost instantaneously, while revealing greatest
proliferation on fibrin gels with the final fibrinogen concentration of 10mg/ml. The enhanced interaction between cell sheet and fibrin indicated a possible integrated composite scaffold for cardiovascular applications. The high cell density from the stacked hMSC layers in addition to the favorable microenvironment provided by the fibrin matrix may lead to improved angiogenesis and myocardium repair, while the preservation of ECM in cell sheets may provide the ability to adhere quickly to host tissue for improved cell retention and engraftment.

4.2 Future Work

4.2.1 Introduction

Future related work to this project can take many directions. The first direction must be to examine the mechanisms behind the rapid degradation of fibrin gels with attached cell sheets when compared to dissociated cells. A preliminary study to examine this behavior was conducted.

Matrix metalloproteinases (MMPs) are a family of Zn$^{2+}$ binding and Ca$^{2+}$ dependent endopeptidases that play a crucial role in ECM breakdown [179]. Since MMPs are responsible for ECM breakdown, they are likely to lead to fibrin degradation. MSCs have been shown to upregulate MMP-2 and MMP-9, leading to increased vessel network formation [180]. MMP-2 and MMP-9 are known as gelatinases due to their ability to breakdown gelatins. They also have the ability to degrade collagen type IV, V, VII, and X, elastin, and stromelysins in basement membranes [181, 182]. Because of previous studies correlating myocardial infarction to increase in MMP-2 and -9 production, we focused our preliminary studies on these specific MMPs [183, 184].

4.2.2 MMP Secretion at Normal Conditions
Combined MMP-2 and -9 release was measured from 1 layer and 3 layer cell sheets on 10 mg/ml fibrin gels (Fig 4.1). High cell density in cell sheets and intact cell-cell junctions are possible contributors to fibrin degradation by causing an increased release of MMP. MMP release from 1 cell sheet layer and 3 cell sheet layers was measured using zymography techniques. A larger MMP release from single layer cell sheets than from individual cells at both 24 hrs and 48 hrs after culture was measured. A similar trend was seen from 3 layer cell sheet stacks, where MMP release was greater from 3 layer cell sheets compared to equivalent amount of dissociated cells. Increased MMP release from cell sheets over dissociated cells indicates cell-cell interactions promote higher cell MMP expression. Three dimensional endothelial cell cultures in fibrin gels have shown to induce expression of MMP-2 and membrane type MMPs (MT1, MT2, and MT3) [179] during tubulogenesis. Similarly, our MSC cell sheet stacks on fibrin gels expressed higher amounts of MMP than dissociated cells, indicating similar behaviors of the two cell types. Although our models did not reveal tubulogenesis, the increased release of MMPs by MSCs may be an indication toward achieving angiogenesis. Increased expression of MT1-MMP is a vital component towards achieving angiogenesis as indicated by several studies [179, 185, 186]. Thus, it will be important to study the expression of this MMP using our models to determine the angiogenesis potential for future cardiovascular applications.
4.2.3 MMP Secretion at Hypoxic Conditions

A similar study to the one described in section 4.2.2 was conducted under hypoxic conditions to test once the cell sheet was transplanted into the ischemic heart how the hypoxic condition would affect the MMP secretion from the cell sheet. In this study, overall MMP release was lower under hypoxic conditions than normal for single cell sheet layers (Fig 4.2). While these differences were not statistically significant due to small sample size, this result could be due to several reasons. Release of MMP is found to be important in modulating cell migration, proliferation, and invasion into substrata [187-189]. MMP-2 and MMP-9 fragment the fibrin ECM to create new integrin-binding sites [190], facilitating new ECM-integrin interactions, which lead to activation of focal...
adhesion kinase and thus, influences migration of cells [188]. MMP release, however, may be modulated by oxygen levels [191, 192]. MSCs investigated under hypoxia downregulate the expression of MMP-2 genes, resulting in lower MMP-2 secretion [193].

In addition to oxygen, MMP release could change with scaffold type [194]. To investigate the effects of the underlying matrix on MMP release from cell sheet stacks, 2.5 mg/ml collagen gels and tissue culture plates were used to compare to 5 mg/ml fibrin gels. Fibrin gel concentration of 5 mg/ml was used because it most closely matched the stiffness of 2.5 mg/ml collagen gels. Collagen gels and 5 mg/ml fibrin gels exhibit a Young’s modulus of ~1kPa (data not shown). Mechanical cues are known to affect cell behavior [177]. By eliminating the stiffness differences between the substrates, MMP release was assumed to be solely affected by the substrates composition. Three layer sheet stacks of MSC cultured on collagen gels had higher MMP release than those cultured on 5 mg/ml fibrin gels (Figure 4.3). Over a 3 day period, MMP release lessened each day. Cell sheets cultured in hypoxic conditions released less MMP than cell sheets cultured at normal conditions. Cell sheets cultured on 5 mg/ml fibrin gels under hypoxia for 24 hrs released more MMP than those cultured at normal conditions (similar to 10 mg/ml gels shown in Figure 4.2). By 48 hours, MMP release was lower under hypoxia than at normal oxygen levels. Different integrins are associated with collagen and fibrin substrates. Activation of integrins on different ECMs trigger signal transduction pathways that lead to different MMP expression levels [195-198]. Overall, the literature supports the changes in MMP release for single cell sheet layers that were seen in this study, but further investigation into why these differences occurred and the mechanism behind the changes must be performed.
Figure 4.2: MMP Release from 1 and 3 Layer hMSC Sheets at Hypoxic and Normal Culture Conditions. MMP release band intensity measured using ImageJ densitometry. Error bars are standard deviations from 2 samples. No error bars indicate a sample size of n=1.

Figure 4.3: MMP Release from 3 Cell Sheet Layers on Different Substrates. MMP release band intensity measured using ImageJ densitometry. Data is from 1 sample for each condition.
4.2.4 Necessary Studies

Other MMPs also play a critical role in cell migration and fibrin degradation including the cell bound MT1-MMP. A knockdown model of endothelial MT1-MMP demonstrated that endothelial cell invasion and subsequent normal vessel formation are dependent on the proteolytic activity of MT1-MMP [186]. Interactions between co-cultures of bone marrow MSCs and endothelial cells in fibrin matrix previously showed to influence vessel formation and invasion within fibrin matrices [199]. Several groups found that MMP-2, MMP-9, and MT1-MMP expression were all upregulated in these cultures [180, 199-201], but only the knockdown of MT1-MMP inhibited vessel invasion [186]. Thus, it may be beneficial to study the expression of MT1-MMP in our cell sheet fibrin models. Studying the release of these MMPs for longer terms (~10 days) may provide better indications toward cell migration. Further MMP studies on collagen are needed to determine whether cells are likely to migrate into the myocardium.

All of these preliminary studies give an indication of cell-matrix interactions but no definite conclusions can be made due to insufficient number of samples. Thus, all MMP experiments conducted in this research must be repeated with enough samples to make the results statistically significant.
This dissertation is based on the following publications and presentations:


REFERENCES


expression favoring esophageal carcinoma migration and invasion via hypoxia inducible factor-1 alpha activation. Diseases of the Esophagus 2013;26:75-83.


APPENDIX A

SAS OUTPUT FOR CELL METABOLIC ASSAY

The SAS System

The GLM Procedure

Class Level Information

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Number of Observations Used  9
The SAS System

The GLM Procedure

Dependent Variable: RFU

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The SAS System

The GLM Procedure

Distribution of RFU

RFU

10mg/ml 20mg/ml 5mg/ml
FibrinGel
The SAS System

The GLM Procedure

Student-Newman-Keuls Test for RFU

Note: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

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The SAS System

The GLM Procedure

![Distribution of RFU](image)
The SAS System

The GLM Procedure

Tukey's Studentized Range (HSD) Test for RFU

Note: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

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### APPENDIX B

**ZYMOGRAPHY GEL FOR 1 CELL SHEET LAYER**

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APPENDIX C

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APPENDIX D

ZYMOGRAPHY GEL FOR 1 CELL SHEET LAYER UNDER HYPOXIA

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**APPENDIX E**

**ZYMOGRAPHY GEL FOR 3 CELL SHEET LAYERS UNDER HYPOXIA**

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APPENDIX F

ZYMOGRAPHY GEL FOR 3 CELL SHEET LAYERS ON COLLAGEN UNDER HYPOXIA

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APPENDIX G

SAS OUTPUT FOR COMPARISON OF VIABILITY OF TRANSFERRED CELL SHEET USING A MICROPETTE VS. DISSOCIATED CELLS

The SAS System

The GLM Procedure

Class Level Information

<table>
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<tr>
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<th>Levels</th>
<th>Values</th>
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<tr>
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Number of Observations Read 6
Number of Observations Used 6
The SAS System

The GLM Procedure

Dependent Variable: RFU

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<th>Mean Square</th>
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R-Square  Coeff Var  Root MSE  RFU Mean
0.000049  7.547688  3481.769  46130.27

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The SAS System

The GLM Procedure

Student-Newman-Keuls Test for RFU

Note: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

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Means with the same letter are not significantly different.

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</table>
The SAS System

The GLM Procedure

![Distribution of RFU](image)

- **RFU**
  - 50000
  - 48000
  - 46000
  - 44000
  - 42000

- **Cells**
  - CellShee
  - Dissocia

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The SAS System

The GLM Procedure

Tukey's Studentized Range (HSD) Test for RFU

Note: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

<p>| | |</p>
<table>
<thead>
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<tbody>
<tr>
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Means with the same letter are not significantly different.

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