I, Hodari-Sadiki L James, hereby submit this original work as part of the requirements for the degree of Master of Science in Biomedical Engineering.

It is entitled: 
Thermo-Responsive Polymers for Cell-Based Therapeutic Applications.

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This work and its defense approved by:

Committee chair: Daria Nermoeva, Ph.D.

Committee member: T. Douglas Mast, Ph.D.

Committee member: Dale Schaefer, Ph.D.
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by
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Abstract

Poly (N-isopropylacrylamide) (PNIPAAm) is a well-known thermo-responsive polymer that has been shown to be biocompatible, with surfaces coated with PNIPAAm supporting the culture of cells. These surfaces support the adhesion and proliferation of multiple cell phenotypes at 37 °C, when surface is hydrophobic, as the polymer chains are collapse and lose their affinity for water. Reducing the temperature below the polymers lower critical solution temperature (LCST) elicits hydration and swelling of the polymer chains and leads to cell detachment. In vitro culture on thermo-responsive surfaces can be used to produce cell sheets for the use of different therapeutic treatments. PNIPAAm coated membranes were used to culture human keratinocyte cells to confluence, with cell release possible after exposing the membranes to room temperature (~25 °C) for 10 minutes. Cell sheet transfer was possible from the coated membrane to cell culture dishes using a protocol that we developed. There was also a trend towards similar cell apoptosis on both PNIPAAm coated and uncoated surfaces.
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Chapter 1: Emerging Technologies for Cell Sheet Engineering using Thermo-Responsive Polymers.

1.1 Introduction:

During a typical cell culture process, cell adhesion to the culture surface is important in order to foster the healthy growth, differentiation, migration, and survival of cells [1], [2]. Adhesion, however, presents a problem when cells grown in vitro need to be transported to an in vivo target area. The conventional procedure involves proteolytic degradation of extracellular matrix (ECM) that holds the cells together via enzymatic action, for example trypsinization. This procedure usually produces a suspension of cells that can then be transported to another culture vessel. However, destruction of the cell sheet's ECM is not very efficient from a tissue-engineering viewpoint, as the cell-cell interactions present are usually integral to their in vivo function [3]. In addition nonspecific proteases can damage crucial cell surface proteins [3], [4]. These major drawbacks have fueled research into alternative cell harvesting methods.

Poly (N-isopropylacrylamide) (PNIPAAm) is a well-known thermo-responsive polymer which has been studied since the late 1960's [5]. Its use as a culture surface for the in vitro growth of intact cell sheets, however, was not studied until the early 1990's [4], [6]-[8]. In recent years, cell culture on PNIPAAm coated surfaces or within PNIPAAm hydrogels, has been explored as a possible solution to the problems presented by conventional cell harvesting methods [9]. A surface grafted with the polymer has the unique property of switching from a hydrophobic to a hydrophilic state when the temperature is decreased below its lower critical solution temperature (LCST) (~32 °C) [10]. A confirmation change in the polymer chains of the grafted PNIPAAm layer, caused by reduced temperature, can elicit detachment of confluent cell sheets as shown by the schematic in Figure 1 [4], [7], [11]–[16].
This chapter will; 1) Review recent cell sheet engineering studies using PNIPAAm, or PNIPAAm copolymers, as a cell culture surface, and 2) Come up with a unique approach of producing keratinocyte cell sheets to be used in the treatment of chronic wounds. Focus will be placed on cell to surface interactions such as, adhesion, proliferation, and detachment. Since cellular responses to PNIPAAm grafted surfaces have been found to be both cell-type specific, and dependent on PNIPAAm grafting thickness in previous studies [20],[25]. Promising applications of cell sheet engineering research using PNIPAAm coated culture surfaces will also be highlighted at the end of this chapter.

Figure 1.1: Schematic illustration of cell sheet on PNIPAm modified surface. A: At 37 °C, cells can adhere on dehydrated PNIPAm layer. B: At 20 °C, cells can detach from hydrated PNIPAm layer with ECM, resulting in an intact and contiguous cell sheet [14].
<table>
<thead>
<tr>
<th>Surface Description</th>
<th>Cell Phenotype</th>
<th>Cell Adhesion &amp; Proliferation</th>
<th>Cell Detachment</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flat (F) and Micro-patterned (MP) PNIPAAm films on TCPS</td>
<td>Rat bone marrow mesenchymal stem cells (BMSC)</td>
<td>Static: ↑proliferation rate at 2 weeks on both F and MP surfaces.</td>
<td>N/A</td>
<td>[17]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dynamic: ↑ Total cell proliferation at 21 days on MP surface.</td>
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<tr>
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<tr>
<td>(PNIPAAm) on Glass substrates</td>
<td>NIH/3T3 fibroblast cells.</td>
<td>Best attachment and spreading on graft thickness ≤ 14nm</td>
<td>Best detachment on thicknesses between 5 and 14 nm</td>
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</tr>
<tr>
<td>(PNIPAAm-r-MPDSAH)) on Glass substrates</td>
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<td>Complete detachment only on surfaces with a with a 75:1 PNIPAAm:MPDSAH mixture.</td>
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<tr>
<td>PNIAM-co-AM on TCPS</td>
<td>Human Chondrocyte Cells</td>
<td>Very good attachment and spreading w/o PVDF 69% detachment</td>
<td></td>
<td>[21]</td>
</tr>
<tr>
<td>PNIAM-co-AM on TCPS PNIPAAmSt microgel films Spin-coated (SC) on glass &amp; Drop-coated (DC) on TCPS.</td>
<td>Human Chondrocyte Cells</td>
<td>Very good attachment and spreading Attachment and spreading similar on both uncoated and DC TCPS</td>
<td>with PVDF100% detachment 90% cell detachment in 2-3 mins from DC TCPS with pipetting.</td>
<td>[21] [22]</td>
</tr>
<tr>
<td>PNIPAAmSt microgel films Spin-coated (SC) on glass &amp; Drop-coated (DC) on TCPS. Gelatin/PNIPAAm-PHB-PNIPAAm Copolymer on Glass cover-slips (GCS)</td>
<td>NIH/3T3 fibroblast cells</td>
<td>Attachment and spreading similar on both uncoated and SC glass substrates</td>
<td>↑ detachment on SC 1.0 wt % microgel films compared to SC 0.5 and 0.75 wt % films</td>
<td>[22] [23]</td>
</tr>
<tr>
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<td>grew to confluence within seven days</td>
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<tr>
<td>Flat (F) and Micro-patterned (MP) PNIPAAm films on TCPS</td>
<td>Rat bone marrow mesenchymal stem cells (BMSC)</td>
<td>Static: Proliferation similar on F and MP to noELP surface.</td>
<td>N/A</td>
<td>[17]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dynamic: ↑ Total proliferation on both F &amp; MP surfaces.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surface Description</td>
<td>Cell Phenotype</td>
<td>Cell Adhesion &amp; Proliferation</td>
<td>Cell Detachment</td>
<td>Refs.</td>
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</tr>
<tr>
<td>CD-90 immobilized-P(IPAAm-co-CIPAAm) on TCPS</td>
<td>I) Thymic carcinoma cell line (Ty-82).</td>
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<td>Ty-82 &amp; ADP cell detachment required application of shear stress &amp; low temperature treatment.</td>
<td>[25]</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>III) Primary Mouse Adipose tissue-derived (ADP) cells</td>
<td></td>
<td>NHDFs detached from all coated surface with low temperature treatment.</td>
<td></td>
</tr>
<tr>
<td>bFGF-bound heparin-fictionalized P(IPAAm-co-CIPAAm) on TCPS</td>
<td>NIH/3T3 fibroblast cells.</td>
<td>Cells reached confluence in 3 days on surfaces with bFGF densities ≥ 20ng cm⁻² compared to 5 days on TCPS dishes.</td>
<td>BFGF-8 and bFGF-20 showed best detachment rate.</td>
<td>[26]</td>
</tr>
<tr>
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<td>NIH/3T3 fibroblast cells</td>
<td>↑ proliferation with increased collagen content.</td>
<td>↓ Cell detachment with increase in collagen.</td>
<td>[27]</td>
</tr>
</tbody>
</table>

**Table 1:** Responses of Multiple Cell Phenotypes Cultured on Thermo-responsive Surfaces.
1.2 Cell interactions with PNIPAAm Coated Surfaces

Multiple studies have shown that cell adhesion and detachment are dependent upon the thickness of the PNIPAAm layer that is grafted onto the underlying base surface [11], [12], [20], [28]. Grafted layers thinner than 14 nm showed good cell adhesion in a study carried out by Kong et al. However, in Kong's study, low-temperature detachment of cells from the PNIPAAm coated glass substrates was only optimal on surfaces with 11 nm and 13 nm thicknesses respectively, cells on detached poorly from layers less than 5nm thick [20]. Different concentrations of the polymer Poly ((3-(methacryloylamino)propyl)-dimethyl(3-sulfopropyl)-ammonium hydroxide) (PMPDSAH) were added to PNIPAAm, creating new polymer composites to improve cell detachment [20]. They observed complete detachment from surfaces grafted with 4 nm thickness of (P(NIPAAm-r-MPDSAH)) which had a 75:1 PNIPAAm to MPDSAH composition [20].

The improvement of cell detachment has been approached in multiple ways. Thai researchers observed improved cell release from a temperature-responsive poly (N-isopropylacrylamide)-co-acrylamide (PNIAM-co-AM) surface with the help of a hydrophilically modified poly(vinylidene fluoride) (PVDF) membrane [21]. The group cultured human chondracyte cells on their co-polymer coated surface, but only observed 69 ± 11.45% cell release when dishes were incubated at 10 °C for 30 minutes [21]. The use of the PVDF membranes along with low temperature treatment, not only improved the cell yield to 100%, but also facilitated cell sheet transfer and decreased cell sheet shrinkage [21].

So far we have only looked at culture on traditional coated base surfaces, such as TCPS and glass substrates, which are known to support in vitro cell culture. There are two studies in Table 1 that explore the use of non-traditional base surfaces coated with PNIPAAm to improve adhesion, growth and detachment of specific cell phenotypes. In 2008 Da Silva et al. used PNIPAAm coated chitosan
membranes as a thermo-responsive culture surface. The group correlated chitosan's biocompatibility, non-toxicity and membrane porosity with improved mass transfer of nutrients and metabolic wastes [18], [29]. Results from the study showed favorable adhesion and spreading on coated surfaces, with ungrafted chitosan membranes showing very poor cell compatibility [18]. Plasma treatment of coated surfaces increases not only the surface's cell proliferation, but also enhances the detachment of confluent cell sheets after 16 °C incubation [18].

More recently, a Korean study, led by Hwan Hee Oh, fabricated thermo-responsive polystyrene (PS) nano-fibrous mats, by the electro-spinning of PS solution. These mats were then grafted with PIPAAm to produce thermo-responsive PS nano-fibrous mats for use in in vitro cell culture [13]. They observed improved adhesion and growth of human fibroblasts that were cultured on coated PS mats as opposed to coated PS dishes [13]. The investigators proposed that the three-dimensional structure of electrospun mats provided better conditions for cell metabolism, which resulted in improved proliferation. There was no cell detachment on uncoated surfaces. However cells detached from both polymer coated PS mats and dishes, with a higher percentage of cell detachment seen on coated PS

![Diagram](image-url)

*Figure 1.2: An illustration of cell sheet detachment by different types of water supply to (a) detachment from a PIPAAm-grafted TCPS (solid) surface and (b) detachment from a PIPAAm-grafted (porous) membrane [64].*
 nano-fibrous mats [13]. The porous structure of the mats was identified as the reason for rapid hydration and improved detachment compared to solid surfaces [13]. The schematic in Figure 1.2 illustrates how different hydration methods can cause variations in cell detachment.

The culturing of cell sheets with specific architectures is desirable since most normal tissue has some structure that is difficult to replicate in vitro. The effect of surface chemistry, mechanical stress and topography on bone marrow mesenchymal stem cells (BMCS) cultured on PNIPAAm-coated surfaces was investigated by Ozturk et al. Micro-patterned PNIPAAm films encouraged cell nuclei alignment and elongation parallel to the direction of trenches fabricated on the surface [17]. Unpatterned surfaces exhibited random growth [17]. Detachment studies were not performed because the goal was to impose mechanical stress on the cells via temperature variation [17].

Williams et al. attempted induced cell alignment by culturing vascular smooth muscle cells (VSMCs) on a thermo-responsive surface micro-patterned with the cell adhesive protein fibronectin. The group maintained alignment within 30° of the fibronectin lanes [30] with successful detachment and transfer of the aligned cell sheets [30]. This group also observed good adhesion and growth of VSMCs on PNIPAAm-grafted micro-textured PDMS surfaces. Hoechst staining revealed elongated nuclei aligned with lanes of the textured surface [19]. Complete cell sheets detached after low temperature exposure, with reduced shrinkage compared to sheets grown on flat PNIPAAm-coated PDMS surfaces [19].

The majority of the studies employed the photo polymerization method. This method allows for chemical bonding between the grafted polymer layer and the underlying base surface, and is believed to foster better cell response and detachment [31], [32]. Thermo-responsive p(NIPAm-co-St) was used to spin-coat glass substrates and drop-coat TCPS dishes in Xia et al's study. Styrene was included in the co-polymer to optimize the amphiphilicity of the microgels in an attempt to improve cell detachment [22]. NIH/3T3 cells showed similar cell adhesion and spreading properties on both spin-coated and
uncoated glass substrates. However there was no cell detachment observed upon low temperature treatment (20 °C) with cells only becoming more rounded, and requiring the application of shear stress to be removed from the surface [22]. Drop coated surfaces only supported good adhesion and spreading with a 0.5 wt % polymer composite coatings [22]. The investigators again reported the need for shear stress along with low temperature to detach cells [22].

Loh et al. also looked at drop coating method for creating a thermo-responsive cell culture surfaces. Loh's team used the tri-block copolymer, poly(N-isopropylacrylamide)- block-poly[(R)-3-hydroxybutyrate]-block-poly(N-isopropylacrylamide) (PNIPAAm-PHB-PNI- PAAm), to drop coat glass coverslips, and then added a layer of gelatin onto the polymer surface [23]. Mouse embryonic stem (ES) cells were observed to proliferate better on the gelatin-triblock polymer coated surface, than on surfaces coated with gelatin/PNIPAAm [23]. Detachment was tested by incubating substrates at 4 °C for 20 minutes, with 91.3% ± 17.6% detaching from gelatin/triblock polymer, compared to 59.2% ± 10.4% from gelatin/PNIPAAm coated surface [23].

Most cell culture protocols employ growth factors, which are soluble stimulants used to accelerate proliferation [33]. Because growth factors induce the degradation of other signaling molecules surface immobilization of growth factors is seen as a promising method to reduce degradation [33], [34]. A Japanese group explored thermo-responsive culture surfaces using poly(N-isopropylacrylamide-co-2-carboxyisopropylacrylamide) P(IPAAm-co-CIPAAm) [25], [26]. They covalently tethered heparin onto the CIPAAm chains, and then added heparin binding proteins, such as CD-90 and basic fibroblast growth factor (bFGF) to the surface [25], [26].

In the first study, CD-90 antibody-immobilized P(IPAAm-co-CIPAAm) TCPS surfaces were used to culture two cell lines, thymic carcinoma cell line (Ty-82), and neonatal normal human dermal fibroblasts (NHDFs), both of which are known to express CD-90 on their cell surface [25]. TY-82 cells adhered better than NHDFs to the antibody-immobilized surfaces. However both cell types formed a
confluent cell sheet after 4 days. There was only partial detachment of TY-82 cells after low temperature treatment (20°C) on all grafted surfaces, with the application of shear stress required to get complete detachment [25]. NHDFs spontaneously detached in response to the same low temperature treatment, indicating that the detachment process is “cell specific” [25].

The same group looked at cell responses to surface immobilization of the growth factor bFGF on the thermo-responsive surface [26]. NIH/3T3 fibroblast cells were cultured on bFGF-bound heparin-fictionalized P(IPAAm-co-CIPAAm) surfaces with varying densities of bFGF. Cells reached confluence in 3 days on surfaces with bFGF densities of 20 ng cm-2 and above, two days faster than cells cultured on non-immobilized surfaces [26]. Density of bFGF also affected cell detachment with cells taking 45 minutes to detach from surfaces with densities of 8 and 20 ng cm-², compared with 120 minutes for densities above 100 ng cm-², with the fastest detachment, 30 minutes, seen on non-immobilized P(IPAAm-co-CIPAAm) surface [26]. The benefit of better adhesion and faster culture time on certain bFGF-bound heparin-fictionalized P(IPAAm-co-CIPAAm) surfaces is helpful despite the drawback of decreased detachment rate [26].

Table 1's final entry is a study of thermo-responsive poly (N-isopropylacrylamide)-grafted polycaprolactone (PCL-g-P(NIPAAm)) films that were immobilized with cell-adhesive collagen to improve cell sheet formation [27]. The higher the content of the immobilized collagen, the higher the density of the attached cells [27]. PCL-g-P(NIPAAm)-collagen with thinner collagen coatings had 100% detachment. The percentage detachment decreased with increased collagen thickness [27].

1.3 Applications of Cell Sheet Engineering

Given the promising results on growth and release of different phenotypes on PNIPAAm substrates or hydrogels it is logical that different research groups have looked into the medical applications of cell sheet engineering.
One such application is the construction of mesenchymal stem cell (MSCs) sheets for use in tissue regeneration without the differentiation of the stem cells. MSCs have self-renewal ability and multi-differentiation potential, making them ideal for treatment of certain debilitating injuries and diseases [35]. In Yang et al's study, primary rat bone marrow-MSC (BM-MSCs) and human adipose tissue-MSC (AT-MSCs) were cultured on PNIPAAm copolymer films in an attempt to reduce the loss of its cell adhesion and differentiation caused enzymatic treatment [36]–[38]. Although the mechanism by which MSCs lose their primitive nature is not fully understood, the deficiency in their plasticity may arises from multiple exposure to proteolytic enzymes that degrade extracellular matrix (ECM) proteins [39], [40]. Using temperature release instead of trypsinization should prolong the primitive nature during in vitro culture [35].

Yang's group cultured intact cell sheets on PNIPAAm copolymer surfaces [35]. They observed greater cell viability, more colonies and stronger differentiation of MSCs harvested via temperature drop in comparison with the trypsinization [35]. This result was linked to the preservation of ECM proteins caused by whole sheet retrieval using the thermo-responsive surface [35]. In Ozturk et al's study demonstrated that rat BM MSCs retain their primitive nature after in vitro culture. Here MSCs cultured dynamically on a PNIPAAm surface showed reduced MSC differentiation when compared to static culture [17].

In contrast to Yang’s work, Peroglio et al sought to foster the differentiation of MSC cells in vitro for treatment of intervertebral disc (IVD) pathologies. Injection of human MSC (hMSCs) into the intervertebral disc (IVD) space is being explored to combat IVD degeneration [41]–[43]. A drawback to this treatment is the harsh conditions present within the disc tissue. Low glucose, oxygen levels, pH adversely impact undifferentiated MSCs [44]. The quality of repair hinges on the ability of the MSCs to differentiate to the disc cell phenotype after injection, and also on the cell carrier that is used to initially support the injected cells. Pre-culture of hMSCs to encourage differentiation toward the IVD cell

17
phenotype before injection is a possible path to improved outcome [46]. Peroglio's ex vivo study, used the thermo-reversible hyaluronan-based hydrogel, hyaluronan-poly(N-isopropylacrylamide) (HA-PNIPAAm) for the pre-culture of hMSCs, and also as a carrier for cell delivery. The hydrogel's reversible gelation caused by its PNIPAAm content, made it more suited for pre-culture of hMSCs before delivery to the target area [47]. The HA-PNIPAAm hydrogel induced hMSC differentiation toward the disc phenotype more effectively than alginate gel, with neither pre-differentiation nor growth factor supplementation required for hMSC disc-like differentiation [47].

Schwann cell transplantation has emerged as an attractive candidate in treating demyelinating diseases resulting from peripheral and central nervous system injuries [48]. Most of the existing methods involve the in vitro culture of schwann cells, which are then harvested and transported to the target site via direct injection or via scaffold seeding [48]. These conventional harvesting and transfer methods often involve proteolytic treatment, which leads to the degradation of cell-surface proteins and also breakdown of important cell-cell interactions [49]. Pesirikan et al explored the construction of schwann cell sheets on polystyrene tissue plates coated by PNIPAAm, which preserves cell-surface proteins and cell-cell interactions through temperature initiated release. The investigators were able to construct sheets of viable schwann cells that maintained cell-cell interactions as indicated by high expression of cadherins as well as the level of neurotrophins [48].

Cell sheet engineering has also been used clinically in the treatment of injured corneas caused by trauma or disease [50]. Japanese scientists constructed epithelial cell sheets by culturing autologous oral mucosal epithelial cells on a thermo-responsive surface, which included the PNIPAAm polymer [50]. Although the sample size of this study was small (4), the group reported a 100% success with complete reepithelization of the previously damaged corneal surface in less than a week after the cell sheets were introduced [50].

In a more recent study, human undifferentiated immature dental pulp stem cells (hIDPSC) were
used to construct cell sheets for implantation on the cornea of rabbits with limbal stem cell deficiency (LSCD). LSCD can be caused by multiple conditions, including genetic disorders, contact lens-induced keratopathy, and iatrogenic multiple ocular surgeries [51]. The pathology causes a persistent corneal epithelial defect or abnormal re-epithelialization by conjunctival epithelial cells, and can be treated by a transplant of tissue from the patients’ non-affected eye [52]–[54]. Gomes et al looked at culturing hIDPSCs cell sheets in vitro on a thermo-responsive surface, and transplanting the sheets onto the cornea of impaired rabbits. Results showed continued improvement in corneal transparency in comparison to the control group that had total opacification [51].

The final application considered is repair and regeneration of skin defects in mice. Cerqueira et al. engineered a construct made by stacking multiple layers of human keratinocytes (hKC) cell sheets, dermal fibroblasts (hDFb) cell sheets, and dermal microvascular endothelial (hDMEC) cell sheets, which were cultured in vitro on thermo-responsive culture dishes [55]. Recognizing the complexity of skin regeneration, the investigators choose hKCs for their wound re-epithelialization, hDFbs for ECM remodeling, and hDMECs for dermal angiogenic signaling [56]–[58]. The rate of wound closure improved in wounds treated with the cell-sheet constructs [55]. The outcome was dependent on the arrangement of the different cell types in the construct, with some stacking configurations showing only minimal wound healing improvement when compared to the control [55].

1.4 Proposal for a Novel Thermo-Responsive Cell Culture Surface

The information gathered from this review of cell interactions with these thermo-responsive surfaces allowed us to construct our own approach for the culture, retrieval, and transfer of KC cell sheets. Spin coating was chosen as our method of grafting PNIPAAm to the base surface due to its shorter processing time and ability to support cell growth based on previous studies [22]. To counteract the issues that were observed with cell detachment from spin coated surfaces however we proposed the
use of a porous PVDF membrane as our base surface.
Chapter 2: Experimental Studies with Thermo-Responsive Membranes

2.1 Background and Introduction:

Tissue engineering is a promising field of medicine which aims to develop biological substitutes that restore tissue functions [59]. Wound closure is a key process during natural wound healing, as the presence of barrier between the affected tissue and outside elements not only reduces the risk of infection, but also creates a stable micro-environment for the regeneration of the extracellular matrix (ECM) by cells [60]. The natural wound closure process is hampered in diabetic patients due to a dysfunctional ECM and deficient blood vessel formation process [61], [62]. A common treatment of diabetic wounds is wound management, which involves the regular cleaning and coverage of the affected area to assist the natural healing process [63].

In the past our group has looked at the novel use of a nanofiber hydrogel as an ECM scaffold to improve healing in the diabetic wound micro-environment [61], [62]. More recently, we have focused on providing protection for unstable region while the healing process is taking place. To do so we have proposed the in vitro growth of a keratinocyte cell sheet to be used in wound coverage, and is illustrated in Fig 2.1 shown below. As explained in Chapter 1, cell sheet recovery using traditional cell culture methods (eg. enzymatic action) is nearly impossible, as it requires the disassociation of the cultured cell layer into a solution during the harvesting process [3]. The growth of KCs on a thermo-responsive surface is a novel approach to produce a transferable cell sheets for use in diabetic wound treatment.
In this study we focused on the development of a method to culture and harvest human KCs on thermo-responsive PNIPAAm coated PVDF membranes. In the first section of the study, we investigated different cell detachment and transfer techniques, using information gained from extensive literature review, as described in Chapter 1. The second section of our study focused on demonstrating that PNIPAAm coating does not negatively affect cell viability, by comparing the levels of KC apoptosis (programmed cell death) between different PNIPAAm substrates and plastic control.

Figure 2.1: Wound healing schematic (left) and the proposed treatment strategy (right). The normal wound shows the presence of growth factors (GFs) and extracellular matrix (ECM) laid down by cells as well as formation of blood vessels to the wound site. In contrast, in diabetes, there is increased inflammation and cell death, degradation of GFs and ECM, leading to non-healing diabetic ulcers. The proposed treatment (right) includes filling the wound with the stable nano-fiber matrix for cell infiltration, covered by keratinocyte sheet layer delivered by smart release technology.
2.2 Cell Detachment and Transfer Study

2.2a Overview

In this first section, porous PVDF membranes housed in plastic inserts, shown in figure 2.2, were spin coated with PNIPAAm before they were used to culture KCs. The ability of these coated membranes to support cell adhesion and proliferation were observed qualitatively. The objectives of this study were to; 1) Conduct a preliminary study to determine optimal PNIPAAm coating parameters for cell attachment and growth; 2) Demonstrate cell sheet detachment from the PNIPAAm coated membrane, and 3) develop a procedure for the transfer cultured sheets to a secondary in vitro site.

![Coated Membrane](image)

*Figure 2.2 Image of the plastic insert which contains the porous PVDF membrane that was spin coated with PNIPAAm. The thin fabric-like membrane is surrounded by a circular plastic barrier, which forms an inner well around the membrane.*

2.2b Methods

**Cell Culture**

Frozen human primary Keratinocytes (KCs) obtained from healthy skin cell donors at the University of Cincinnati Hospital were thawed and cultured in 75cm² (T-75) cell culture flasks (*Fisher brand*) in Medium 154 (Life Technologies, Grand Island, NY), supplemented with 0.1% CaCl₂ 0.2mM (Life Technologies, Grand Island, NY), 1% antibiotic/antimycotic (Atlanta Biologicals, Lawrenceville, GA)
and Human Keratinocyte Growth Supplement (Life Technologies, Grand Island, NY). Cell cultures were maintained at 37°C in 100% humidified air containing 6% CO₂. Cells of passage 1-3 were used in all experiments, in order to maintain the phenotype of the KCs.

**Determination of Coating Parameters that Supported Cell Attachment and Growth.**

To determine the best PNIPAAm casting parameters for PVDF membranes to support KC cell growth, membranes were spun and drop coated with three different concentrations (0.5 % wt, 1.0 % wt, and 2.0 % wt) of PNIPAAm. The schematic in figure 2.3 shows an overview of this preliminary study.

**Cell Culture on Thermo-Responsive Membranes**

From culturing of KCs on the different PNIPAAm coated membranes described above, we observed that cells attached and proliferated best on surfaces spin-coated with 2%wt PNIPAAm. Therefore, 2-wt % PNIPAAm was used to spin-coat the membranes held within the inserts (30mm diameter, 0.4µm pore size, 4.2 cm² effective membrane; Millipore, Billerica, MA), followed by pretreatment with formaldehyde. All coating procedures were carried out by our collaborator Dr Naiping Hu in Prof. Dale Schaefer’s laboratory. Keratinocytes (KC) harvested from the T-75 culture flasks, via trypsinization, and seeded on PNIPAAm coated membranes (n=2) that were sterilized with ethanol. The cells were seeded at a density of 4.5×10⁴ cells/cm².
Cell Sheet Detachment and Transfer:

After 4 days, cells were stained with Orange Cell Tracker Dyes, CMTMR (Invitrogen, Carlsbad, CA).

Two approaches for cell detachment were attempted. The first involved the transfer of the membrane to cell culture plates (Fisher Scientific, Pittsburg, PA) with cells inverted onto the surface of the dish. These sections were then left at room temperature with a drop of media for 10 minutes to allow for cell detachment, after which the membrane was peeled off the surface using sterile tweezers. In the second approach, illustrated in Figure 2.4, cells were covered with 2.2 mg/ml collagen solution (Inamed Biomaterials, Fremont, CA) mixture containing 5% 0.1M DPBS, 5% 0.2M HEPES and 55% PBS, and incubated for 1 hour. The membrane from this insert was then transferred at room temperature to cell culture plates with the collagen-cell layer inverted onto the surface of the well. After 10 minutes at room temperature the membrane was peeled off using sterile tweezers. The detached cells in both approaches were fixed with 2% formaldehyde, rinsed with PBS, and aqueous mounting medium (Vector Laboratories, Burlingame, CA) was added afterwards. Images of detached cells were then taken using an inverted fluorescent microscope (Olympus IX81; Olympus America, Center Valley, PA).

Figure 2.4: Schematic of the cell detachment and transfer approach using collagen. Cells cultured on PNIPAAm coated surface for 4 days before a layer of collagen is used to cover the cells. The membrane, cell, and collagen structure is then inverted onto a cell culture dish at room temperature, before the membrane is peeled off.
2.2c Results

The KC cells adhered and proliferated well on the 2% wt PNIPAAm coated membranes. After 4 days in culture a uniform layer of KCs were present, with cells bearing the same cell morphology as those cultured in cell culture flasks. Fig 2.5 shows 4X images of KCs after being detached from the PNIPAAm coated membranes using both transfer methods. The left image shows transfer cells from the coated membranes directly, where cells were observed to detach in clumps. In contrast, cells transferred after the addition of collagen layer showed more uniform cell-sheet detachment from the coated membrane, shown in the right image.

Figure 2.5. (Right) Addition of collagen layer over cultured human keratinocytes on PNIPAAm substrate resulted in a better and more uniform sheet release, as compared to the more clump-like cell release without the collagen support (Left). Bar-150um.

Cell detachment from the coated membranes was observed after the membranes were left at room temperature for very short time periods, between 5 and 10 minutes. The cell sheet could be viewed floating in media without the use of a microscope, after detaching from underlying membrane surface. This made it difficult to transfer cell sheets without the addition of collagen, as cells would be detached from the membrane before inversion onto the cell culture dish. However with collagen added to the layer of cells prior to membrane transfer, we observed detachment of cells on the well surface after the
membrane was peeled off. The difference in cell detachment using these two transfer methods is shown in the 4x images in figure 2.5. Figure 2.6 shows a 20x image of cells after transfer using collagen, with the cells maintaining a 3d structure in the collagen after transfer.

Figure 2.6: 20x image of keratinocyte cells (stained red) held in collagen (stained green) after being transferred from coated membranes. The image shows that the cell/collagen construct was able to maintain a 3D structure after transfer. Bar 5 μm.
2.3 Comparison of Keratinocyte Apoptosis on Different Culture Surfaces

2.3a Overview and Objective

The objective of the study was to determine if the PNIPAAm coated surface does not negatively affect KC cell viability. Based on our observations from the carrying out the cell detachment and transfer study (Section 2.2) we hypothesized that there would be no detectable difference in KC apoptosis between PNIPAAm coated surfaces (membranes and dishes) and uncoated surfaces. This hypothesis was tested by measuring the percentage of caspase-3 positive KC present on PNIPAAm coated membranes, PNIPAAm coated dishes and uncoated dishes after 24 and 72 hours of culture. Figure 2.7 shows an overview of this study.

![Diagram showing KC cultured at 37 °C and stained with DAPI and Caspase-3](image)

Figure 2.7: Schematic showing an overview of KC cell apoptosis study. KC are seeded on three different surfaces, PNIPAAm coated membranes, PNIPAAm coated dishes and uncoated dishes. Cells were then cultured for 24 and 72 hours before staining with DAPI to identify all cell nuclei, and Caspase-3 to identify apoptotic nuclei (red shaded cells).

2.3b Methods

Cell Culture

Same as in Chapter 2.2b

Cell Culture on Thermo-Responsive Membranes

Same as in Chapter 2.2b except the cell seeding density for each membrane was changed to $10^5$ cells/cm² with one measurement ($n = 1$) for each time point (24 hours and 72 hours). One measurement was viewed as sufficient since the area of a membrane was more than three times that of
the individual coated and uncoated wells.

**Cell Apoptosis Detection**

Three experimental groups were established: Cells seeded on (1) PNIPAAm-coated PVDF membranes 

\( n = 2 \), (2) a PNIPAAm-coated 24-well plate \( n = 4 \) per time point (UpCell\textsuperscript{TM} Thermo Fisher Scientific, Roskilde, Denmark), and (3) uncoated 24-well plate \( n = 4 \) per time point serving as the control experiment, at a seeding density of \( 5 \times 10^3 \) cells/cm\(^2\) for both 24 h and 3 days of culturing \( n = 4 \) per experimental group). At the end of the respective culture periods, cells cultured on all three surfaces were fixed with 2% formaldehyde and immunostained afterwards with active caspase-3 monoclonal antibody (Life Technologies, Grand Island, NY) followed by goat anti-rabbit Alexa Fluor 488 antibody (Invitrogen, Eugene, OR) and DAPI.

**Fluorescent Microscopy and Cell Counting**

An inverted fluorescent microscope (Olympus IX81; Olympus America, Center Valley, PA) was used to take images of the fixed cells on all three surfaces at 4X magnification. With DAPI staining fluorescing at a different wavelength than Caspase-3 and allowing quantification of percentage cell death. Figure 2.8A shows the result of the staining process at high magnification (20x), with the nucleus of a dead cell colored both blue and red. Images were randomly taken at five different areas around the well or membrane. The cells were then counted using Adobe Photoshop\textsuperscript{™} image software. Figure 2.8B shows a 4X image randomly taken of a section of an uncoated dish, with all cell nuclei stained blue and apoptotic cell nuclei also stained red.

**Statistical Analysis**

A univariate ANOVA with two factors – time (2 levels) and substrate (3 levels) was carried out on the data set after the counting procedure was finished.
2.3c Results

During cell culture it was observed that the KCs attached and proliferated better in the uncoated dishes than on the PNIPAAm coated dishes. Good cell growth was also observed in the coated inserts, mimicking what was seen in the first section of the study, an indication that successful culturing of KC on this surface is a repeatable process.

DAPI and Caspase-3 staining showed that percentage cell death after both 24 and 72 hours of culture was greatest on PNIPAAm coated dishes. With Fig 2.6 illustrating 4.5 ± 0.4% and 5.5 ± 0.3% cell apoptosis after day 1 and day 3 respectively on coated dishes. The figure also shows similar percentages for apoptosis on uncoated dishes and coated membranes after one day of culture. After day 3 apoptosis was less on the coated membrane than both the uncoated and coated dish, with only 1.7 % cell death. It is possible that here again, the porous nature of the membranes allowed for improved nutrition and waste management between the KCs and the culture media, or better attachment of PNIPAAm polymer to the surface.

Statistical analysis carried out on our data set showed that these results were not statistically
significant (two-way ANOVA testing for the effects of substrate and time in culture). Post-hoc comparison with Bonferroni correction demonstrated a trend ($p$-value of 0.14) for the increased apoptosis levels for PNIPAAm coated dishes (UpCell$^\text{TM}$) compared to PNIPAAm coated membranes and uncoated dishes, as also evidenced by the standard deviation bars in Figure 2.9.

Figure 2.9: Culture of human keratinocytes (epithelial cells) on PNIPAAm-coated porous membrane did not increase cell apoptosis (Caspase 3 marker), as compared to standard plastic dish control. In contrast, there was a trend ($p=0.14$) for the increased apoptosis for keratinocytes cultures using commercial UpCell$^\text{TM}$ PNIPAAm-coated dishes.
Chapter 3: Conclusions and Future Directions

From Chapter 1 we can clearly see that PNIPAAm grafted surfaces and hydrogels can be used for the successful culture of multiple cell phenotypes. With specific cell responses such as adhesion, growth, alignment, and differentiation being controlled via the composition, thickness, density and topography of the PNIPAAm surface. Promising applications of cell sheet engineering in medical and tissue engineering fields are also being explored given the biocompatibility of thermo-responsive PNIPAAm surfaces and their ability produce viable cell sheets.

Our group’s exploratory studies into using KCs cultured on a thermo-responsive membrane as a part of a treatment method for diabetic wounds are promising. As qualitative observations showed good cell adhesion and proliferation of KCs on the PNIPAAm coated membranes in both sections of the study. The rapid cell detachment, which was seen in Chapter 2.2c, being attributed to the membranes porous nature. Transfer of the cell sheets was the main challenge of our study. The plastic barrier around the insert made the removal of the membrane a demanding process. For future studies it would be advantageous to remove the inserts circular border after spin coating with PNIPAAm. The use of a slightly flexible “transport surface” to transfer the KCs from the coated membrane to a desired final location, would also be an improvement on our current experimental design. Maneuvering the membrane when it is moist is currently very challenging, so the use of this intermediary would improve the efficiency of the process. This solution would be analogous to Pasuwat et al's hydrophilically modified (PVDF) transfer membrane, which allowed the in vitro transfer of chondrocyte cell sheets [21].

The second section of the study also produced promising results, as the trend that was observed via caspase-3 staining did not show a difference between KCs cultured on PNIPAAm coated and uncoated surface. Here we would like to extend the study to quantitatively look at KC proliferation on
all three surfaces (uncoated dishes, coated dishes, coated membranes). Qualitative analysis from the experiments conducted thus far show superior cell growth on our PNIPAAm coated membranes.

After the completion of the above modifications, experiments incorporating an animal model will be more manageable. Our group has considerable experience studying the diabetic wound healing process in the rat animal model [61], [62]. Fusing our current KC cell sheet work with the previous *in vivo* wound healing studies, would prove a huge step forward towards the creation of a therapeutic smart band aid for the treatment of diabetic wounds, as illustrated earlier in Figure 2.1. Hopefully, the PNIPAAm coated membranes cell compatibility is not limited to KC, as we can name a multiple other therapeutic applications from Chapter 1 alone where improved cell viability and increased cell release would be greatly advantageous.
Bibliography


