ABSTRACT

BARCIO, SARAH ANN. Thermally Responsive Surfaces for Tissue Engineering and Apparel Applications. (Under direction of Dr. Marian G. McCord.)

Thermally responsive surfaces were created by grafting poly (N-isopropylacrylamide) (pNIPAM) onto polyester (PET) film and fabric using atmospheric pressure plasma treatment, which provided a quick, simple means of grafting that sufficiently sterilized the samples for cell culture. Grafting was achieved by a two-step process of surface activation with atmospheric pressure plasma followed by exposure of the substrate to a monomer solution in the presence of atmospheric pressure plasma. The plasma exposure time and monomer solution volume were optimized using cell culture studies. The graft was characterized by surface analysis techniques and cell culture studies. Contact angle measurements at different temperatures verified the thermally responsive nature of the graft on the PET film and fabric. Atomic force microscopy (AFM) was used to examine the surface topography and the effects of an aqueous environment on the surface. Scanning electron microscopy (SEM) was also used to examine the surface of the films and fabrics and to confirm the presence of the pNIPAM. AFM images showed the surface become significantly rougher and more variable when placed in water as the polymer chains became hydrated and a gel structure formed. The decrease in surface roughness seen with the grafted film and the SEM images confirm the graft coating the untreated film. The graft thickness on the PET film was found to be between 30 and 100 nm with AFM measurements. An acid dye test verified the presence of the graft on the filtration fabric. Cell culture studies were completed using human epidermal keratinocytes (HEKs), human lung fibroblasts (HFLs), and human

hepatocellular carcinoma (Hep G2) cells to demonstrate thermally modulated cellular adhesion, growth and detachment on the films and fabrics. Viable cell sheets were successfully released from atmospheric plasma grafted pNIPAM on polyester film. Although no detachment was achieved with the grafted PET fabric, the treated fabrics could potentially be useful for tissue engineering scaffolds in bioreactors or for large-scale cell sheet engineering.

Thermally responsive textiles were created using coat- and spray-grafting of pNIPAM onto woven cotton, nylon, and polyester with atmospheric pressure plasma treatment. Fourier transform infrared spectroscopy (FTIR) was used to examine the surface chemistry and confirm the presence and washfastness of the grafts produced from the two methods. Vertical wicking tests showed an increase in wettability with increasing temperature. Coat-grafted fabrics had the greatest resistance to wicking, and spray-grafted fabrics had the greatest wicking. An acid dye test also confirmed the presence of the graft showing the greatest uniformity and washfastness from the coat-grafting method. Once fully characterized, these fabrics could be used as responsive textiles for apparel applications.

THERMALLY RESPONSIVE SURFACES FOR TISSUE ENGINEERING AND APPAREL APPLICATIONS

by

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DEDICATION

This work is dedicated to my parents, sister, and fiancé who have been so supportive and encouraging throughout this whole experience. I love you guys!

BIOGRAPHY

Sarah Ann Barcio was born April 16, 1982 in Racine, Wisconsin to Gayle and Dave Barcio. She also has a younger sister Rachel and fiancée Chris Boyd. Sarah completed her B.S. degree in Polymer and Textile Chemistry with a minor in Bioengineering in December of 2003 from Clemson University. She began a M.S. in Biomedical Engineering in August of 2004 at North Carolina State University.

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1. INTRODUCTION

Responsive polymers exhibit functional changes in response to a stimulus giving them dual and reversible properties. These polymers have received much attention for many applications including biotechnology and smart textiles. Particularly attractive responsive polymers are those stimulated by temperature. They are easy to control and allow for careful modulation of the property changes of the polymer. Poly (N-isopropylacrylamide) (pNIPAM) is a thermally responsive polymer that displays a phase change at 32^{o}C, which is between room and body temperatures.[1] This transition temperature allows it to be useful for biological applications such as drug delivery, bioseparation, biosensors, gene delivery, and cell sheet engineering.[5] Above 32^{o}C in water, the polymer becomes dehydrated and hydrophobic allowing cell adhesion; when the temperature is dropped below 32^{o}C, the polymer chains rapidly hydrate and cells detach from the surface.[37] The potential for releasing cell sheets with intact junctions is important for the future of tissue engineering as it does not introduce any of the problems with current approaches such as compliance mismatch, the inflammatory response, and the inability to repair large areas of tissue.

Grafting pNIPAM onto other substrates imparts the thermoresponsiveness to the substrate surface. PNIPAM grafting can be achieved through reactions induced by chemical reagents, UV, electron beam irradiation, γ-irradiation, and plasma treatment. Each method has advantages and disadvantages, but atmospheric plasma treatment is a novel method of grafting pNIPAM that is quick, easy, and can be used in a continuous process.

This work evaluates atmospheric plasma grafting of pNIPAM on polyester film and optimization of treatment parameters with cell culture studies intended as a model for cell attachment/detachment on polyester filtration fabric. It also examines two methods of pNIPAM grafting on woven cotton, nylon, and polyester fabrics through surface characterization and wettability testing. The goals of the research are to create thermally responsive textile substrates for tissue culture that allow for control of the attachment and release of individual cells or cell sheets and provide new surfaces for large-scale cell sheet engineering or tissue engineering scaffolds in bioreactors.

2. LITERATURE REVIEW

2.1. Thermoresponsive Polymers

"Smart" polymers are those that demonstrate reversible sharp property changes in response to environmental cues such as pH, electric field, light, and temperature. Temperature is one of the most commonly used stimuli in responsive polymers due to the ease of control. There are many thermoresponsive polymers used for drug delivery and biotechnology applications. However, poly (N-isopropylacrylamide) (pNIPAM) is one of the most widely studied thermoresponsive polymers because of its acute phase transition near human body temperature. In water, pNIPAM undergoes a phase separation with an increase in temperature at a lower critical solution temperature (LCST) of 32^{o}C. PNIPAM chains hydrate to form an expanded, hydrophilic structure in water when the solution temperature is below its LCST and dehydrate to form a compact, hydrophobic structure when heated to above the LCST.[1] This behavior is shown in Figure 2.1 using wettability and contact angle measurements.

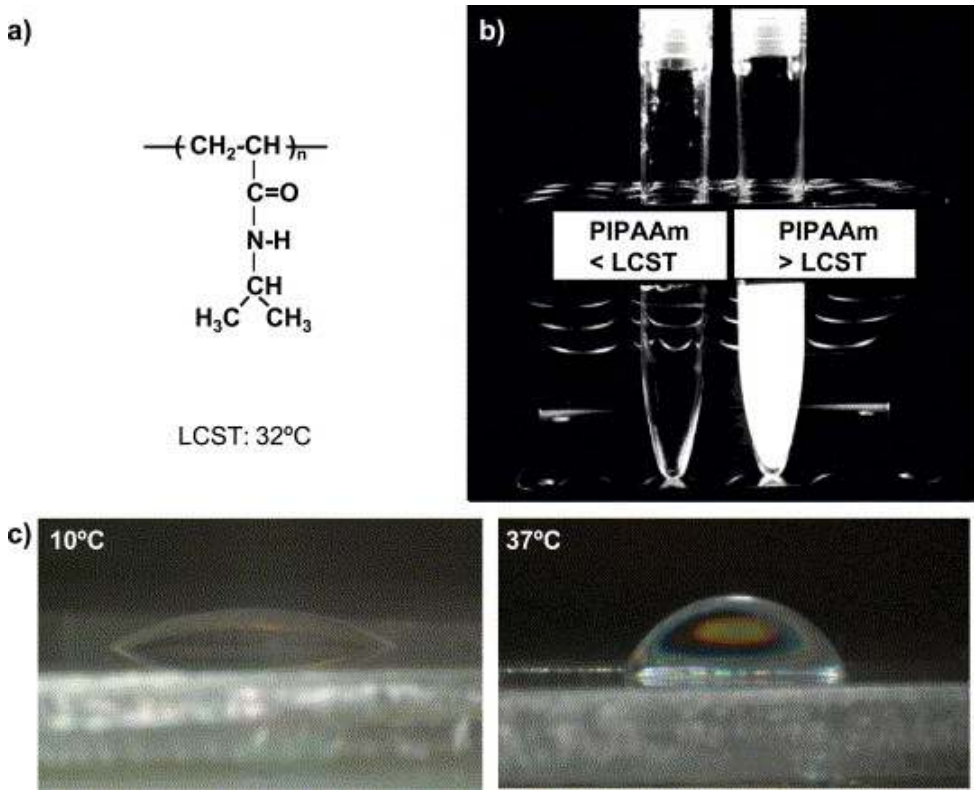


Figure 2.1: Structure and properties of thermoresponsive PIPAAm in solution and on surfaces. (a) Structural formula for PIPAAm. (b) Thermoresponsive soluble/insoluble changes for PIPAAm in aqueous solution. (c) Temperature-dependent wettability changes for PIPAAm-grafted surfaces at 10 and 37 °C.[2]

The LCST of pNIPAM is significant because it falls between room and body temperatures making it beneficial for medical applications.

As seen in Figure 2.1 A, the pNIPAM structure contains hydrophilic groups (NH and C=O) and a hydrophobic group (isopropyl).[3] The phase transition that occurs in water is due to the temperature-dependent hydrogen bonding and intermolecular hydrophobic interactions.[3] The intermolecular hydrophobic interactions within the polymer are mainly responsible for the dehydration of the structure above the LCST; hydrogen bonding and intermolecular interactions between the polymer and water are responsible for the hydration of the polymer chains.[3,4]

2.2. Applications of pNIPAM

Grafting pNIPAM onto surfaces such as textiles or plastics endows the new material with the same thermoresponsiveness of the polymer. This grafting allows for greater potential applications and usable forms. The proximity of the LCST to body and cell culture temperatures and its responsive nature make it valuable for use in biotechnology and medical applications such as drug delivery, chromatography, tissue engineering, and cell sheet engineering.[2,5]

For drug delivery, it has been used to make core-shell microcapsules with thermo-responsive gates as shown in Figure 2.2, drug loaded thermo-responsive micelles or in water-diffusion controlled release hydrogels.[7- 9]

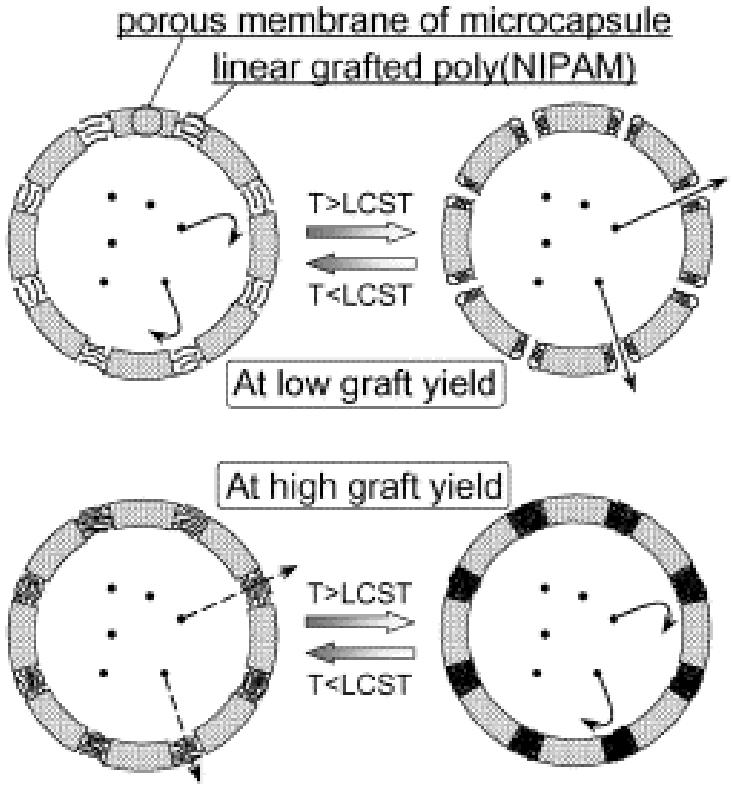


Figure 2.2: A schematic representation of the thermo-responsive release principle of core-shell microcapsules with a porous membrane and thermo-responsive polymeric gates.[7]

For chromatography, it has been used to make modified column matrices with grafted beads.[10,11] Figure 2.3 shows how the thermoresponsive beads respond to temperature.

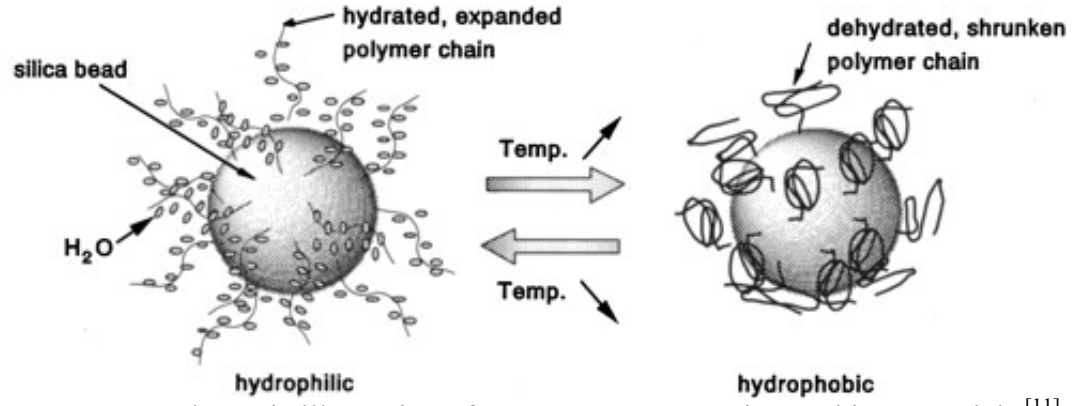


Figure 2.3: Schematic illustration of temperature-responsive packing materials.[11]

It has also been found that the polymer can be used to coat surfaces for cell growth where the hydrophobic phase acts as an adhesive surface for cells and the hydrophilic phase acts as a releasing surface.[12,37] As the temperature drops below 32°C, the pNIPAM chains rapidly hydrate causing the cells to detach from the surface.[13] It has also been found that, like active adhesion, cell detachment requires metabolic energy and includes a cell shape change.[13,14] Figure 2.4 shows cell attachment and detachment to grafted surfaces.

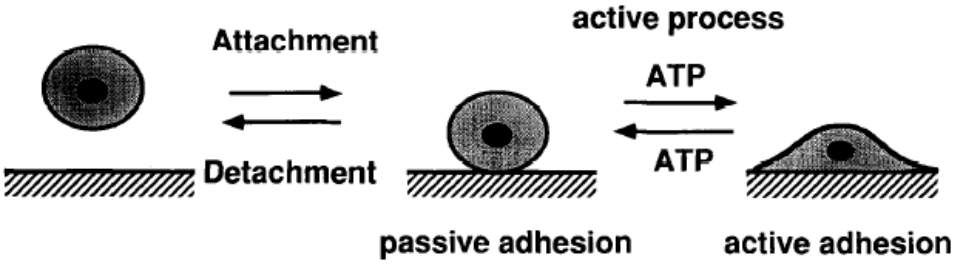


Figure 2.4: Mechanism of cell attachment to and detachment from material surfaces.[13]

This behavior is advantageous because it eliminates the need for enzymatic or mechanical detachment of cells allowing them to retain their morphology and function as shown in Figure 2.5.

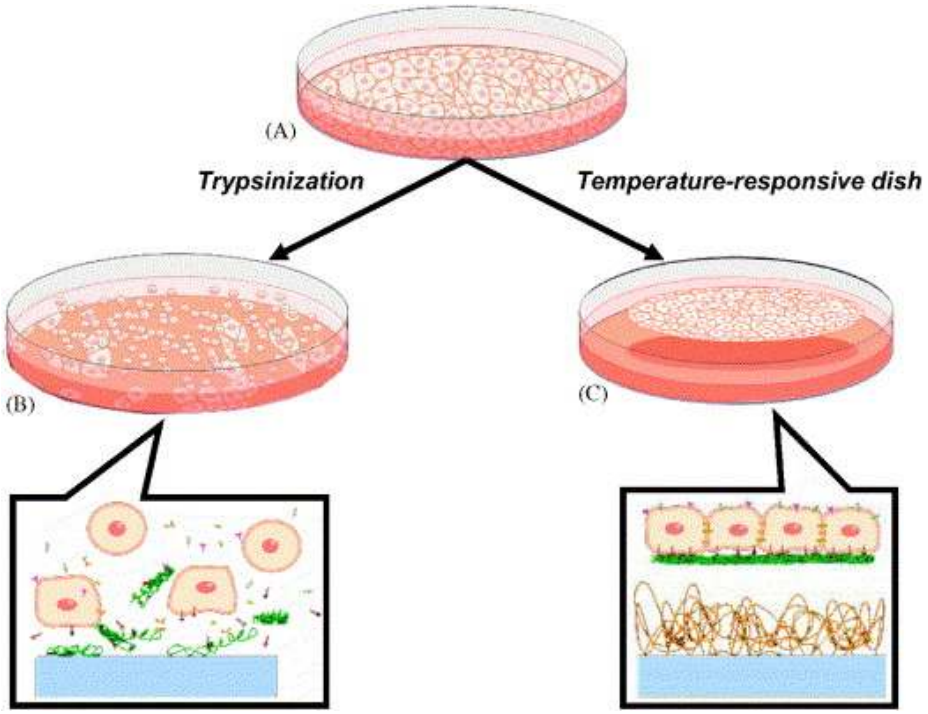


Figure 2.5: Temperature-responsive culture dishes. (A) During cell culture, cells deposit extracellular matrix (ECM) molecules and form cell-to-cell junctions. (B) With typical proteolytic harvest by trypsinization, both ECM and cell-to-cell junction proteins are degraded for cell recovery. (C) In contrast, cells harvested from temperature-responsive dishes are recovered as intact sheets along with their deposited ECM, by simple temperature reduction.[6]

It has been found that enzymatic and mechanical detachment can disrupt the cell membrane and cause a change in cellular activity.[15]

As mentioned above, pNIPAM grafted surfaces have already been used extensively for cell culturing being used with urothelial, vascular smooth muscle, retinal, and lung cells to list a few.[16-19] This has led it to be looked at for tissue engineering and more specifically cell sheet engineering use. Traditional methods for tissue engineering are based on isolated cell suspensions or biodegradable scaffolds.[6] These two methods have shown limited success and improvement of them has been slower than expected leaving room open for other options. Okano's group recently identified cell sheet engineering as another option.[6] This type of engineering is advantageous because it yields cell sheets that retain their native extracellular matrix, which is responsible for the intrinsic adhesion.[2] Tissue reconstruction can be performed with different types of cell sheets. Single cell sheets can be used for the cornea; and multilayer sheets can be used in the heart as shown in Figure 2.6.

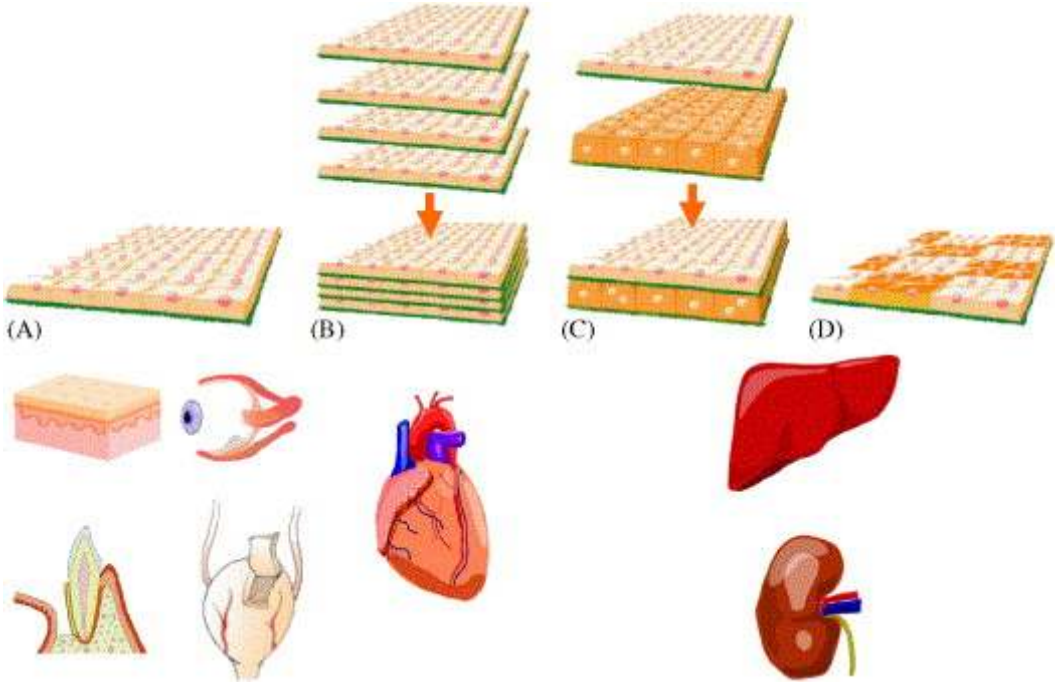


Figure 2.6: Tissue reconstruction using cell sheet engineering. (A) By transplanting single cell sheets directly to host tissues, skin, cornea, periodontal ligament, and bladder can be reconstructed. (B) Using homotypic layering of cell sheets, 3-D myocardial tissues can be created. (C) With heterotypic stratification of cell sheets, laminar structures such as liver and kidney can be fabricated. (D) Additionally, the creation of co-cultured cell sheets from dishes with dual temperature-responsive domains, also allows for the re-creation of higher-order structures such as the kidney and liver.[6]

Unlike biodegradable scaffolds, they do not induce the foreign body response and result in more uniform cell growth along the entire length of the construct.[6] The use of single cell suspensions for integration with native tissue does not address the need for a connected cell sheet in applications such as smooth muscle.[6] Figure 2.7 shows how detached cell sheets released using pNIPAM grafted surfaces can be stacked in vitro and adhere quickly in vivo.

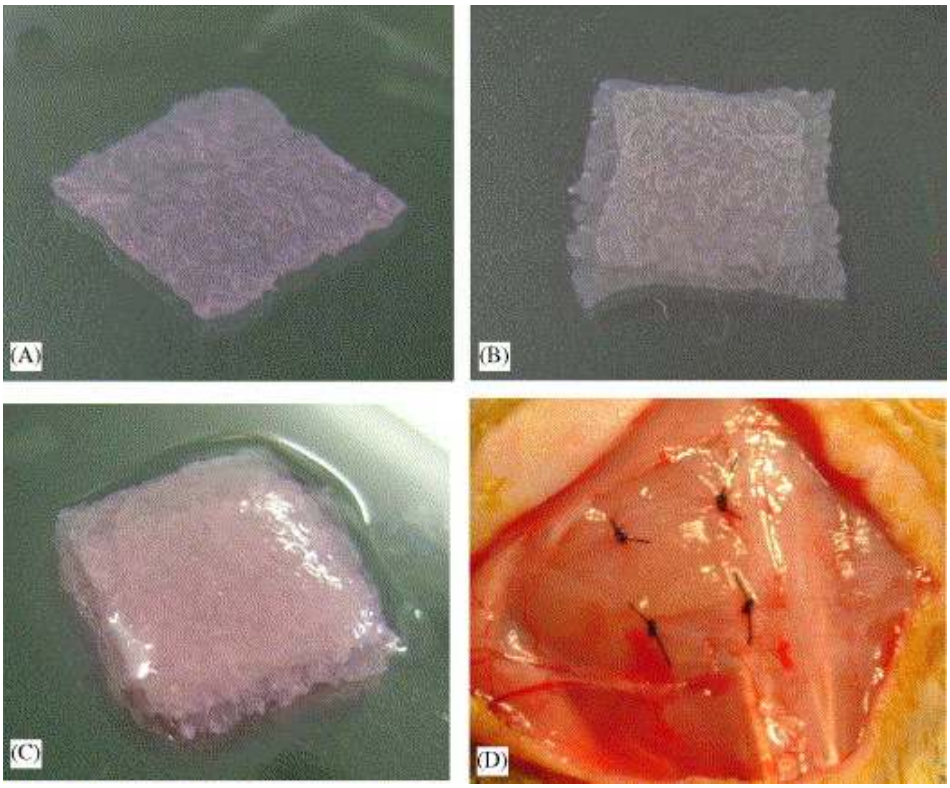


Figure 2.7: Smooth muscle cell sheets. (A) Smooth muscle cells cultured on temperature-responsive dishes can be harvested as intact sheets by simple temperature reduction. (B) Two or (C) five smooth muscle cell sheets can be layered in vitro. (D) Five-layer smooth muscle cell constructs can be transplanted subcutaneously and adhere after 5 min due to the presence of deposited extracellular matrix. Note: In panel (D), sutures are used only to mark the borders of the transplanted constructs.[6]

Advantages to using pNIPAM coated surfaces in cell sheet engineering include their ability to grow co-culture systems, to be modified with peptide sequences, and to form micropatterns.[2,6]

2.3. Grafting Methods

PNIPAM is already being used in many applications. The most frequently used methods for pNIPAM graft polymerization are γ-irradiation, vacuum plasma, and electron beam. Other methods include the use of chemical reagents, UV, and ozone treatment.

2.3.1. Traditional Grafting Methods

Most of the recent work with pNIPAM grafted surfaces for tissue engineering purposes has been by Okano et al. and Ratner et al. Okano has used chemical and electron beam irradiation grafting.[20-23] Although these methods have been very successful and well characterized, chemical grafting requires the use of numerous toxic reagents and gases with lengthy preparation times; and electron beam irradiation introduces radiation into the process. Overall, these two methods lead to a lengthy, expensive, and potentially hazardous preparation of grafted surfaces.

Ratner has used vapor-phase deposition of pNIPAM by plasma polymerization.[1] This process is useful because it is one-step and does not require the use of solvents making it safe and environmentally friendly. Plasma polymerization itself also has many advantages including the creation of uniform, adhesive, and pinhole-free grafts.[1] Like Okano, they have extensively characterized the resulting pNIPAM graft. However, the vapor-phase plasma polymerization requires the use of vacuum, high temperatures, and low chamber pressure. Overall, this method is still expensive and does not allow for the production of large grafted surfaces.

2.3.2. Atmospheric Plasma Grafting

Plasma is an ionized gas consisting of electrons, ions, radicals, atoms, and molecules created with electrical or thermal stimulation.[32,35] Plasma treatment has already been used extensively for surface modification by bombarding the surface with the species created from the ionized gas and with the creation of free radicals on the substrate.[33,34] It allows for reaction with the surface from depths of 100 Å to 10 μm

without changing the bulk material properties.[33,35] Figure 2.8 shows how plasma treatment can be used to modify surfaces through crosslinking, etching, grafting, and functionalization.

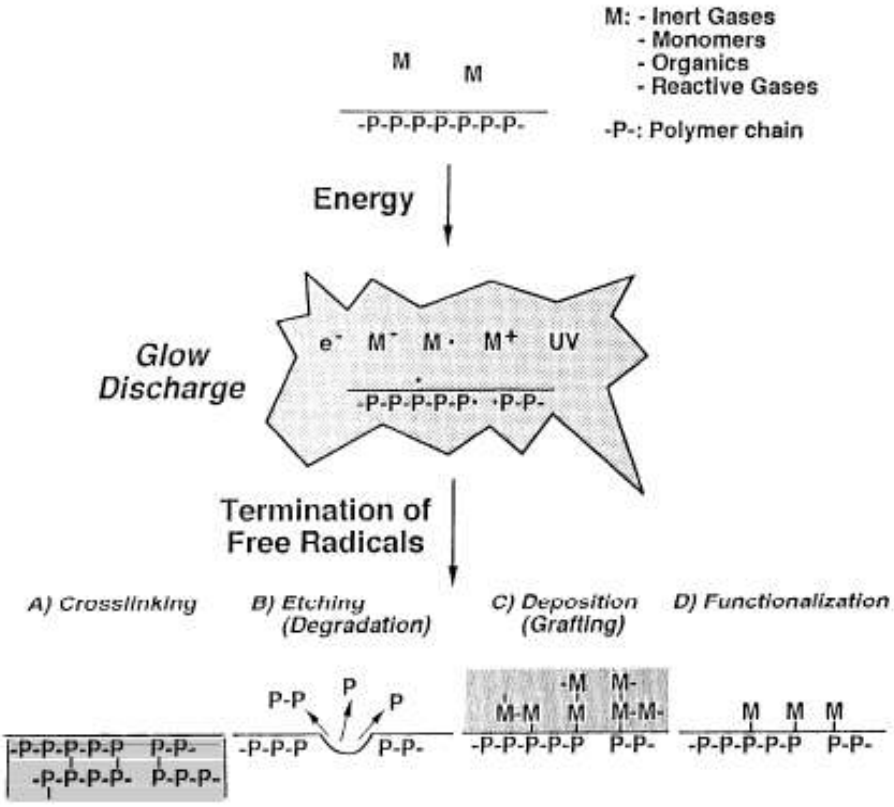


Figure 2.8: Schematic of the reaction mechanisms of plasma surface modifications.[33]

Many different gases including argon, helium, oxygen, nitrogen, and fluorine can be utilized with plasma.[35] The two gases chosen for this study were helium as the seed gas and oxygen as the treatment gas. Helium is an inert gas that is highly stable and produces a uniform plasma. Oxygen creates functional groups at the surface and aids in crosslinking and sterilization.[35]

The traditional methods used above are successful in grafting pNIPAM onto substrates, but atmospheric plasma polymerization provides a simple and quick way to

graft that does not require extensive chemical use or expensive equipment. It is also effective in producing a sterile substrate for cell culture.[33] Atmospheric plasma treatment can be performed at room temperature and pressure without extra chemical reagents and can accommodate the processing of large surface area and continuous production lines. The large scale capabilities of this method will enable production of a fabric substrate that can be used to grow significant amounts of cells. Since the fabric is pliable, it can be treated and then packed into a smaller space with large cell growth capabilities per area. By being able to graft onto fabrics, we will be able to use them for scaffolds in bioreactors and create large cell sheets.

2.4. Previous Work with pNIPAM Grafted Surfaces

PNIPAM grafted surfaces have been extensively characterized and studied for use with cell culture. Successful cell sheet engineering has been achieved with cardiac myocytes,[40-43] keratinocytes,[44] lung cells,[19] urothelial cells,[16] periodontal ligament cells,[45] and tracheal epithelial cells.[46]

Since 1990, Okano et al. have evaluated pNIPAM grafted surfaces by testing cell proliferation and detachment with bovine aortic endothelial cells and rat hepatocytes on tissue culture polystyrene (TCPS) plates and PET membranes.[4,12,13,23,36-38] Okano et al. have also studied the mechanism of cellular activity with grafted surfaces through immunohistochemistry and transmission electron microscopy finding that cell detachment is mediated by intracellular signal transduction and the fibronectin matrix is recovered with the detached cell sheets.[13,14,37,39] Characterization of the pNIPAM grafted surfaces has been performed with Fourier transform infrared spectroscopy (FTIR), contact angle,

electron spectroscopy for chemical analysis (ESCA), and atomic force microscopy (AFM) to examine the thermoresponsiveness, graft thickness, and surface chemistry and morphology.[4,20,23] It was found that electron beam irradiation successfully grafted pNIPAM onto porous cell culture membranes making them more smooth than the ungrafted membrane.[4] It was also found that, with electron beam irradiation grafting, the thermoresponsiveness of the polymer and cell attachment and detachment is affected by the graft thickness.[23] The thickness is thought to affect the polymer chain mobility and interactions. The graft thickness that was most successful in cell culture studies was 8-23 nm.[23] The thin grafts have a lower chain mobility below the LCST which promotes aggregation and enhances dehydration; however, the thicker grafts have increased hydration even below the LCST which does not allow for cell adhesion.[23]

Recently, Ratner has evaluated plasma polymerized pNIPAM surfaces. Characterization of the grafted surfaces has been examined with FTIR, AFM, ESCA, contact angle, time-of-flight secondary-ion mass spectroscopy (ToF-SIMS), and sum frequency generation vibration microscopy (SFG) to examine surface wettability, film thickness, and surface chemistry and topography.[1,5] It was found that vacuum plasma treatment successfully grafted pNIPAM with retention of the monomer structure and thermoresponsiveness.[1,5] The changes in wettability and mechanical properties of the graft that occur with changes in temperature were due to conformational transformations of the side chain groups.[5] Cell proliferation and detachment has also been examined with bovine aortic endothelial cells on TCPS plates; and cellular activity on the surfaces has been examined with immunohistochemistry, X-ray photoelectron spectrometry (XPS), and

ToF-SIMS.[15,47] It was found that cell detachment using pNIPAM grafted surfaces is less harmful to extracellular matrix proteins than enzymatic and mechanical processes.[15] Although this type of cell detachment results in an intact extracellular matrix, some of the extracellular matrix proteins are left on the surface after release.[47]

Other successful cell attachment/detachment has been demonstrated with corneal endothelial cell sheets[48] and neural cells;[49] however, a minimal amount of research has been published on the tissue engineering potential of pNIPAM grafted surfaces besides the work by Ratner and Okano. Most other cell culture studies utilize pNIPAM in copolymer systems with polymers such as gelatin or acrylic acid.

Preliminary work characterizing pNIPAM grafted on PS plates and nylon film using a novel atmospheric plasma method demonstrated successful grafting of pNIPAM and its retention of thermoresponsiveness. This was confirmed through FTIR, contact angle measurements, and cell attachment and detachment with Hep G2 cells.[24] The effects of grafting parameters such as plasma pre- and post-treatment times and monomer solution volume were examined as well.

The application of responsive polymers has also led to interest in the development of smart textiles. In the textiles industry, pNIPAM has been studied mostly with nonwovens or as part of a copolymer system,[50] but on a few occasions it has been grafted onto woven cotton fabric as well.[24,51] However, these surfaces have not previously been proposed for use with cell culture.

3. MATERIALS AND METHODS

3.1. NIPAM Solution Preparation

N-isopropylacrylamide (NIPAM, 99%) was purchased from Acros Organics (Hampton, NH) and was recrystallized for further purification using hexane (95%) from Acros Organic (Hampton, NH). To recrystallize the NIPAM, 10.33 g was added to 125 mL of hexane. The mixture was dissolved on a hot plate while stirring. The solution was allowed to cool and recrystallize before being filtered to remove impurities and washed with hexane then left to dry in a fume hood. The NIPAM monomer solution was a 45% solution with 2-propanol. The 2-propanol used was purchased from Sigma-Aldrich (St. Louis, MO).

3.2. pNIPAM Grafting

3.2.1. Atmospheric Plasma Parameters

The grafting was performed in an atmospheric glow discharge device (APGD) plasma treatment system.[25] A schematic of the apparatus is shown in Figure 3.1. The atmospheric plasma was generated using 99% He/1% O_2 gas at flow rates of 10 and 0.14 L/min respectively, power level of 4.8 kW, and frequency of 5 kHz.

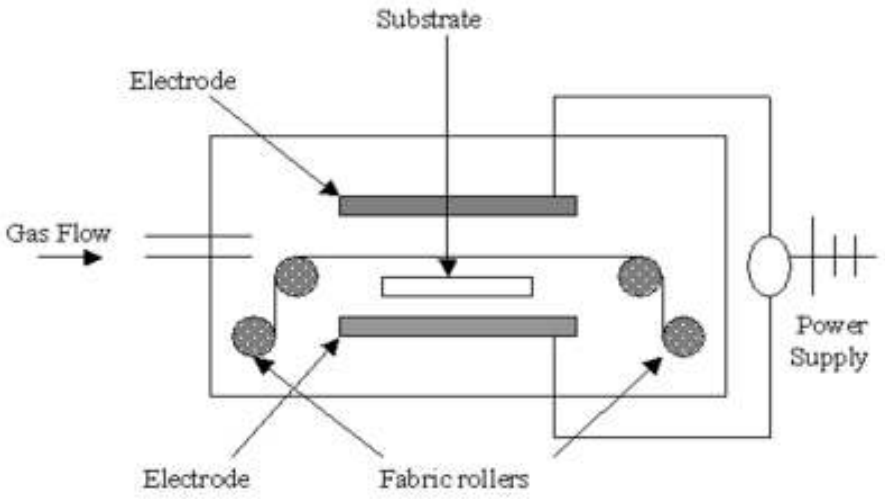


Figure 3.1: Schematic of the atmospheric pressure plasma device.[25]

3.2.2. Polystyrene Plate and Polyester Film and Filter Fabric Grafting Method

Non-tissue culture treated 24-well polystyrene (PS) plates were purchased from Falcon (Becton Dickinson, Franklin Lakes, NJ). The non-treated 60 mm PS culture dishes were purchased from Corning (Corning, NY). Polyethylene terephthalate (PET) film used was purchased from McMaster-Carr (product number 8567K52). The film is made from ethylene glycol and dimethyl terephthalate, and it is a thermoplastic film comparable to Mylar®. The PET filtration fabrics (5 μm and 21 μm pore sizes) used were generously provided by Spectrum Laboratories (Rancho Dominguez, CA). The film samples were prepared by gluing a piece of film washed with 70% ethanol to the bottom of 24-well PS plates and 60 mm PS plates. The fabric samples were simply placed in a 60 mm PS plate for plasma polymerization and later cut to size according to the test application. The glue (Silastic A-100-S) used was purchased from Dow Corning (Midland, MI). The film and fabric used for cell culture studies were cut to the size of a 24-well plate well (1.6 cm diameter). The samples were pretreated in the APGD and then coated with the NIPAM

solution. The volume of monomer solution used is shown in Table 3.1 according to the type of plate used. The solution was spread evenly with gentle agitation by hand. After coating, the polymer was grafted onto the surface of the samples with post-treatment in the APGD. The samples were then vigorously washed with cooled distilled water to remove any unpolymerized NIPAM and left to dry. The samples prepared for cell culture studies were washed with sterile water and dried in a sterile hood. Figure 3.2 shows a schematic of the pNIPAM grafting process.

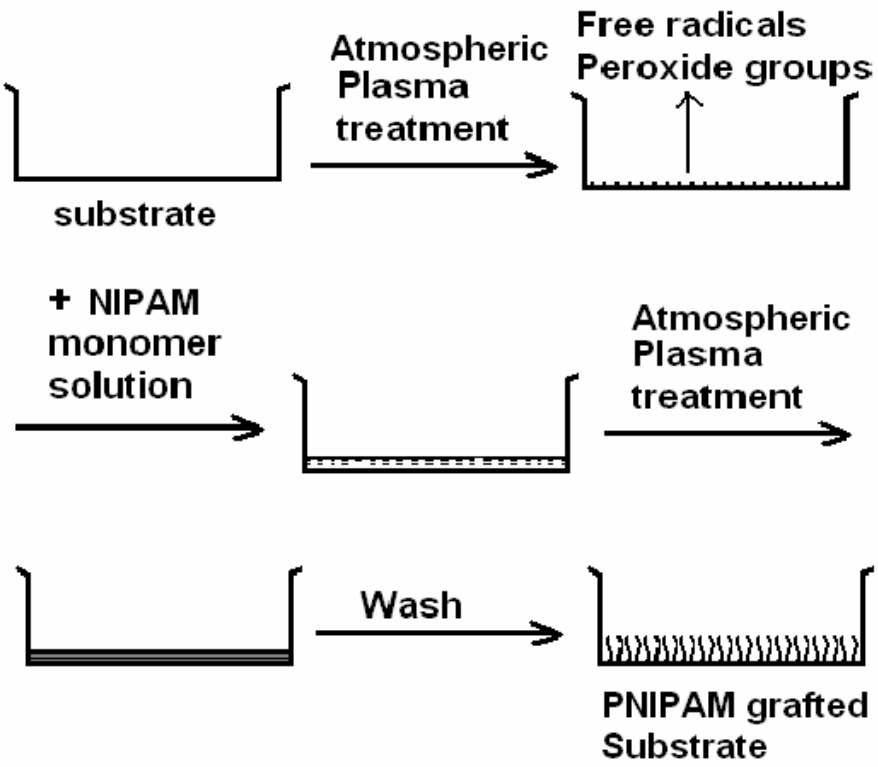


Figure 3.2: The schematic of graft polymerization of NIPAM.[24]

The amount of monomer solution and the pre- and post-treatment times were chosen based on previous studies and optimized during the tests.

Table 3.1: Volumes of monomer solution used based on plate type.

Plate Type	Well Area (cm^2)	Volume Tested (μL)				
		5.14 μl/cm^2	10.3 μl/cm^2	15.4 μl/cm^2	20.6 μl/cm^2	25.7 μl/cm^2
6-well	9.72	50 ul	100 ul	150 ul	200 ul	250 ul
24-well	1.98	10.2 ul	20.4 ul	30.5 ul	40.8 ul	50.9 ul
60 mm	28.27	145.3 ul	291.2 ul	435.4 ul	582.4 ul	726.5 ul

3.2.3. Fabric Grafting Method

Woven cotton (greige, 122 g/m^2), nylon (spun Dupont Type 200, 124 g/m^2), and polyester (batiste, 72 g/m^2) fabrics from Testfabrics, Inc. (West Pittston, PA) were provided in the lab. Three sets of samples were made with each of the fabrics according to Table 3.2.

Table 3.2: Grafting methods for the coated and sprayed fabrics.

Fabric Set	Monomer Solution Application Method	Amount of Solution Applied	Plasma Pretreatment Time (min)	Plasma Post-treatment Time (min)	Washing Method	Drying Method
Coated	Dipped	completely submerged (excess padded off)	3	5	agitated in cool distilled water	oven
Sprayed 1	Sprayed w/airbrush	until entire surface visibly wet	3	5	agitated in cool distilled water	oven
Sprayed 2	Sprayed w/spray bottle	until entire surface visibly wet	3	5	agitated in cool distilled water	oven

3.3. Surface Characterization

3.3.1. Contact Angle

The water contact angles of the pNIPAM-grafted PET film and filter fabric samples were measured in air at 25°C and 40°C using the sessile drop method with a goniometer (Model A-100, Ramé-Hart). An environmental chamber with a heater was used for the

warm tests. Deionized (DI) water (8 μL) was dropped on the samples using a syringe. The contact angle was read 10 seconds after the water drop contacted the sample.

3.3.2. Atomic Force Microscopy

The surface topography of the PET films was examined with an atomic force microscope (JEOL JSPM-5200) under tapping mode in dry and wet phase (DI water). The original and plasma pretreated films were scanned in the dry phase at room temperature (24°C), and the grafted film was characterized in the dry phase (24°C) and wet phase at room temperature and 37°C. Measurements were carried out with a BS-Multi 75Al silicon probe at varying scan areas. The thickness of the graft was determined with the Digital Instruments D3000 under tapping mode in dry phase at room temperature with a BS-Multi 75Al silicon probe. An xy- and x-scan were done to confirm results. The sample for this test was a templated grafted 60 mm PS plate. The template was created by placing a piece of static cling film to the bottom of the plate prior to plasma treatment. The film was peeled off to allow a vertical distance to be recorded between the grafted and ungrafted portions.

3.3.3. Scanning Electron Microscopy

The surface topography of the PET film and filter fabric was examined with a scanning electron microscope (Hitachi S-3200N) under vacuum at room temperature at 5 kV. The samples were gold plate coated by sputtering for 180 seconds with argon.

3.3.4. Fourier Transform Infrared Spectroscopy

The surface chemistry of the grafted PET filter fabric was examined with Fourier transform infrared spectroscopy (FTIR) with the Nexus 470 FTIR spectrometer used in

conjunction with the Avatar Omni Sampler, which is an attenuated total reflection (ATR) accessory with a 45° KRS-5 crystal. The spectra were collected at 4 cm^{-1} resolution with an FTIR microscopic spectrometer over 64 scans at room temperature.

3.4. Cell Culture Studies

3.4.1 Human Epidermal Keratinocyte Studies

Human epidermal keratinocytes (HEKs) were cultured in keratinocyte basal medium (KBM-2) (Cambrex, East Rutherford, NJ) supplemented with 0.1 ng/mL human epidermal growth factor, 5 mg/mL insulin, 0.4% bovine pituitary extract, 0.1% hydrocortisone, 0.1% transferring, 0.1% epinephrine, and 50 mg/mL gentamicin/50 ng/mL amphotericin-B (Single Quots, Cambrex). The cells were seeded at a density of approximately 20,000 cells/well in 1 mL of media in treated 24-well PS plates. The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. A media change was performed on day 2 and day 5 if the cells had not reached at least 75% confluency at that time. The cells were removed from the incubator and allowed to remain at room temperature (25°C) for 1.5 hours.

3.4.2 Human Lung Fibroblast Studies

Human lung fibroblasts (HFLs) were cultured in Kaighn's modification of Ham's F-12 medium (F12K) (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and antibiotics. For the film samples, the cells were seeded at a density of 125,000 cells/well in 1 mL of media in treated 24-well PS plates. For the fabric samples, the cells were seeded at a density of approximately 500,000 cells/well on an inverted 6-well cell culture insert with a polyester membrane (Becton Dickinson, Franklin Lakes, NJ) with the

grafted fabric glued to the underside in a 6-well PS plate. Figure 3.3 shows a layout of the insert for the fabric study.

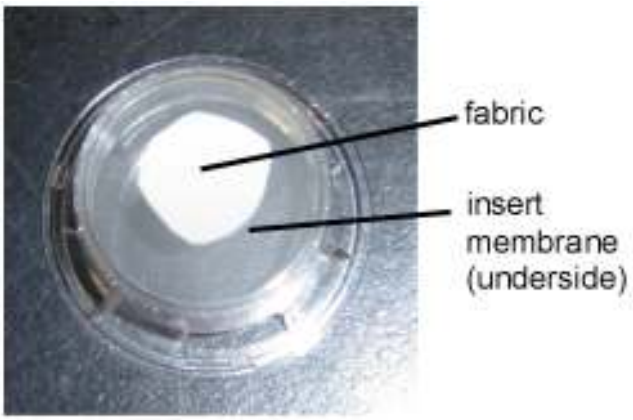


Figure 3.3: Layout of the cell culture insert for filtration fabric seeding.

The inserts were left inverted for the cells to attach for 2-4 hours and then turned over to have 1.5 mL of media added. The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. A media change was performed on day 2, and the cells were photographed on day 4. For better visualization, the cells on the fabric samples were stained with Coommassie Blue for 1-2 minutes then rinsed with phosphate buffered saline (PBS) several times. After removal from the incubator, the plates were allowed to reach room temperature (25°C) and checked periodically over a period of one hour.

3.4.3. Viability Test

The viability of the releasing HFLs was evaluated using 0.4% Trypan Blue Vital Stain (Cambrex, East Rutherford, NJ). The Trypan Blue was added 1:1 with medium in the 24-well plate (1 mL) and allowed to sit for 5 minutes at room temperature. The wells were then carefully washed with PBS. The control wells were a non-tissue culture treated well and a grafted PET film well. The Trypan Blue was added immediately to these wells.

The test wells were a non-tissue culture treated well and a grafted PET film well. These cells were left out at room temperature for 2 hours to allow for release, and then Trypan Blue (1 mL) was added and allowed to sit for 5 minutes. The wells were then carefully washed with PBS. The viable and dead cells were visually noted and photographed in the wells.

3.4.4. Proliferation Test of Released Cells

The proliferation of the releasing HFLs was evaluated by allowing the cells to grow to confluency on grafted film in a 24-well plate. The cells were seeded at 250,000 cells per well in 1 mL of growth medium. After being allowed to release at room temperature for 60 minutes, the cell aggregates and medium were transferred with a pipette to a new non-tissue culture treated well. The cells were then incubated for 3 days and observed.

3.4.5. Human Hepatocellular Carcinoma Cell Studies

Human hepatocellular carcinoma (Hep G2) cells were purchased from ATCC (Mannasas, VA) and cultured in Eagle's minimum essential medium (EMEM 1X) (Cellgro, Herndon, VA) supplemented with 1 mM sodium pyruvate (Cellgro) , 0.1 mM MEM non-essential amino acids (Cellgro), 10% fetal bovine serum (FBS) (Cambrex, East Rutherford, NJ) , pen/strep (Cellgro), and 2 mM L-glutamine (Cellgro). For the fabric samples, the cells were seeded at a density of approximately 20,000 cells/well in 2 mL of media in a 6-well PS plate. The fabric was seeded with 100 μL of the cell suspension and left to attach for 1 hour before having 2 mL of media added. The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. No media change was performed, and the cells were photographed on day 4.

3.4.6. Live/Dead Viability Assay

A Live/Dead Viability Assay kit was purchased from Molecular Probes (Eugene, OR). Small pieces of grafted PET filtration fabric (5 μm pore size) were put in a non-tissue culture treated 6-well plate. Three wells were seeded at a density of 20,000 cells in 50 μL of medium, and three wells were seeded at a density of 40,000 cells in 100 μL of medium. The cells were incubated and allowed to adhere to the fabric for 1 hour after which 2 mL of growth medium was added. The cells were grown for 1, 2, and 6 days. After allowing the cells to grow for the specified period of time, the fabric pieces were inverted and washed twice with PBS. A 4 μM ethidium homodimer-1 and 2 μM calcein AM solution was prepared, and 2 mL was added to the wells and incubated for 10 minutes at room temperature. The fabric was viewed on an inverted fluorescent microscope (Leica DMIL) with a standard fluorescein bandpass filter for the live cells (Chroma 41001) and a standard rhodomine bandpass filter for the dead cells (Chroma 41002).

3.5. Fabric Characterization

3.5.1. Vertical Wicking

The wettability of the cotton, nylon, and polyester fabrics was evaluated using a vertical wicking test. Samples were cut into strips (7 cm long x 2 cm wide) and taped vertically with a string with about 2 cm of the strip immersed in water and weighted with a paper clip. Figure 3.4 shows a schematic of the test setup.

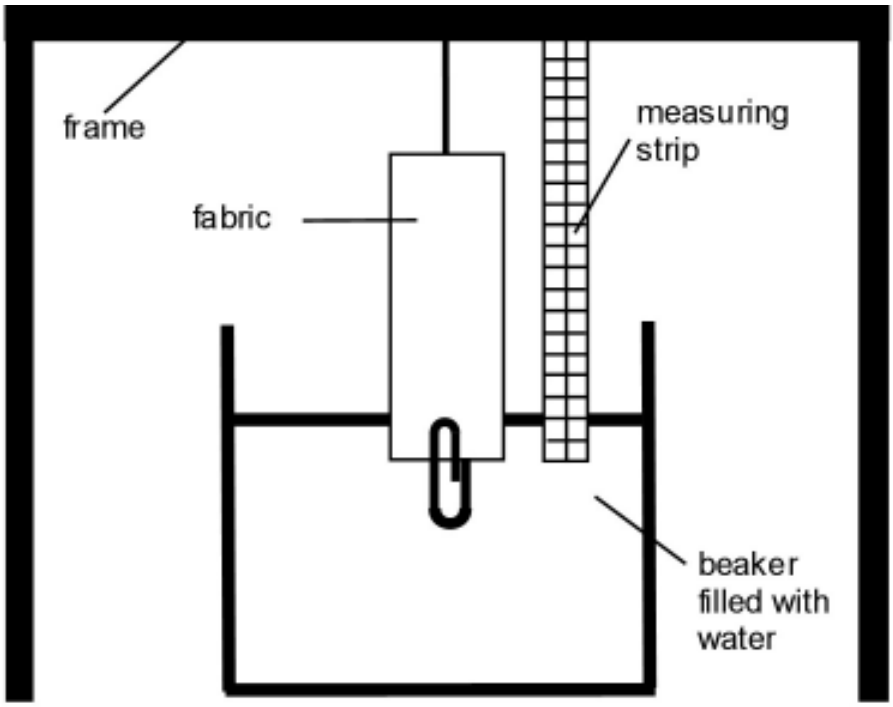


Figure 3.4: The schematic of the vertical wicking test.

The tests were performed with room temperature water (20^{o}C) and water warmed with a hot plate (50^{o}C). The height to which the water was taken up along the strip was measured at 5, 15, and 30 minute intervals.

3.5.2. Fourier Transform Infrared Spectroscopy

The surface chemistry of the grafted nylon, polyester, and cotton fabrics was examined with Fourier transform infrared spectroscopy (FTIR). The Nexus 470 FTIR spectrometer was used in conjunction with the Avatar Omni Sampler, which is an attenuated total reflection (ATR) accessory with a 45° KRS-5 crystal. The spectra were collected at 4 cm^{-1} resolution with an FTIR microscopic spectrometer over 64 scans at room temperature.

3.5.3. Acid Dye Test

Dystar Acid Blue 264 dye was used to examine the uniformity of the graft on the polyester and cotton fabrics. To make a dye solution of 3 g/L, 0.6 g of dye was added to 200 mL of water and 2 drops of acetic acid. The solution was stirred with the fabric samples for 15 minutes and then rinsed vigorously and dried in an oven.

3.5.4. Washfastness

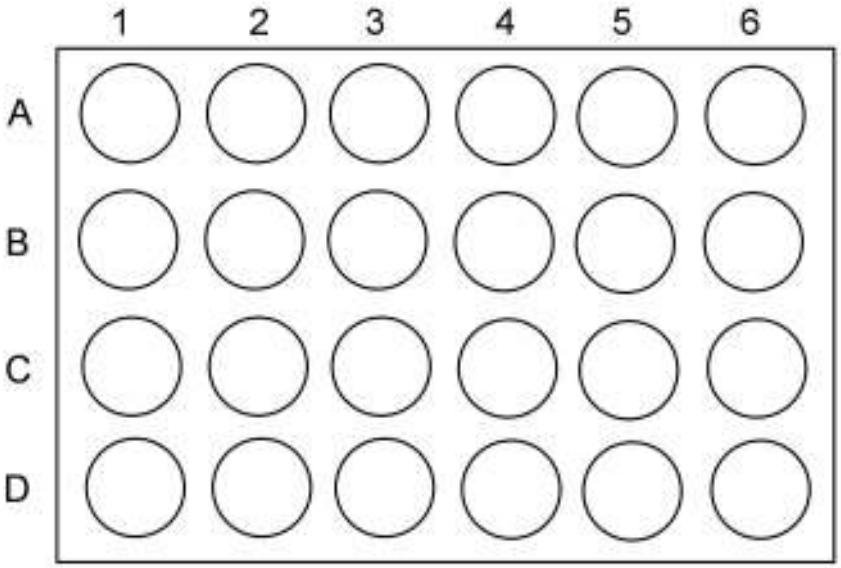
The washfastness of the grafted pNIPAM on the cotton, nylon, and polyester fabrics was examined according to a modified version of AATCC Test Method 61-1993. The samples, cut to no specific size, were conditioned and weighed prior to the grafting procedure and after the grafting procedure. The edges of the samples were sealed with Dritz® Fray Check (Prym Consumer USA, Spartanburg, SC) to prevent fraying. A 2% detergent solution was prepared as the wash liquor with AATCC detergent. Each sample was placed in an individual metal canister containing 150 mL of the prepared wash liquor with 50 stainless steel balls. The canisters were sealed and locked into the Atlas Model LHT Launder-Ometer rack. The canisters were tumbled in the Launder-Ometer for 45 minutes with the water bath temperature set at 120^{o}F, which simulates 5-10 home launderings. After the cycle was complete, the samples were removed and rinsed thoroughly with warm water and dried in an oven.

4. RESULTS AND DISCUSSION

4.1. Optimization of Plasma Treatment Times and Monomer Solution Volume on Grafted Polyester Film

Polyester film was chosen as a preliminary surface for testing due to the ease of grafting and potential for use as a model for surface characterization to predict effects on grafted polyester filtration fabric. Based on previous work, the plasma pretreatment time was set at 3 minutes. Although previous cell culture tests were successful with plasma post-treatment times of 3, 5, and 10 minutes, longer plasma exposure times with polyester film result in increased etching and similar surface activation as shorter times.[24,26] Thus, the decision was made to try to minimize the plasma exposure time. Using data from previous studies on the effect of monomer solution volume on graft yield, 50 and 100 μL monomer solution volumes (in a six-well plate) were chosen.[24] Plasma post-treatment times were 0.5 and 2 minutes. Since the ultimate goal is to produce surfaces for efficient cell sheet engineering, the parameters were optimized through cell culture and comparison to controls.

The first test was done with HEKs on two non-tissue culture treated 24-well plates, one plate grafted for 0.5 minutes in the plasma and the other grafted for 2 minutes in the plasma. Figure 4.1 shows the plate layout.

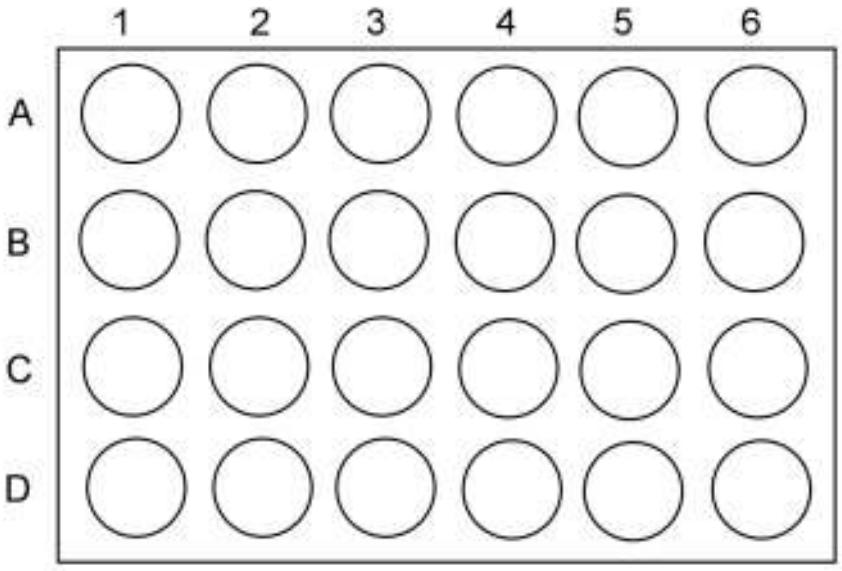


1A,B: Plasma treated PS
1C,D: Grafted PS, 10.2 uL monomer solution
2A,B,C,D: Untreated PET film
3A,B,C,D: Plasma treated PET film
4A,B,C,D: Grafted PET film, 10.2 uL monomer solution
5A,B,C,D: Grafted PET film, 20.4 uL monomer solution
6A,B,C,D: Grafted PET film, 30.5 uL monomer solution

Figure 4.1: Plate layout for first HEK test.

Two wells were seeded in a tissue culture treated 24-well plate as controls. After 7 days, the controls reached 100% confluency. Both plates had spotty growth in all columns with about 50% confluency. The cells looked stressed and stretched in some wells on both plates. After 1 hour at room temperature, there was no complete cell detachment observed, but column 6 (30.5 μL monomer solution) had the most partial detachment. The decreased cell growth was thought to be due to toxicity of the glue used, residual unpolymerized NIPAM, and the treatment parameters.

To address these problems, the washing was increased, and the toxicity of the glue was tested. The post-treatment time was increased to 5 minutes, and the lower monomer solution volume was discarded. Figure 4.2 show the plate layout.



1A,B: Plasma treated PS
1C,D: Grafted PS, 30.5 uL monomer solution
2A,B: Glue
4A,B: Plasma treated PET film
5A,B,C: Grafted PET film, 20.4 uL monomer solution
6A,B,C: Grafted PET film, 30.5 uL monomer solution

Figure 4.2: Plate layout for second HEK test.

Two wells were seeded in a tissue culture treated 24-well plate as controls. After 4 days, the controls reached 100% confluency. The test wells were all about 60% confluent; and although growth on the glue was minimal, it did not hinder growth in the well. After 90 minutes at room temperature, there was no complete cell detachment observed, but column 6 (30.5 μL monomer solution) had the most partial detachment and the best growth. Figure 4.3 shows cell growth and detachment on the plate.

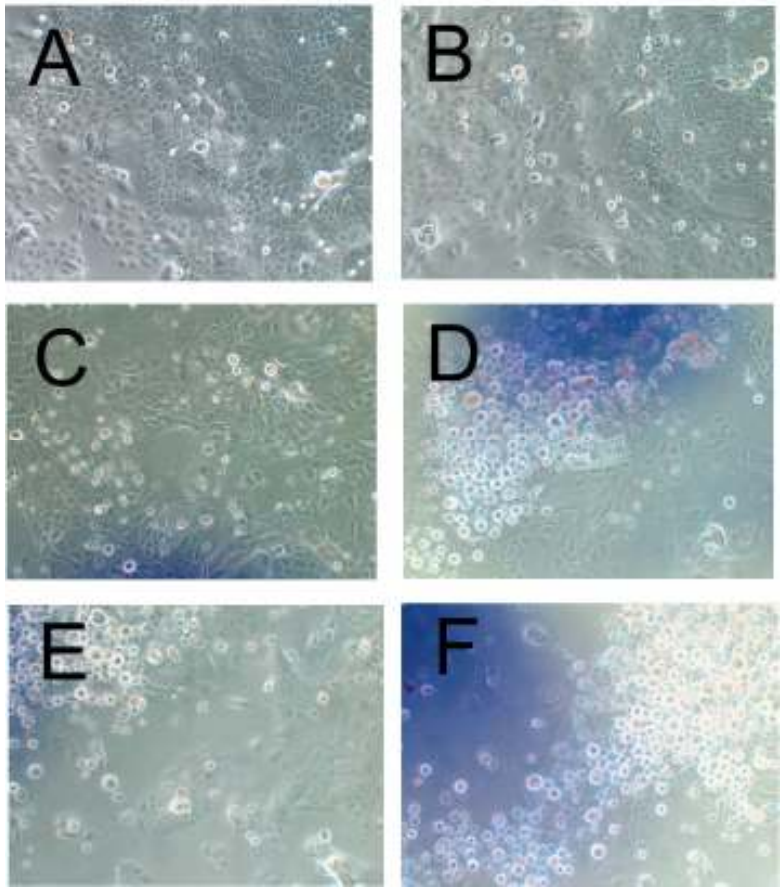
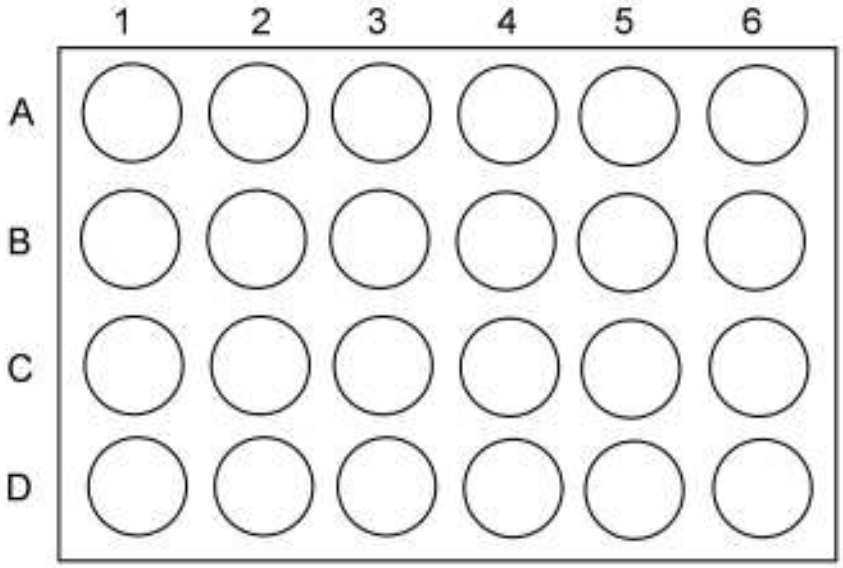


Figure 4.3: HEK growth on the control (A), glue coated well (B), 20.4 μL grafted film (C), and 30.5 μL grafted film (E); partial release and aggregation of cells after 45 minutes on 20.4 μL grafted film (D, and 30.5 μL grafted film (F).

It was obvious that the cells did not like growing on the treated or film surfaces as much as the tissue culture treated PS plates designed for cell culture. However, a slightly slower growth rate does not dismiss its use for tissue engineering purposes. Although Okano et al. previously found that an increase in thickness graft thickness led to a significant reduction in cell growth adhesion and detachment, the increase in monomer solution volume does not seem to have the same effect on the PET film using atmospheric plasma.[23] In fact, it results in improved cellular growth and detachment. Ratner's group also found that cell growth and release was not affected by the graft thickness using plasma polymerization.[5]

The increased cell growth with the second test encouraged testing of a slightly higher monomer solution volume and further testing of the plasma post-treatment time. One plate was made with a 3 minute post-treatment time, and the other plate was made with a 5 minute post-treatment time. Figure 4.4 shows the plate layout.



1A: Plasma treated PS
1B: Grafted PS, 30.5 uL monomer solution
1C: Grafted PS, 40.8 uL monomer solution
1D: Grafted PS, 50.9 uL monomer solution
2A: Plasma treated PET film
4A,B: Grafted PET film, 30.5 uL monomer solution
5A,B: Grafted PET film, 40.8 uL monomer solution
6A,B: Grafted PET film, 50.9 uL monomer solution

Figure 4.4: Plate layout for third HEK test.

After 4 days, the cells were observed and left at room temperature for 60 minutes to induce cell release. The 3 minute post-treatment plate had good growth with 65-90% confluency. The 5 minute post-treatment plate also had good growth with 70-85% confluency. However, neither plate showed any kind of cell detachment after 60 minutes. Figure 4.5 shows cell growth and lack of detachment on the plate.

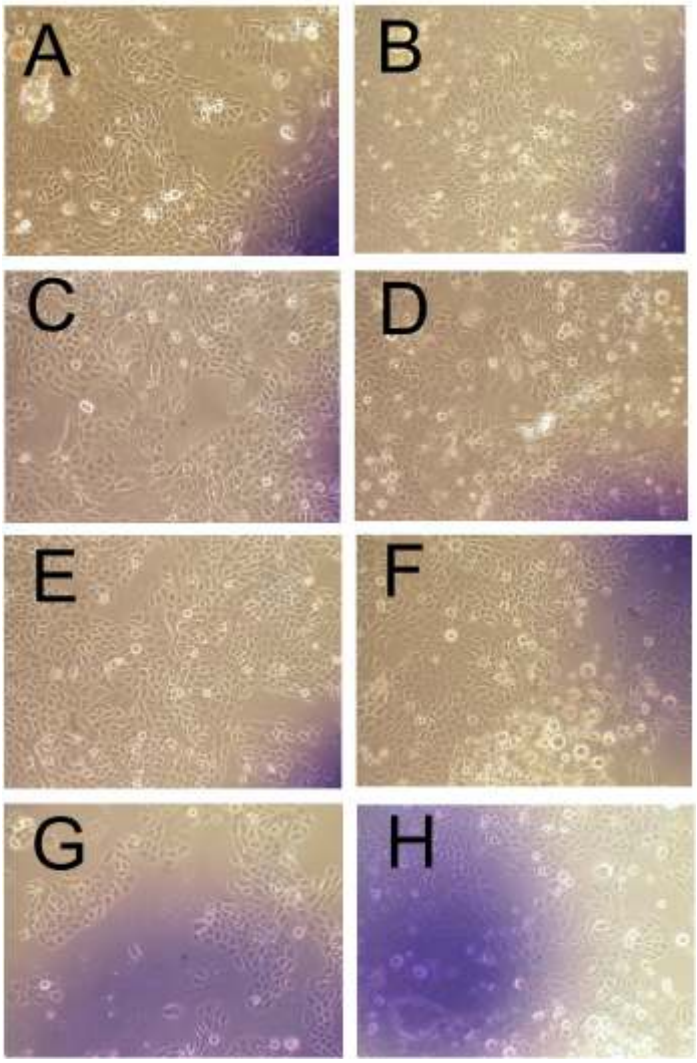


Figure 4.5: HEK growth on the 3 minute post-treatment plate with a 30.5 μL graft at 0 minutes (A) and 60 minutes (B) and with a 50.9 μL graft at 0 minutes (C) and 60 minutes (D); growth on the 5 minute post-treatment plate with a 30.5 μL graft at 0 minutes (E) and 60 minutes (F) and with a 50.9 μL graft at 0 minutes (G) and 60 minutes (H).

Since the uniformity of the plasma and grafting was not yet known, it was thought the pNIPAM might not be evenly distributed across the surface of the film. The difference between the two plates was not significant enough to change the previously successful 5 minute plasma post-treatment time; and the difference in adding more monomer solution was not significant enough to warrant the use of extra chemicals. Thus, the optimal

grafting parameters for cell growth and detachment are 3 minute plasma pretreatment, 5 minute plasma post-treatment, and 30.5 μL of monomer solution in a 24-well plate.

4.2. Characterization of Optimally Grafted Polyester Film

With the optimal grafting parameters found, the films needed to be characterized to determine the uniformity, surface morphology, and efficiency of the graft. Several different test methods were chosen to characterize the grafted polyester film. All samples prepared from this point on were plasma pretreated for 3 minutes, coated with 15.4 $\mu L/cm^2$ NIPAM monomer solution, and plasma grafted for 5 minutes.

4.2.1. Effects of Temperature on Surface Wettability

Contact angle testing is a quick way to determine the changes in wettability of the treated surface and confirm the presence of the graft. A summary of the contact angle results for the PET films is shown in Table 4.1.

Table 4.1: Average contact angle results for PET films in degrees.

Temperature	Original	Plasma Pretreated	Grafted
Warm (40°C)	73.8 ± 1.9	61.4 ± 7.7	77.4 ± 6.6
Cool (25°C)	69.0 ± 5.5	49.1 ± 2.5	64.5 ± 3.6

The contact angles on the grafted film change from 77.4 ± 6.6° at 40°C to 64.5 ± 3.5° at 25°C. It is also interesting that the contact angles changed with the temperature changes on the original and pretreated samples; however, they were not as great as the grafted film. The significantly higher contact angle at 40°C on the grafted film shows that the surface becomes more hydrophobic above the LCST. These results confirm the grafting of pNIPAM onto the surfaces and its thermoresponsiveness.

4.2.2. Cell Growth and Detachment with HFLs

To make sure the grafted surfaces were viable for use with different types of cells, grafted films were tested with human lung fibroblasts. The first optimal cell culture test was done with HFLs on a non-tissue culture treated 24-well plate. Figure 4.6 shows the plate layout.

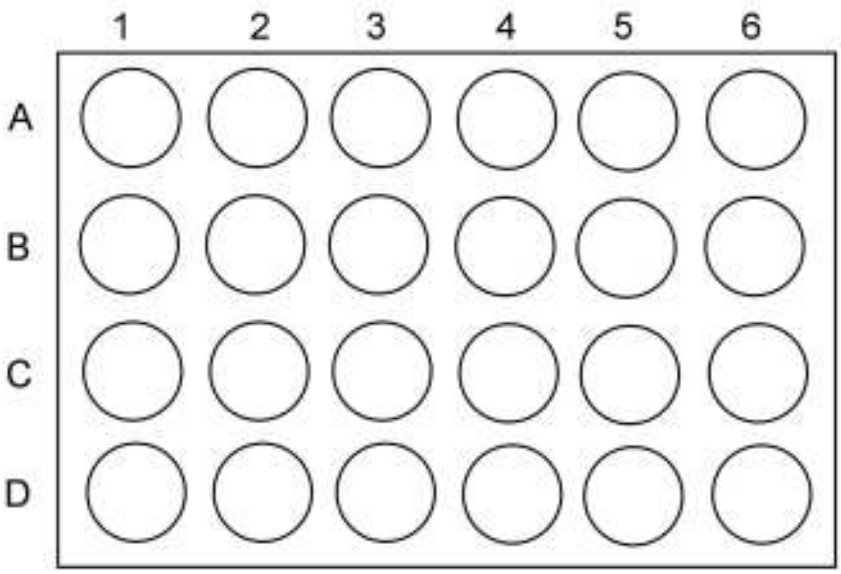


Figure 4.6: Plate layout for first HFL test.

Ample cell growth was observed on the grafted films. The cells reached 100% confluency four days after seeding. The culture dishes were removed from the incubator and kept at room temperature to observe cell detachment. The cell sheet edges were seen peeling up from the plate in Figure 4.7. The grafted films and PS wells (not shown) both showed sheet release around the edges. Actual sheet lifting was observed in real time. About 35% of the edge of the cell sheet was detached on the grafted film sample after 60 minutes.

These results confirm both the sufficient growth on the hydrophobic surface and partial sheet release of HFLs on the hydrophilic surface of the grafted films. It also demonstrates the ability of the grafted films to be used with various cell types.

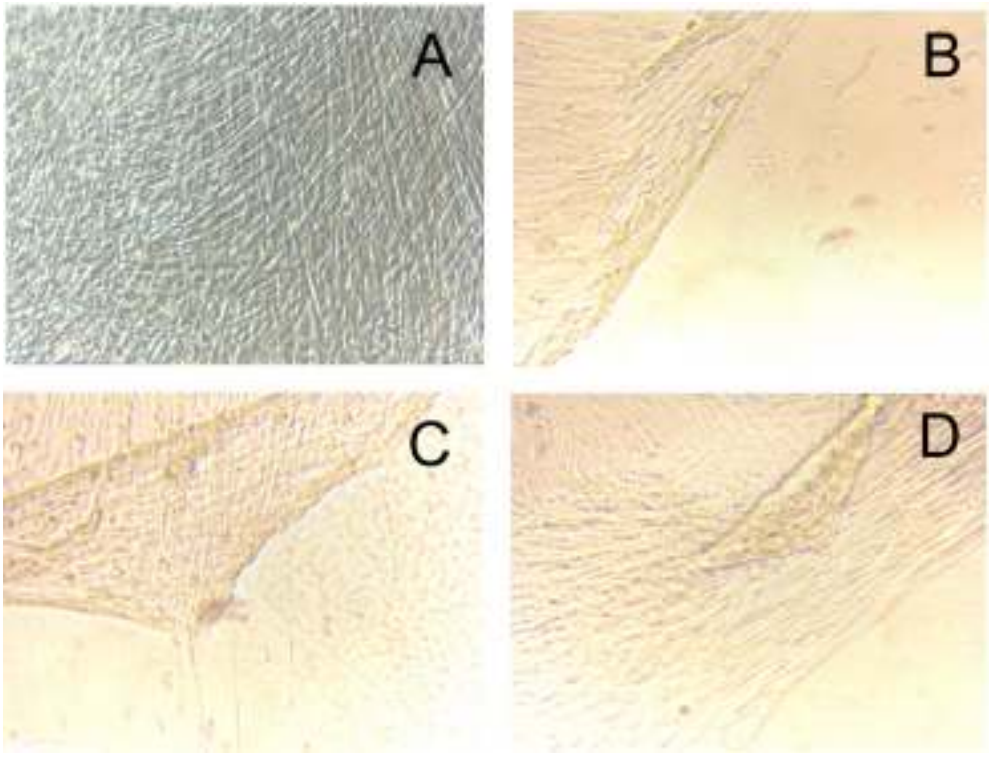


Figure 4.7: HFL release on grafted film edges after 0 (A), 40 (B), 55 (C), and 57 minutes (D).

4.2.3. Surface Roughness

Atomic force microscopy (AFM) allows a better view at the atomic level of the surfaces and an idea of how the surface roughness changes. A liquid cell with controlled temperature was used to examine the changes in surface topography and surface mechanical properties of the grafted polymer in response to changes in temperature. It is well known that grafted pNIPAM becomes hydrophilic and takes up water in an aqueous environment below its LCST. It has also already been shown that atmospheric plasma

treatment induces significant changes in the roughness of a polyester film substrate due to etching.[26] With these two things in mind, we can account for the changes in the appearances of the surface with each treatment.

The results from the dry and wet scans are shown below in Figure 4.8. Plasma pre-treatment has an obvious effect on the surface roughness as indicated by Figure 4.8 A and B. The plasma pretreated film shown in B showed a reduction in the mean-square roughness (RMS) from 11.2 to 7.72 nm when compared to untreated film. Grafting on top of the plasma pre-treated films induces further changes in surface topography (Figure 4.8 C) but no significant change in surface roughness.

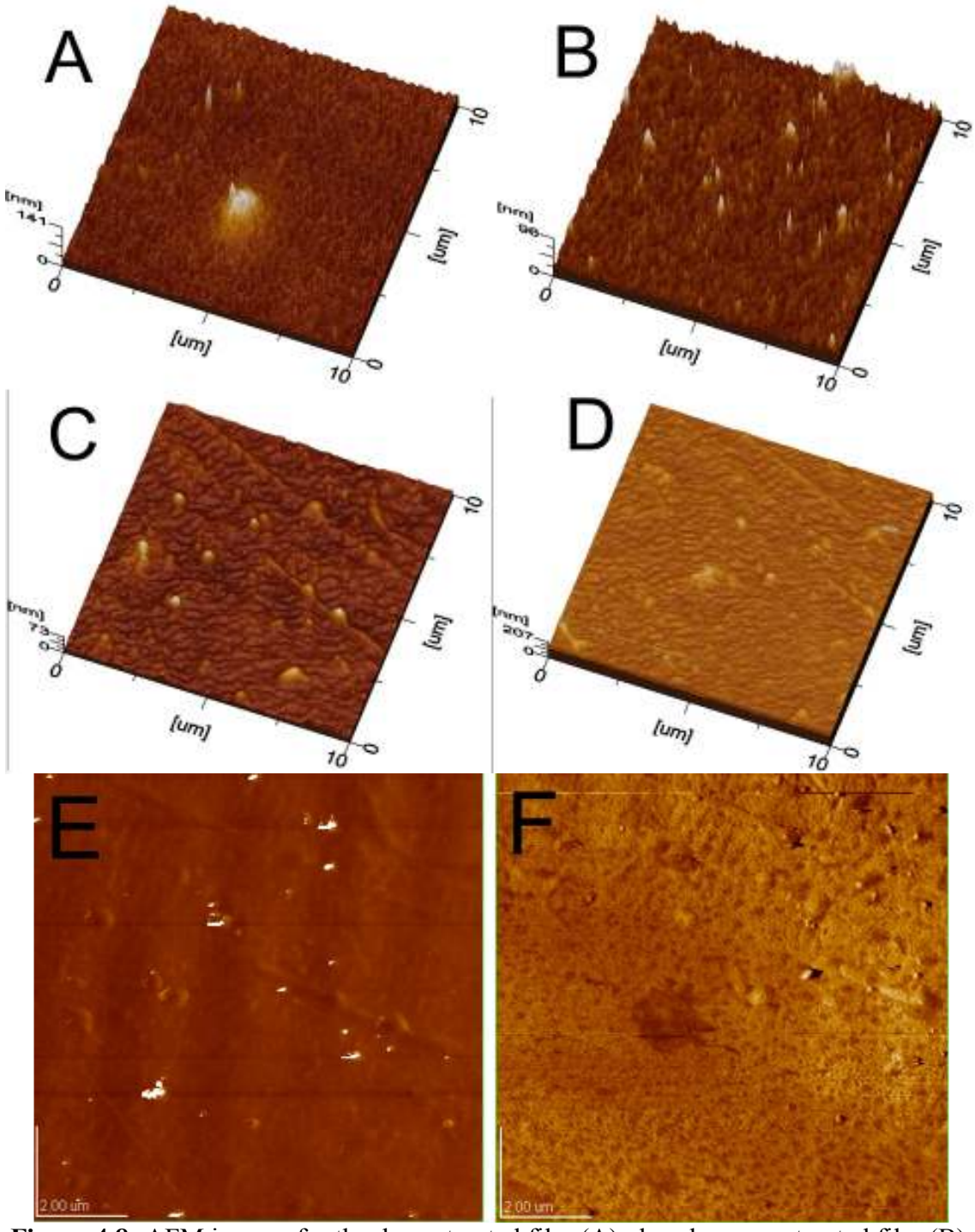


Figure 4.8: AFM images for the dry untreated film (A), dry plasma pretreated film (B), dry pNIPAM grafted film (C), and wet pNIPAM grafted film (D) at room temperature with a 10 μm^2 scan area; phase scans of the dry (E) and wet (F) grafted films are shown at a 10 μm^2 scan area.

When placed in water at room temperature, the surface becomes significantly rougher and more variable. At 37ºC, the surface shows a large increase in roughness. Phase images of the grafted polymer reveal a relatively uniform surface for the dry graft, and an orange peel type phase variation on the wet graft surface. The nanometer-size dots in the wet phase image (F) may be the result of changes in the polymer crosslink density over the surface.[5,27] The surface transformation in the hydrated state leads to a change in average roughness (Ra) from 4.72 ± 0.71 nm (dry) to 5.90 ± 1.51 nm (wet, 24^{o}) and 12.24 ± 5.32 nm (wet, 37^{o}). This can be seen in Figure 4.9. The RMS roughness also increased from 7.15 ± 1.52 nm (dry) to 9.47 ± 3.32 nm (wet, 24^{o}) and 31.65 ± 13.22 (wet, 37^{o}). This increase in roughness was expected and consistent with findings of Cheng et. al.[5]

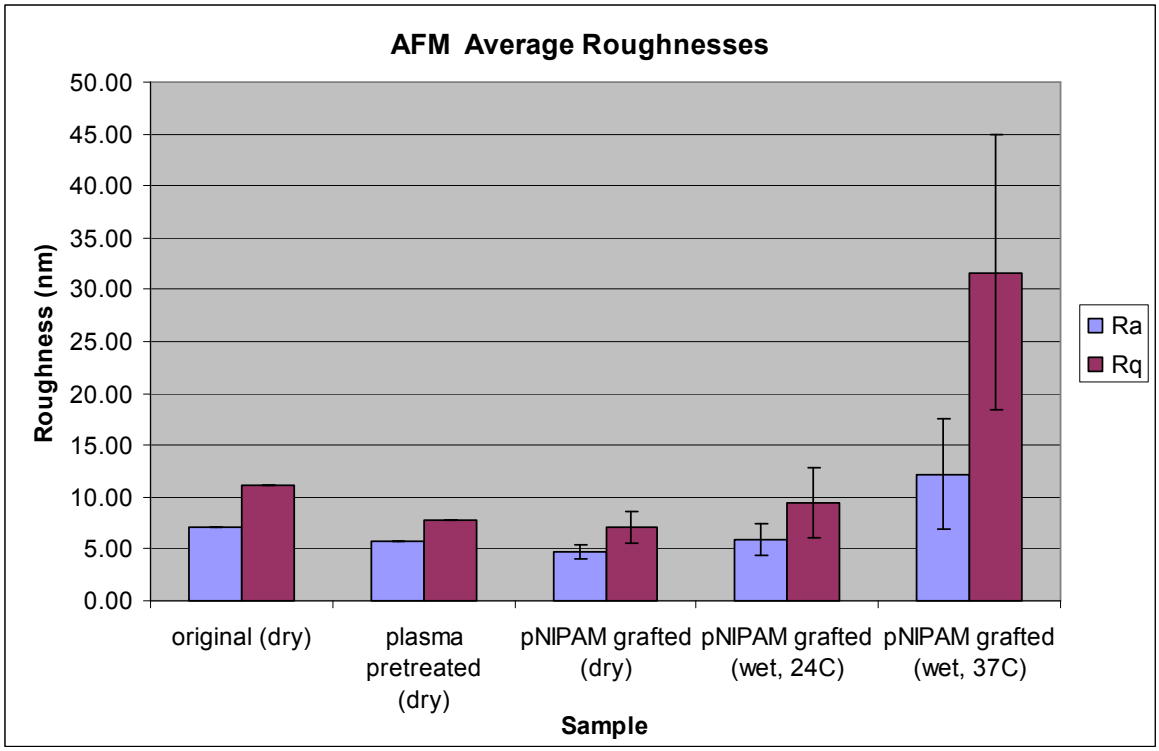


Figure 4.9: Average roughness (Ra) and RMS roughness (Rq) averages shown for the 10 μm^2 area scans of film samples.

The peak-to-valley roughness change is also significant. As shown in Figure 4.10, there is a large increase in peak-to-valley roughness for the wet samples at 37ºC when compared to the dry grafted samples. The average and peak-to-valley roughnesses both decreased on the dry grafted sample as compared to the original and plasma pretreated samples. This indicates the coating of the pNIPAM on the surface for the grafted samples, which corresponds with the data found from the SEM and findings of Kwon et al.[4]

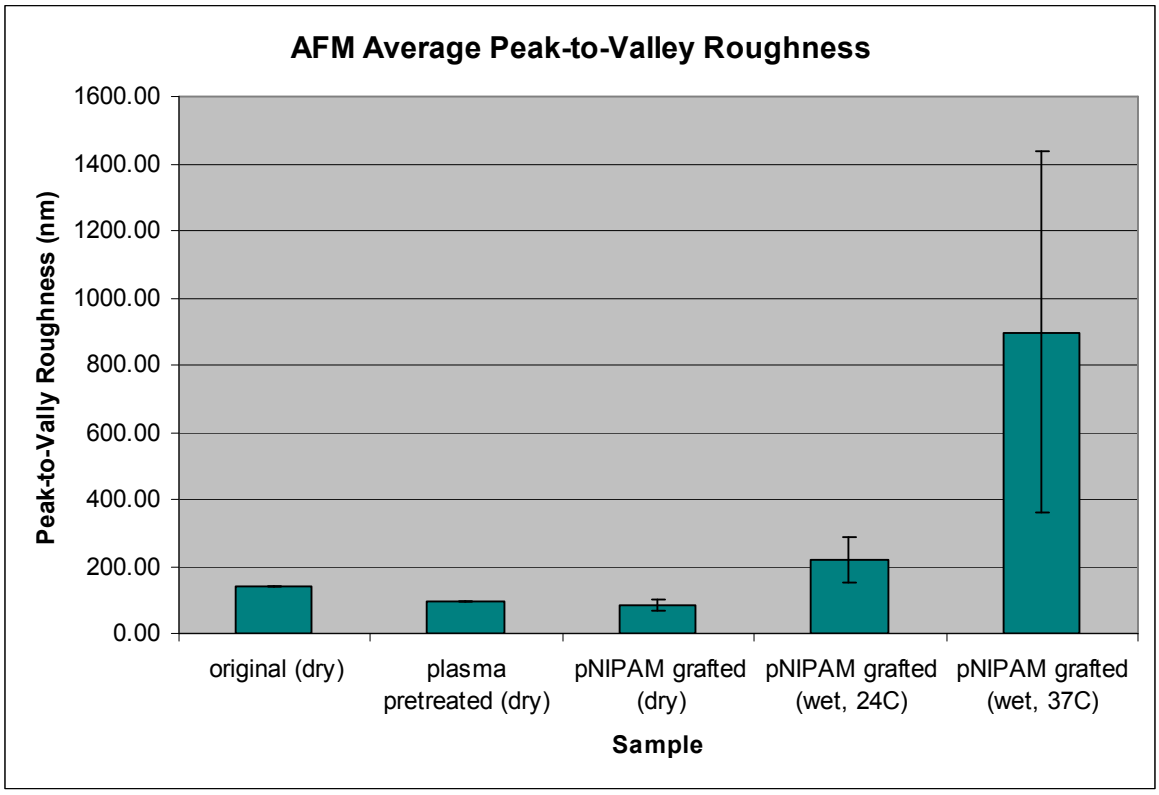


Figure 4.10: Peak-to-valley roughness (Rz) averages shown for the 10 μm^2 area scans of film samples.

4.2.4. Surface Morphology

Scanning electron microscopy (SEM) was used to examine the plasma treated and grafted surfaces and to characterize the uniformity of the grafted pNIPAM. The SEM images for the films are shown in Figure 4.11.

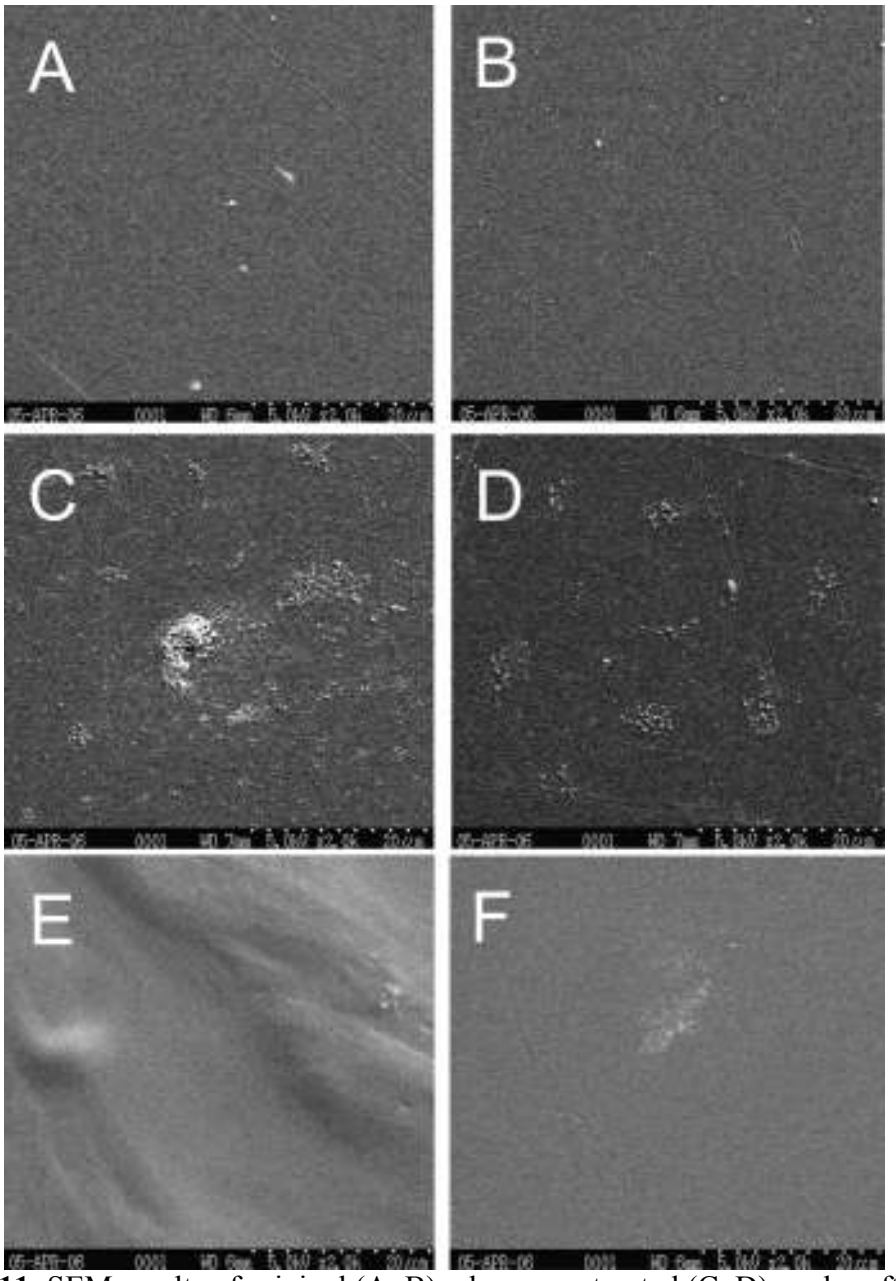


Figure 4.11: SEM results of original (A, B), plasma pretreated (C, D), and grafted (E, F) film.

There were clear differences in the film surface as a result of plasma pretreatment only and further changes resulting from the pNIPAM grafting. The original film is fairly smooth and uniform. The plasma pretreated sample shows isolated areas of roughness. This was expected as the plasma has been shown to etch the surfaces of treated substrates.[28] The

grafted polymer is apparent on the films as shown in Figure 4.11 E and F. The graft fills in the natural cavities on the original film. This finding is also consistent with the results of Kwon et al. and confirms the presence of the graft.[4]

4.2.5. Graft Thickness

The thickness of the graft was previously thought to be very small and immeasurable through weight and graft yield. AFM was performed on a grafted and templated 60 mm PS plate because it was too difficult to resolve an adequate edge between the ungrafted and grafted areas due to the irregularities of the original PET film on that scale. The results from the thickness test are shown in Table 4.2. The maximum and minimum thicknesses were taken due to the discretionary component of the measuring software. The averages for the sample gave a good estimate of the thickness and confirm the thin graft suspected on the samples. From this data, it can be said with confidence that the graft is between 30 and 100 nm. Although the found thickness is considerably higher than was found by Okano et al. to allow for cell adhesion and proliferation, the graft thickness does not seem to have the same effects when using atmospheric plasma polymerization.

Table 4.2: Average maximum and minimum graft thicknesses in dry phase.

	Average Max Thickness (nm)	**Average Min Thickness (nm)**
Dry	69.8 ± 22.8	52.0 ± 22.0

4.2.6. Cell Viability

A quick cell viability test was performed using Trypan Blue, which is a vital dye that does not interact with a cell unless the membrane is damaged. The test was done to ensure the cells were not releasing due to cell death and dying after being out of the

incubator environment for detachment. The viability test was done with HFLs on a non-tissue culture treated 24-well plate. Figure 4.12 shows the plate layout.

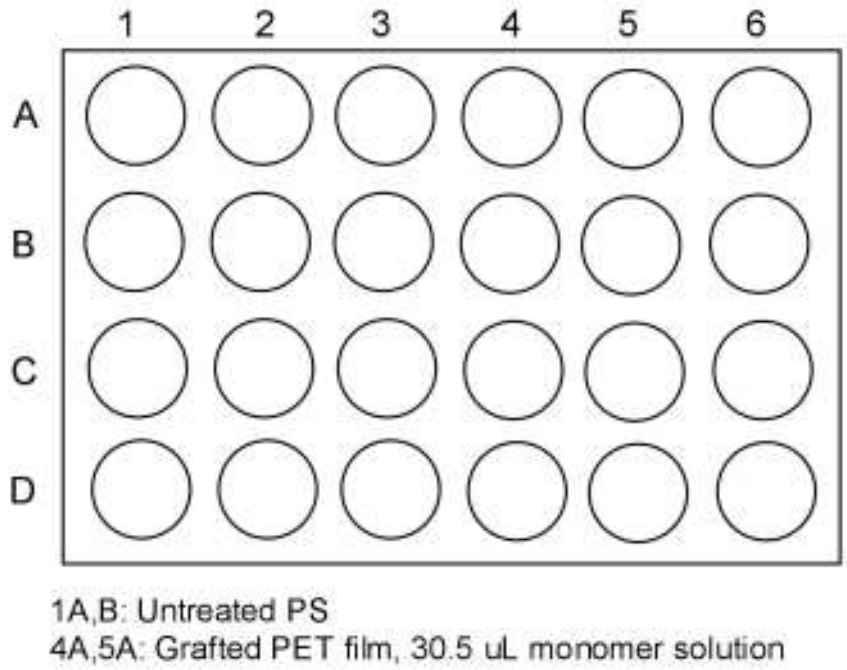


Figure 4.12: Plate layout for the viability test with HFLs.

After 4 days, the cells were removed from the incubator and kept at room temperature to observe cell detachment and viability. The two wells (1A and 4A) were immediately stained to use as controls of initial cell viability. No dead cells were visible in these wells. In the two test wells (1B and 5A), the stain was added after 120 minutes at room temperature. A minimal amount of dead cells were observed in these wells showing that the environmental changes required for release were not fatal to the cells. Figure 4.13 shows the results of the viability test.

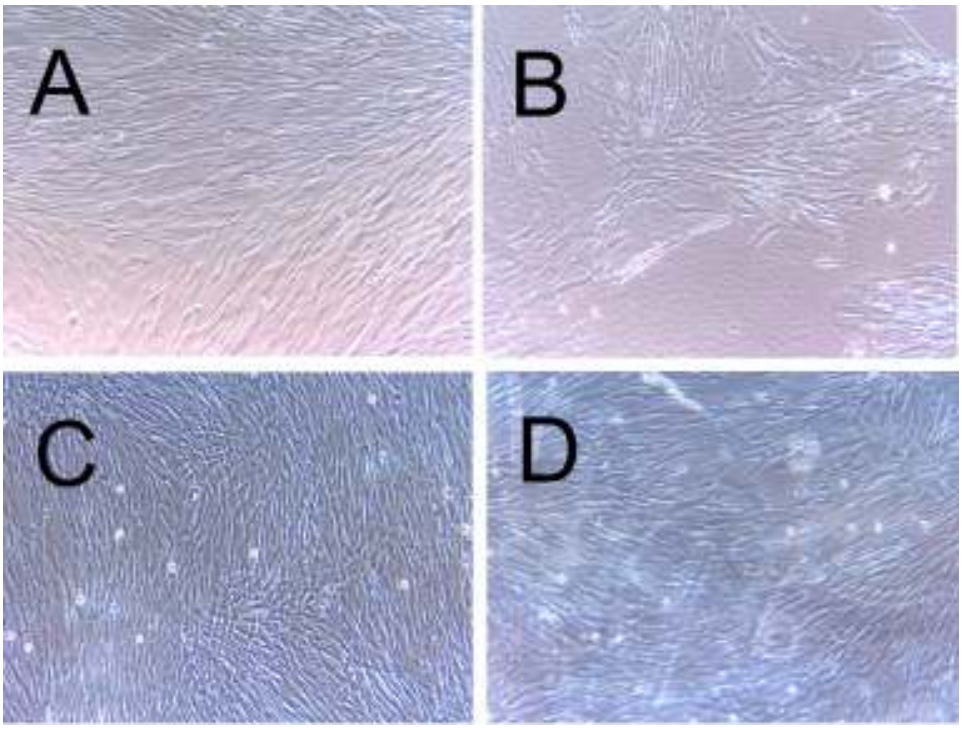


Figure 4.13: PS (A) and grafted film (B) controls after 0 minutes and staining; PS (C) and grafted film (D) after 120 minutes and staining.

The test was also a second cell detachment study to confirm the reproducibility of the previous results. After 120 minutes at room temperature, little release was seen. The plate was then placed in a refrigerator for 30 minutes to aid in the release process. After 150 minutes, still little release was observed. Finally after a few taps on the side of the plate, cell sheet pieces were seen releasing from the surface of the grafted wells; no cells were seen releasing from the control well. Figure 4.14 shows the results of the viability test and cell detachment.

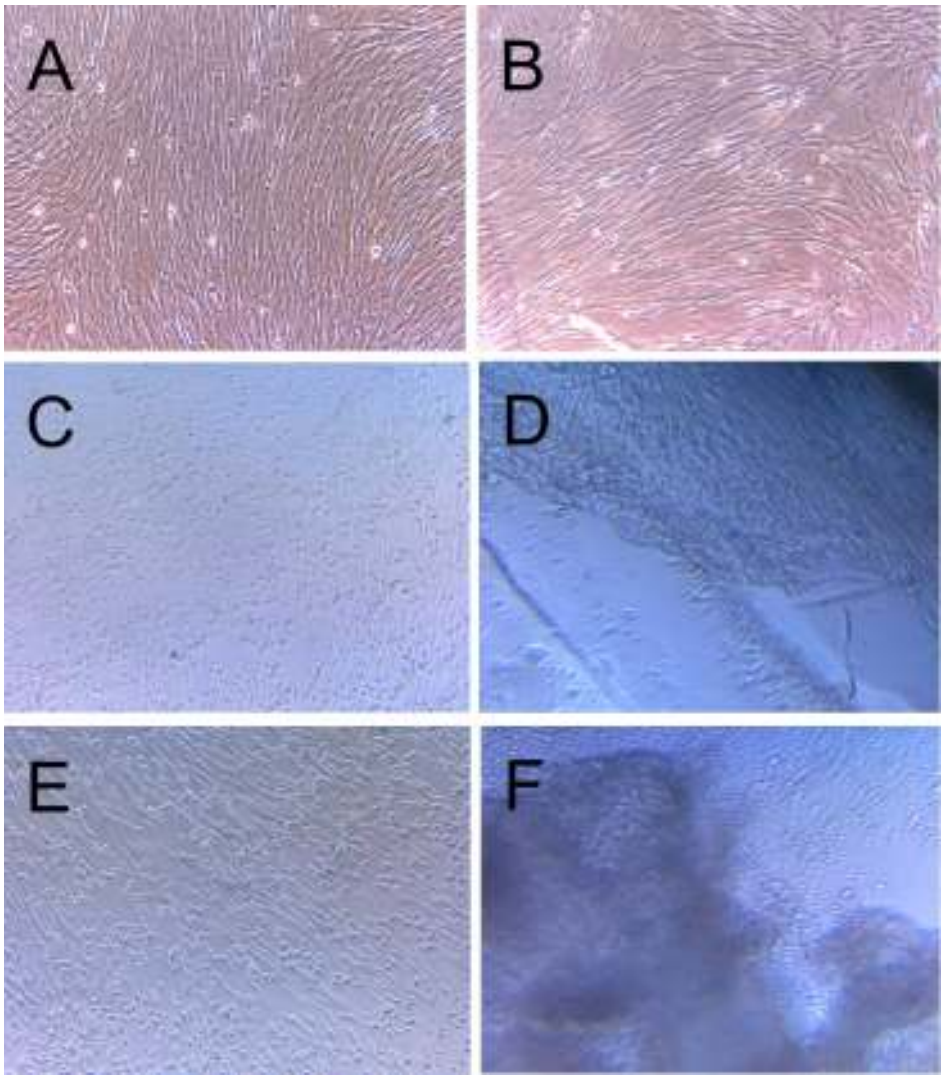


Figure 4.14: HFL release on grafted film after 55 (B), 150 (D), and 160 minutes (F); no cell release observed on non-tissue culture treated PS plate after 55 (A), 150 (C), and 160 minutes (E).

4.2.7. Cell Proliferation after Release

In order to be useful for coculture and tissue applications, the cells being released from the grafted surface need to be able to continue to grow after detachment. The regrowth test was done with HFLs on a non-tissue culture treated 24-well plate. Figure 4.15 shows the plate layout.

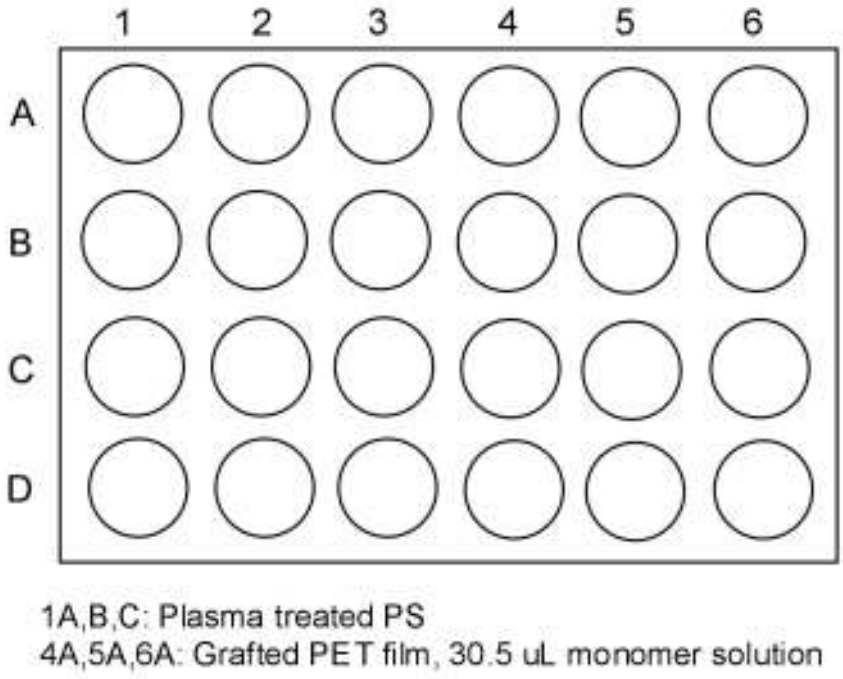


Figure 4.15: Plate layout for the proliferation test with HFLs.

After two days, the cells were observed and left at room temperature for 60 minutes to induce cell release. The control wells were all 100% confluent. The cells on the grafted film were about 60% confluent and had cell ball aggregates form prior to release. These were thought to be due to the increased number of cells used for seeding.

Although the main purpose of the test was to examine proliferation, it was also another opportunity to observe the effectiveness of cell release from the grafted films. The cell detachment was very effective even after 30 minutes at room temperature. The cell ball aggregates mentioned above increased in number, and the cell sheet edges peeled up almost to the center of the well. Actual sheet lifting was seen in real time again. Figure 4.16 shows the detachment and folding of a cell sheet in sequential images taken at the same location of a grafted well. The cells can actually be seen moving out of the field of

view. The contraction and aggregation of the detaching cells is similar to what was seen by Kwon et al. and is due to cell-cell connections.[4]

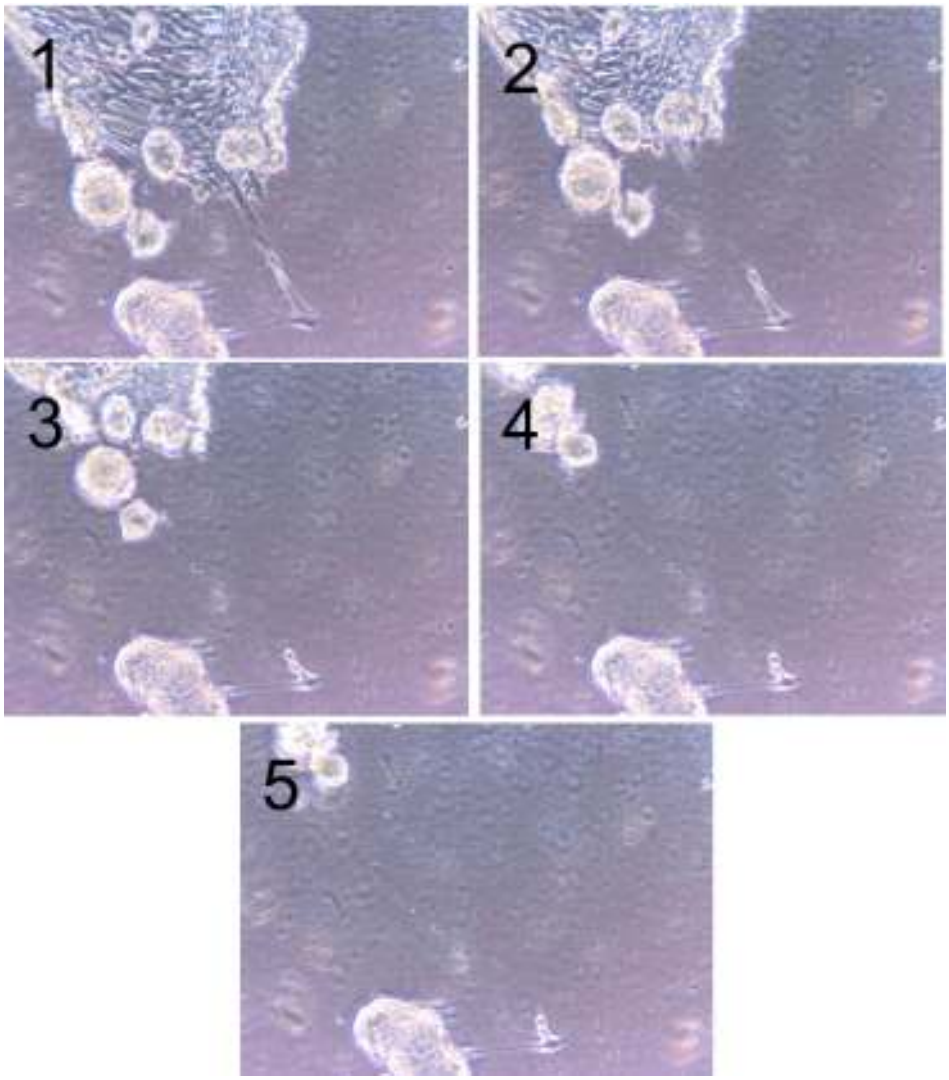


Figure 4.16: Sequential images of an HFL cell sheet detaching from grafted film.

After allowing the cells to detach for 45 minutes, a portion of the released cells and medium were pipetted into a new well. After 3 days, the cells were checked for proliferation. The cell ball aggregates seen above laid down on the bottom of the new wells, and new growth was observed growing from these areas. The cells looked healthy

and had grown to at least 50% confluency. The cell sheets seen rolled up above left in their original wells with medium also laid back down on the plate and displayed new growth. The results of the regrowth test are shown in Figure 4.17. This observation demonstrates the reversible nature of the graft as the cells reattached after the pNIPAM was warmed back up to 37^{o}C. The regrowth of released cells and reversibility of graft properties corresponds with the findings of Kwon et al. confirming the health and functionality of the detached cells.[4]

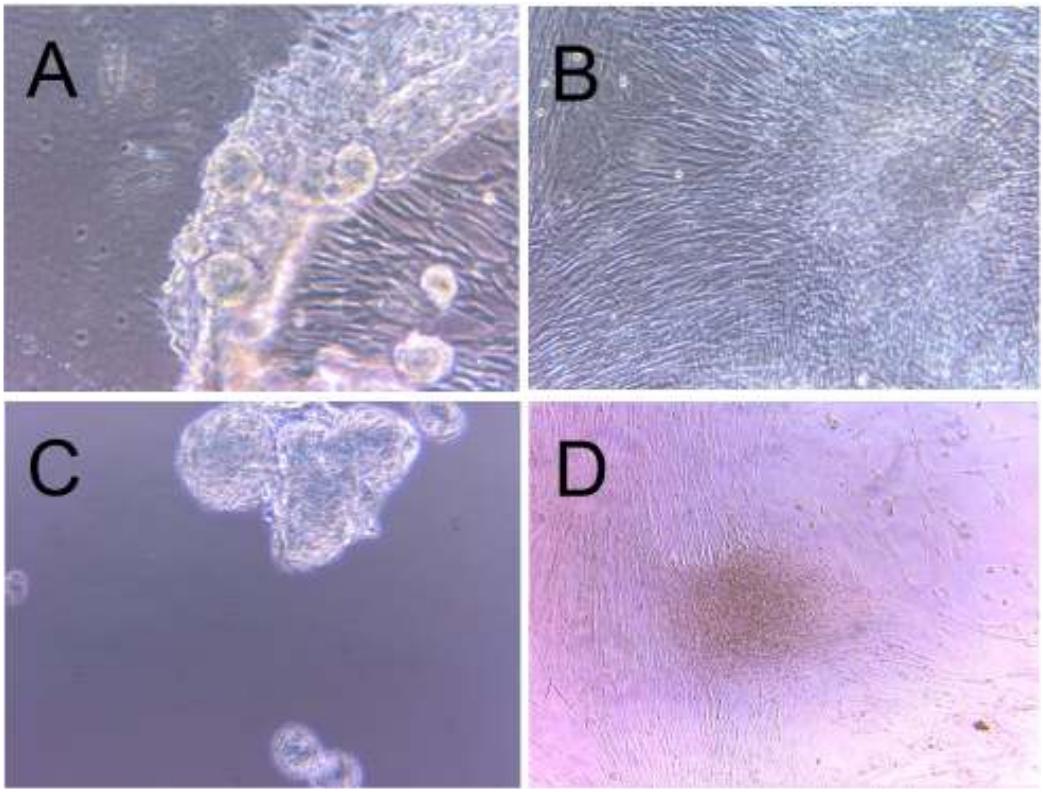


Figure 4.17: Releasing (A) and reattached cells after 3 days (B); reseeded detached cell aggregates on day 1 (C) and day 3 (D).

4.3. Characterization of Optimally Grafted Polyester Fabric

After obtaining sufficient success with the optimally grafted polyester film, grafted polyester fabric was tested and characterized. The polyester filtration fabric was chosen

due to the defined and uniform porosity. Kwon et al. have found that an increase in the hydration of the polymer chains can lead to faster cell sheet detachment by allowing greater water movement between the cells and substrate.[4] This principle is shown in Figure 4.18.

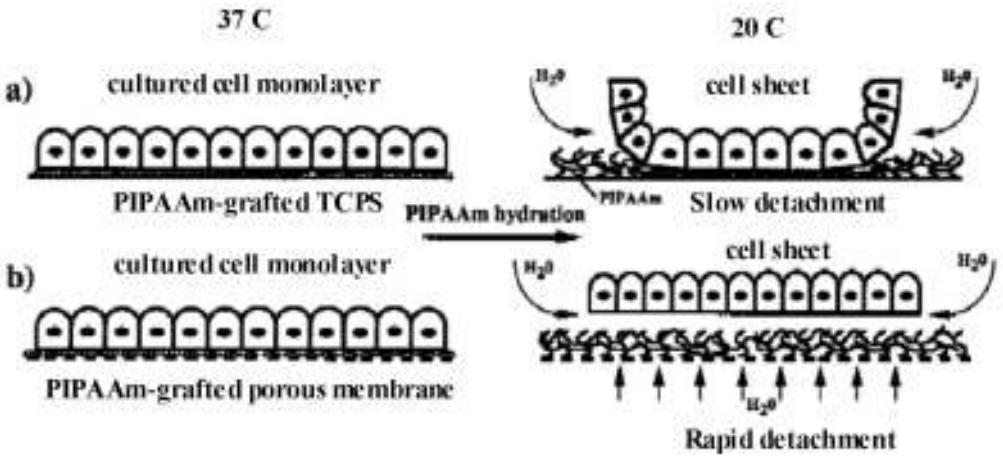
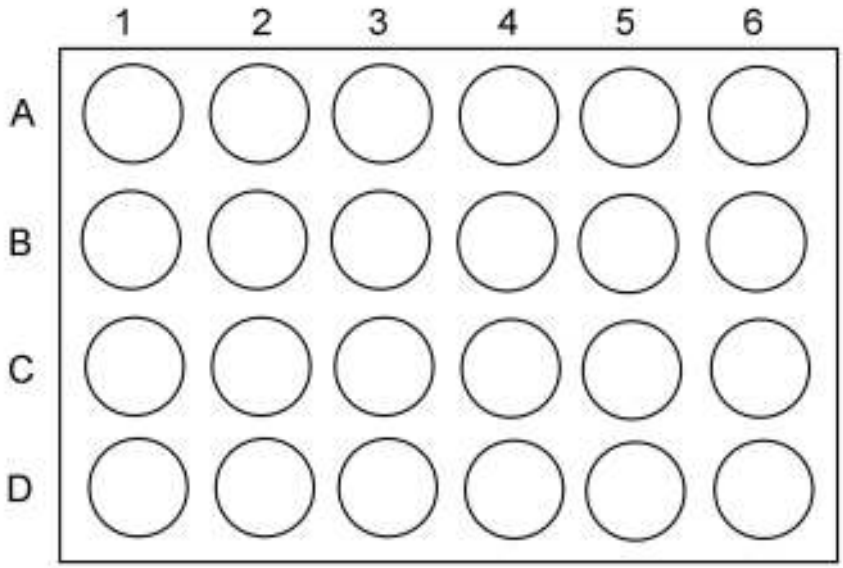


Figure 4.18: An illustration of cell sheet detachment by different types of water supply to (a) the PIPAAm-grafted TCPS surface and (b) the PIPAAm-grafted porous membrane.[4]

Their work with a microporous polyester membrane showed a significant decrease in detachment time when compared with PS plates.[4] However, no previous work has been done with pNIPAM grafted fabrics, and its success with cell growth and detachment holds possibilities for currently unavailable large-scale cell growth and cell sheet engineering.

4.3.1. Cell Growth and Detachment with HEKs

Two different polyester filtration fabrics were tested first, a 5 μm and a 20 μm pore size. These were chosen to determine which pore size would be best for cell growth. The fabrics were cut to the size of the well and weighted down with a sterile stainless steel washer. Figure 4.19 shows the plate layout.



1A: Plasma treated PS
1C: Grafted PS, 30.5 uL monomer solution
2A: Untreated PET fabric, 5 um pores
2B: Untreated PET fabric, 21 um pores
3A: Plasma treated PET fabric, 5 um pores
3B: Plasma treated PET fabric, 21 um pores
4A,B: Grafted PET fabric, 5 um pores
4C,D: Grafted PET fabric, 21 um pores

Figure 4.19: Plate layout for HEK test with fabrics.

After 4 days, the cells were observed and left at room temperature for 60 minutes to induce cell release. The fabric could only be seen with an inverted microscope due to accessibility requiring observation of the cells through the pores. As a result, it was very difficult to see if any cells were growing on the fabric. However, cells were seen on the bottom of the wells with the 21 μm fabric, which means they were able to fall through the pores. The test was inconclusive for cell growth and release but did lead to the decision to use the 5 μm pore size fabric for all remaining tests.

4.3.2. Effects of Temperature on Surface Wettability

Contact angle testing is a quick way to determine the changes in wettability of the treated surface and confirm the presence of the graft. A summary of the contact angle results for the PET fabric is shown in Table 4.3.

Table 4.3: Average contact angle results for PET fabric in degrees.

Temperature	Original	Plasma Pretreated	Grafted
Warm (40°C)	50.0	55.0	54.7 ± 5.3
Cool (25°C)	NA	NA	35.5 ± 0.1

The contact angle changes on the grafted fabrics were dramatic. They demonstrated a change from 54.7° at 40°C to 35.5° at 25°C. The original and pretreated fabrics were only tested at warm temperature as no change was expected. In previous work in our laboratory, it was found that the wettability of plasma treated polyester samples increased, and the contact angle decreased due to the extra –OH groups on the surface after treatment with oxygen gas plasma.[4] However, it was later found that the fabric was treated with an anti-static chemical during manufacturing making it more hydrophilic than normal polyester. Regardless, these results confirm the grafting of pNIPAM onto the surfaces and its thermoresponsiveness.

4.3.3. Surface Chemistry

Fourier Transform Infrared Spectroscopy (FTIR) produces information on the chemical composition of a specific chemical species. It is a very powerful tool used to identify specific chemical bonds simply by interpreting the infrared absorption spectrum. The characteristic peaks that correspond to the chemical structure of pNIPAM are observed at 1386 cm^{-1} (methyl group deformation), 1458 cm^{-1} (-CH_3 and –CH_2- deformation), 1540

cm^{-1} (secondary amide N-H stretching), 1650 cm^{-1} (secondary amide C=O stretching), 2970 cm^{-1} (-CH_3 asymmetric stretching), and 3300 cm^{-1} (secondary amide N-H stretching).[1,3] The test was done to further confirm the presence of the graft and give an indication of the amount of pNIPAM being grafted. The results found are shown in Figure 4.20 and do not show any of the characteristic peaks normally found from pNIPAM. Since the crystal on the spectrometer penetrates to a depth of 0.5 μm, it is likely that it penetrated through the thin coating and is only showing the spectra of the polyester.

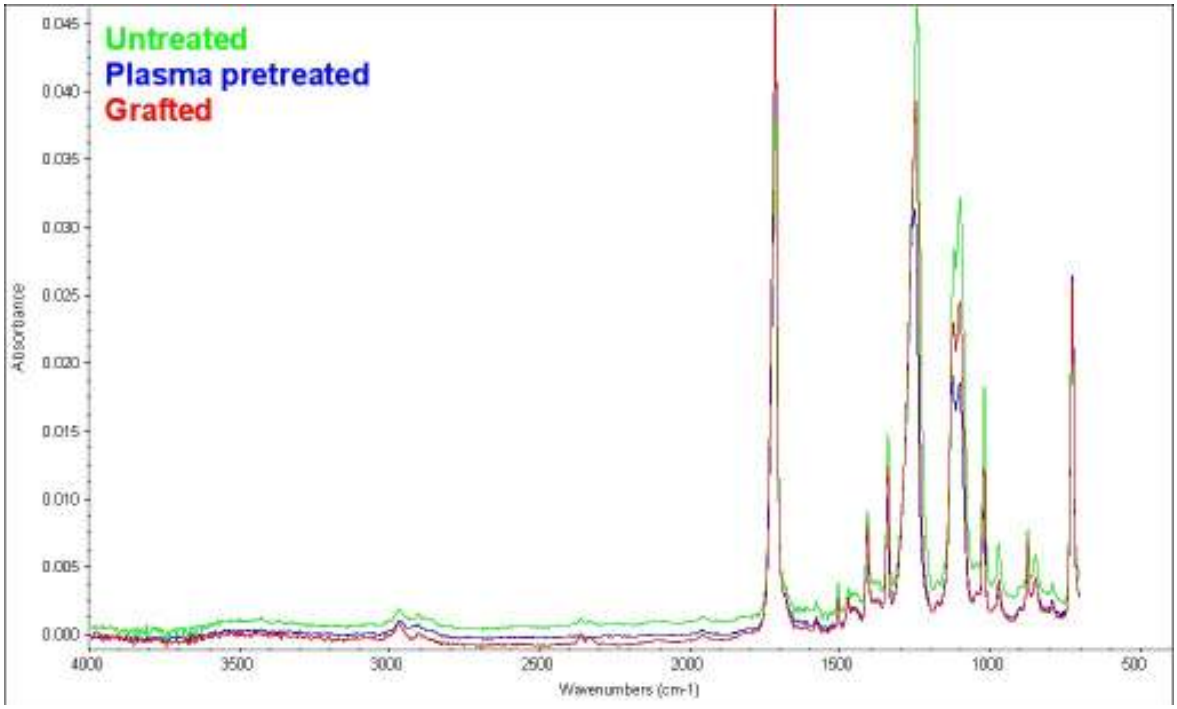


Figure 4.20: FTIR spectra of the untreated, plasma pretreated, and grafted 5 μm PET filter fabric.

4.3.4. Cell Growth and Detachment with HFLs

A second test for cell growth was performed using HFLs. This time the fabric was glued to the bottom of a well insert, and the cells were seeded on top of the fabric to be observed with an inverted microscope. One of each of the untreated, plasma pretreated,

and grafted fabric samples were tested. The cells were checked four days after seeding and kept at room temperature to observe cell detachment. Once again, the cells were difficult to distinguish, so Coomassie Blue was used to stain the cells prior to viewing. The cells were approximately 90% confluent on the surrounding insert, but it is difficult to determine the exact confluency on the filtration fabrics. Based on the dye patterns, the untreated and plasma pretreated fabrics both displayed 60% confluency or higher. The elongated shape and nuclei of the fibroblasts were noticeable on these surfaces as well. The grafted fabric showed cell growth to be approximately 50% confluent. However since we were unable to view the cells before staining and washing, it was possible that some cells were washed off during this process. The unseeded fabrics tested for staining with the Coommassie blue both showed little or no dye pick-up. This finding further confirms the presence of cells on the fabrics. These results demonstrate the ability of the cells to grow on the fabric. Figure 4.21 shows the results of the cell culture study with HFLs on the fabric.

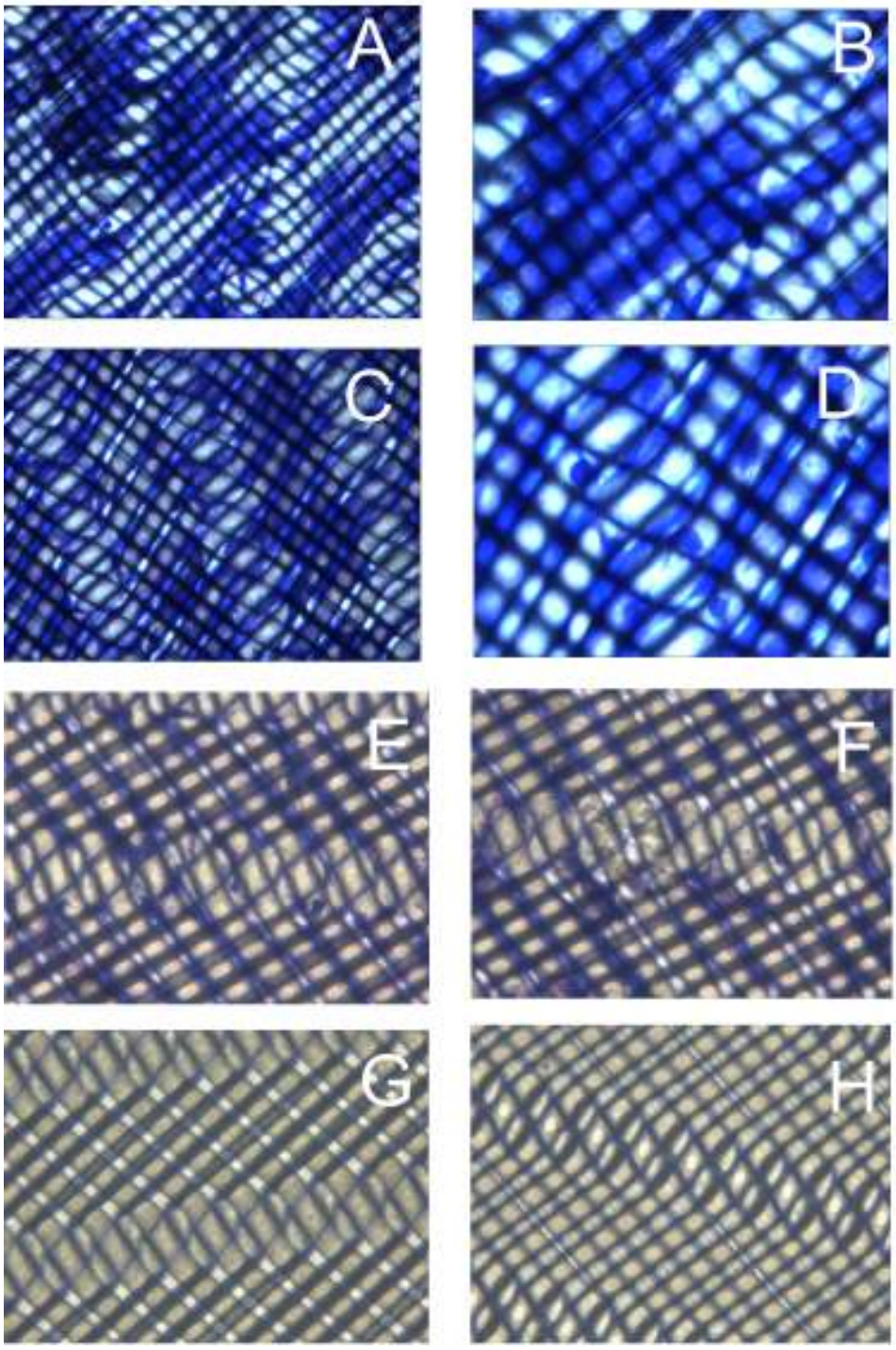


Figure 4.21: HFL growth on the untreated fabric at 10x (A) and 20x (B), on plasma pretreated fabric at 10x (C) and 20x (D), and on grafted fabric at 10x (E, F); stained untreated (G) and grafted (H) fabrics with no cells.

4.3.5. Surface Morphology

Scanning electron microscopy was used to examine the plasma treated and grafted surfaces and to characterize the uniformity of the grafted pNIPAM. The SEM images are shown in Figure 4.22. The grafted polymer was not readily apparent on the surfaces of the fabrics, although the grafted fabric filaments do appear smoother as though the pNIPAM filled in the surface blemishes. However, as suspected, the thickness of the graft was insufficient to be detected using SEM.

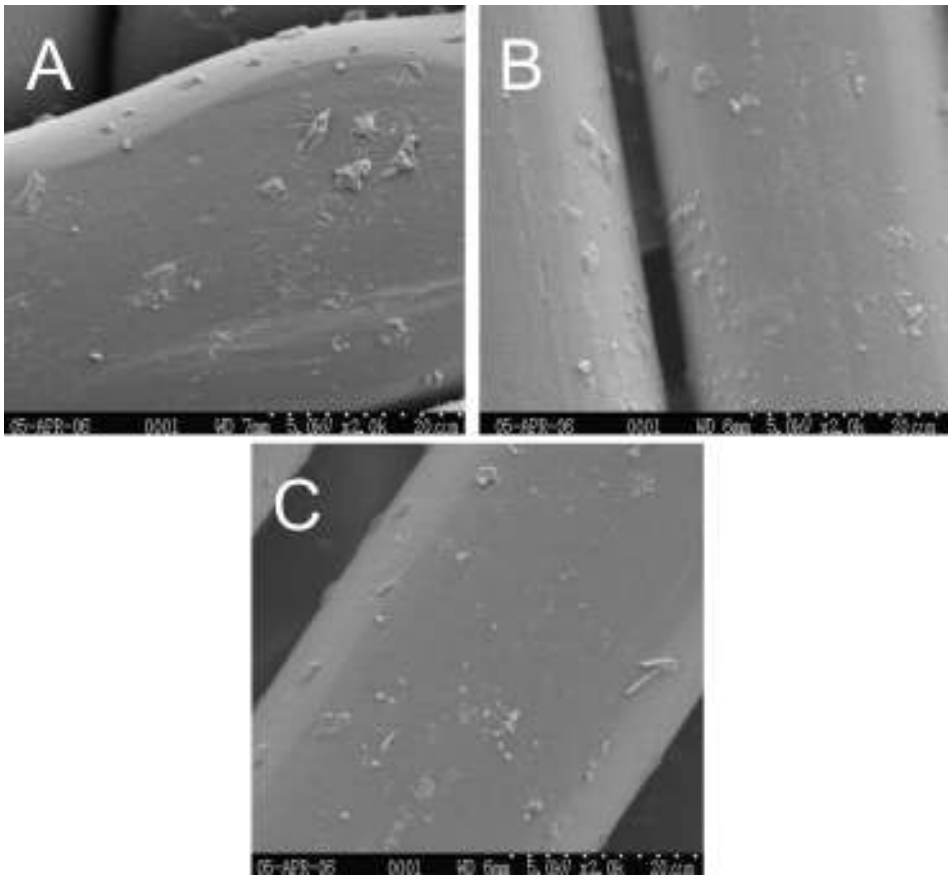


Figure 4.22: SEM results of original (A), plasma pretreated (B), and grafted (C) fabric.

4.3.6. Graft Uniformity

Although the contact angle measurements on the grafted fabric demonstrated the thermoresponsiveness of the pNIPAM, further confirmation of the uniformity of the graft was necessary to ensure efficiency of the plasma treatment. A simple dye test using an acid dye, which is known to color the amino end groups of nylons, was used since pNIPAM is an amide.[29] A piece of untreated, plasma pretreated, and grafted fabric was tested. The results are shown in Figure 4.23.

Figure 4.23: Acid dye test results from untreated (A), plasma pretreated (B), and grafted PET filtration fabric.

The blue coloring on the grafted fabric was clearly visible and was not seen on the untreated and plasma pretreated fabrics. However, the dye does look darker in some spots possibly indicating a thicker graft in those areas. This test confirmed that the pNIPAM was successfully grafted to the filtration fabric in a fairly uniform manner with some patchy areas.

4.3.7. Cell Growth, Detachment, and Viability with Hep G2 Cells

A third test for growth was performed using Hep G2 cells. This time grafted fabric pieces were placed on the bottom of a non-tissue culture treated 6-well PS plate, and the cells were seeded on top of the fabric to be observed with an inverted microscope.

Because of previous problems viewing the cells, a fluorescent nuclear stain was used to view cell growth and detachment; and a fluorescent assay was performed to confirm the viability of the cells on the fabric. The fabric fluoresces naturally but not enough to present a problem when viewing the cells.

For the viability test, the cells were observed one, two, and six days after seeding. The results of the viability test are shown in Figures 4.24 and 4.25. The cells proliferated well on the fabric. The cells stained after only 1 day of incubation clearly preferred growing on the natural grooves in the fabric as seen in Figure 4.24 A and B. However after 2 and 6 days of incubation, the cells spread out over the surface of the fabric and grew to at least 60% confluency. A small number of dead cells were seen on each day, but overall the cells attached and proliferated successfully on the PET filtration fabric.

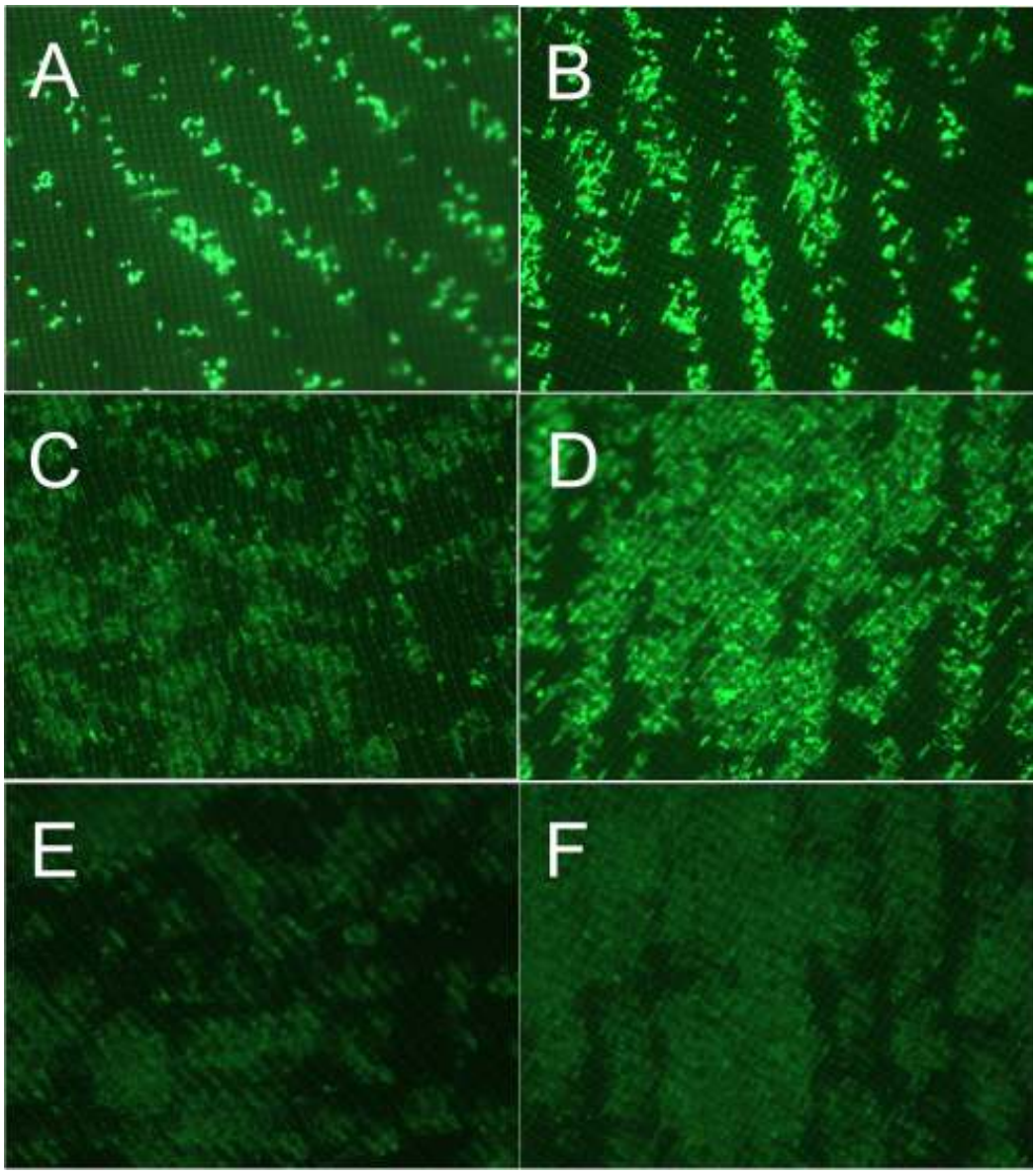


Figure 4.24: Live cells seen with the viability test on grafted PET filtration fabric seeded at a density of 20,000 cells after 1 (A), 2 (C), and 6 (E) days and seeded at a density of 40,000 cells after 1 (B), 2 (D), and 6 (F) days.

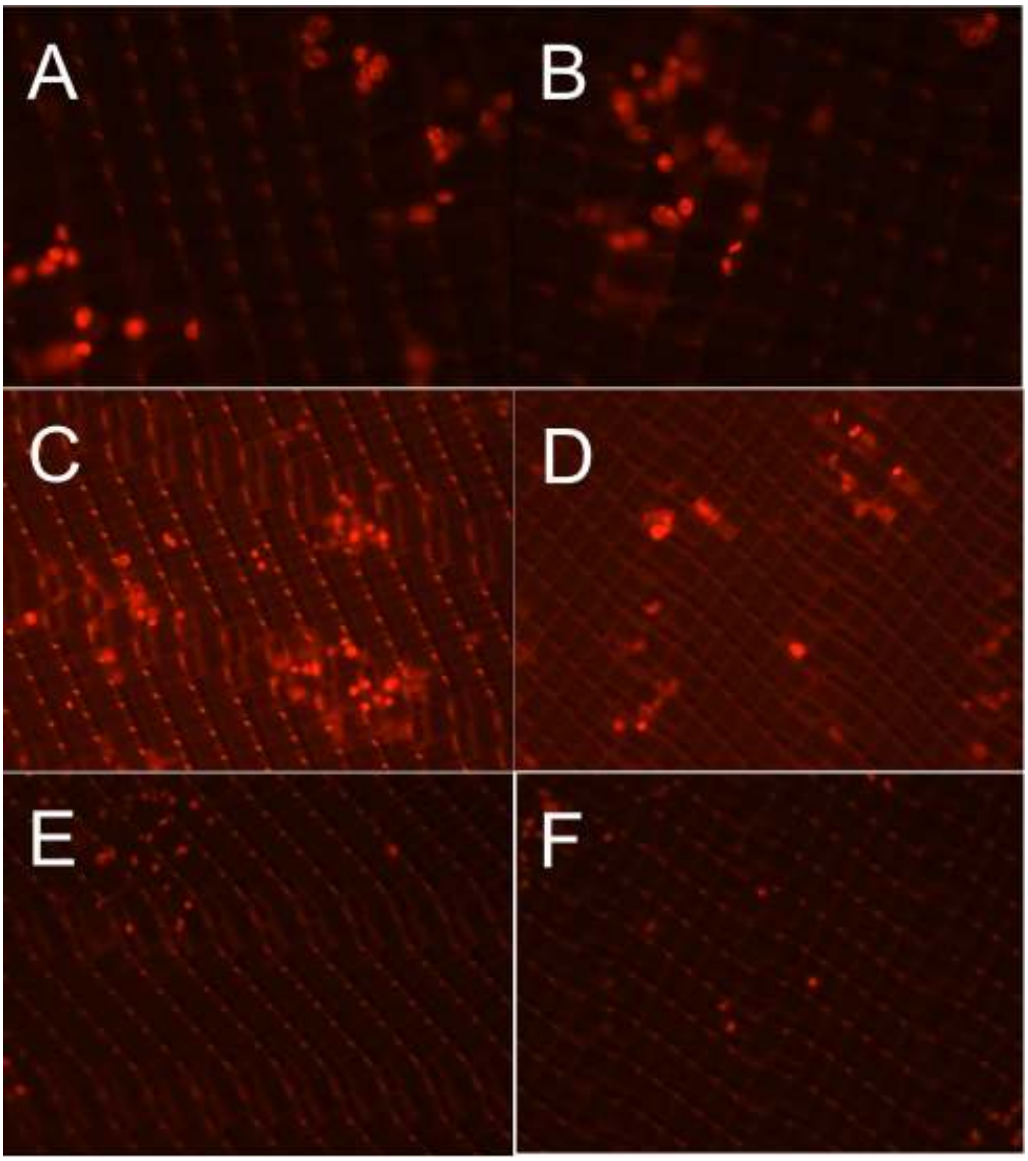


Figure 4.25: Dead cells seen with the viability test on grafted PET filtration fabric seeded at a density of 20,000 cells after 1 (A), 2 (C), and 6 (E) days and seeded at a density of 40,000 cells after 1 (B), 2 (D), and 6 (F) days.

For the cell growth and detachment test, the cells were observed and left at room temperature for 60 minutes to induce cell release after growing for 4 days. Two fabric samples were tested. The cells grew to about 50% confluency after 4 days on both samples. The fluorescent viability stain was used to visualize the cells on the fabric. After 60 minutes at room temperature, the cells were still attached to the fabric and little change

was visible. To aid in detachment, the samples were placed in a refrigerator and cooled further for 30 minutes. Even after 90 minutes, the cells remained attached to the fabric. These results were very unexpected since Kwon et al. noted a 50% reduction in release time when the cells were grown on a porous membrane compared to the PS plates.[4] The results of the detachment test are shown in Figure 4.26.

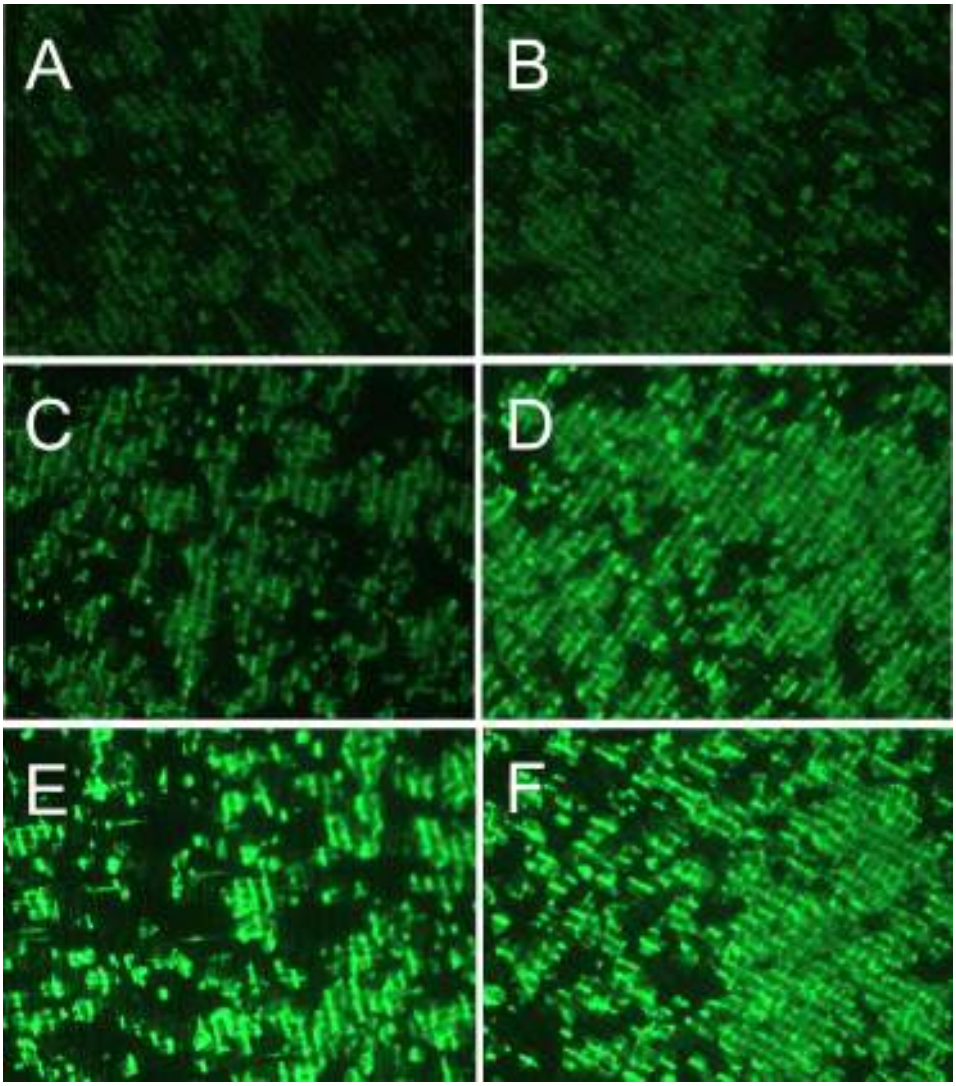


Figure 4.26: Live cells seen on grafted PET filtration fabrics after 0 (A, B), 60 (C, D), and 90 (E, F) minutes at 4x.

Even with the fluorescent stain, clear visualization of the cells on the fabric was difficult. The cells clearly proliferated well on the grafted fabric; but after no detachment was observed after cooling for 90 minutes, the cells were examined further. A closer look at the cells revealed possible growth in the pores or between the twill weave of the fabric shown in Figure 4.27. The circled areas highlight cells that come into or out of focus with the change in focal point on the fabric. This suggests cells are growing in both focal planes.

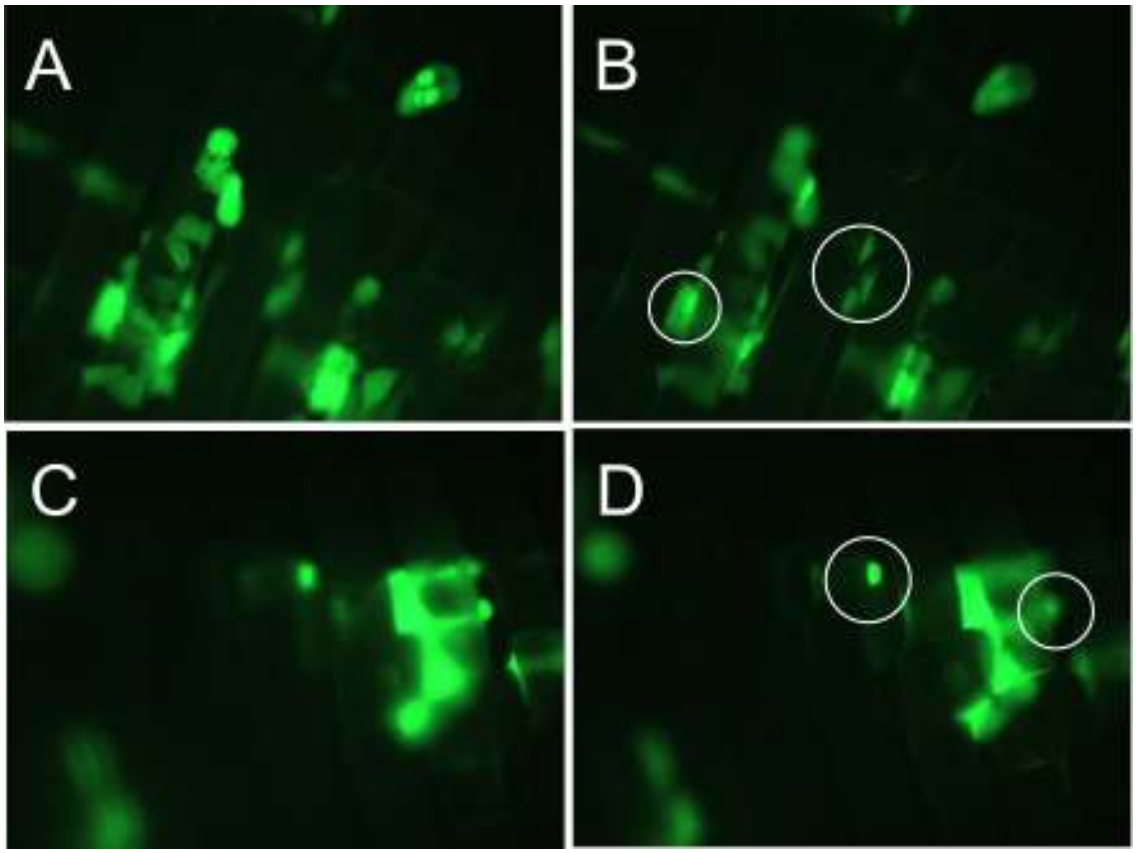


Figure 4.27: Possible cell growth between the weave or in fabric pores shown with focal point changes on same area in two parts of the fabric at 20x.

Lee et al. found that a pore size equal to or greater than 5 μm hindered the adhesion and growth of fibroblasts on polycarbonate membranes suggesting the difficulties of growing

cells on the PET filtration fabric.[30] Although the Hep G2 cells and HFLs grew well on the fabric, it does pose questions about the effects of the large pore size. Since the pores are larger, they allow for cells to grow around the edge of the filament and into the pore.[31] If the cells are growing in the pore space, the unsuccessful detachment of the cells is likely due to two reasons. First, cells growing on edges must apply a larger adhesive force to hang on than those on a flat surface.[30] Second, it is possible that there is a reduction in the amount of pNIPAM grafted on the inner sides of the filaments due to inability of the plasma to efficiently access those areas and a reduction in the monomer solution volume as it flows through the pores during the coating procedure.[4] It has been found that the monomer solution solvent type and concentration has an effect on the grafting rate of a porous membrane.[35,52] Although it was found that methacrylate can be grafted into submicron pores, this was not evident in our fabric when viewed with SEM; and the pores of our fabric may be too large for the polymer chains to bridge.[52] If the cells are growing within the weave of the fabric, the unsuccessful detachment may be due to the large variations in the surface and subsequent irregular cell sheet formation or entrapment of cells between the filaments and thus attachment of cells to ungrafted areas of the fabric. However, it still remains unclear as to why the cells did not detach. Further characterization of the grafted fabric needs to be examined; and a flatter fabric with a smaller pore size should be tested to evaluate the effects of the weave and pore size on cell attachment and detachment.

4.4. Characterization of Coat- and Spray-Grafted Fabrics

PNIPAM grafted fabrics have possible use in apparel applications as well. Woven cotton, nylon, and polyester fabrics were grafted with a coating method or spraying method described in the Materials and Methods section. The spraying method is more practical for commercial use because it require less resources and preparation time. The characteristics of the fabrics from the two methods were compared and contrasted with the testing. Washfastness was considered with each test as it is an essential component in the apparel industry. Table 4.4 explains the abbreviations for the fabrics used in the following figures.

Table 4.4: List of abbreviations used for fabric testing.

Abbreviation	Sample
C	untreated cotton
CW	washed untreated cotton
CP	plasma pretreated cotton
CPW	washed plasma pretreated cotton
CC	coat-grafted cotton
CCW	washed coat-grafted cotton
CS	spray-grafted cotton
CSW	washed spray-grafted cotton
N	untreated nylon
NW	washed untreated nylon
NP	plasma pretreated nylon
NPW	washed plasma pretreated nylon
NC	coat-grafted nylon
NCW	washed coat-grafted nylon
NS	spray-grafted nylon
NSW	washed spray-grafted nylon
P	untreated polyester
PW	washed untreated polyester
PP	plasma pretreated polyester
PPW	washed plasma pretreated polyester
PC	coat-grafted polyester
PCW	washed coat-grafted polyester
PS	spray-grafted polyester
PSW	washed spray-grafted polyester

4.4.1. Surface Chemistry

Fourier Transform Infrared Spectroscopy (FTIR) produces information on the chemical composition of a specific chemical species. It is a very powerful tool used to identify specific chemical bonds simply by interpreting the infrared absorption spectrum. The characteristic peaks that correspond to the chemical structure of pNIPAM should be observed at 1386 cm^{-1} (methyl group deformation), 1458 cm^{-1} (-CH_3 and –CH_2- deformation), 1540 cm^{-1} (secondary amide N-H stretching), 1650 cm^{-1} (secondary amide C=O stretching), 2970 cm^{-1} (-CH_3 asymmetric stretching), and 3300 cm^{-1} (secondary amide N-H stretching).[1,3] The test was done to give an indication of the amount of pNIPAM being grafted on the fabrics and compare the washfastness of the graft from the two methods. The results for the cotton fabrics are shown in Figure 4.28.

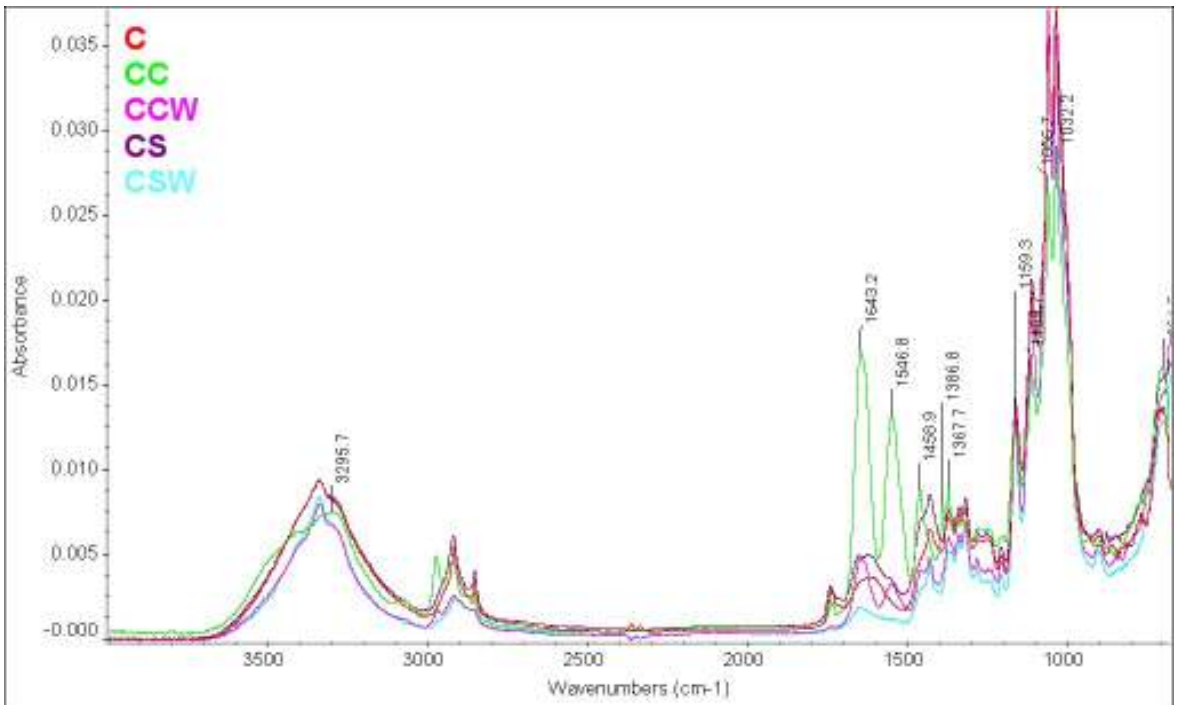


Figure 4.28: FTIR spectra of the cotton fabrics.

The characteristic peaks that correspond to the chemical structure of pNIPAM were seen at 1386 cm^{-1}, 1458 cm^{-1}, 1540 cm^{-1}, 1650 cm^{-1}, and 2970 cm^{-1} for the coat-grafted cotton indicating the successfulness of the coat-graft method. These peaks were also seen on the coat-grafted and washed fabrics although at a lower absorbance band. However, it does confirm the washfastness of the coat-grafted cotton fabrics. The spray-grafted fabrics did not show any of the characteristic pNIPAM peaks and mimicked the spectra of the untreated cotton. These results were not surprising due to the reduced monomer solution volume used and suspected thinness of the graft with the spray method.

The results for the nylon fabrics are shown in Figure 4.29.

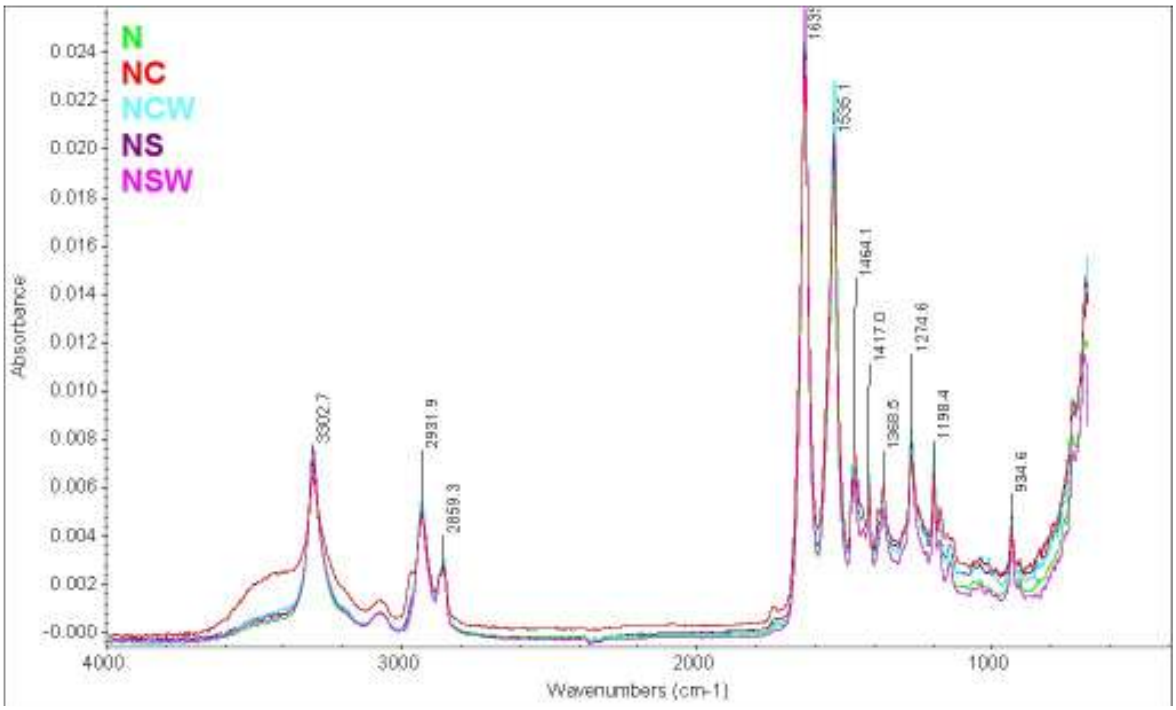


Figure 4.29: FTIR spectra of the nylon fabrics.

Since nylon also has amide groups, absorption bands at 1540 cm^{-1}, 1650 cm^{-1}, and 3300 cm^{-1} could not be used to identify pNIPAM on the surface of the fabric. Therefore, the

peak occurring at 2970 cm^{-1} was used to evaluate grafting. This peak appeared only on the coat-grafted nylon demonstrating the effectiveness of the coat method. The small peak was not visible on coat-grafted and washed nylon. However since a decrease in absorbance was seen with the washed cotton fabrics, it is likely that the already small peak was diminished on the washed nylon sample. Once again, the spray-grafted fabrics did not show the characteristic pNIPAM peak and mimicked the untreated nylon spectra. This was likely due to the thinness of the graft using the spray method.

The results for the polyester fabrics are shown in Figure 4.30.

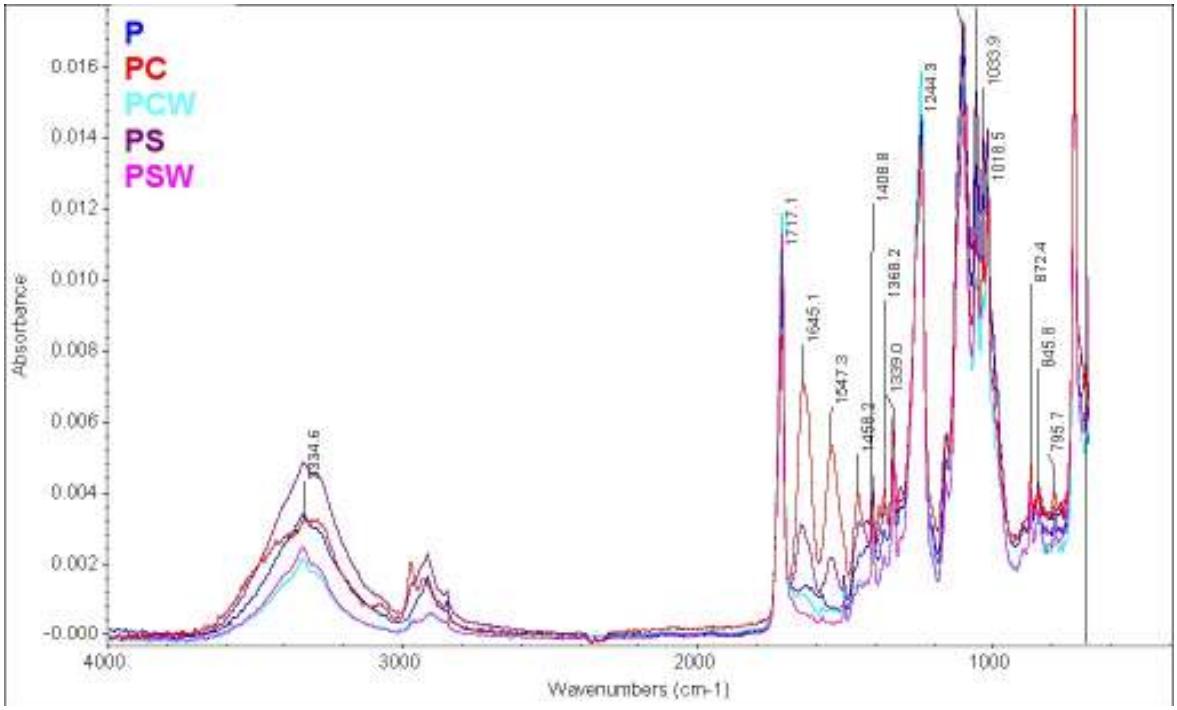


Figure 4.30: FTIR spectra of the polyester fabrics.

The characteristic peaks that correspond to the chemical structure of pNIPAM were seen at 1458 cm^{-1}, 1540 cm^{-1}, and 1650 cm^{-1} for the coat-grafted and spray-grafted polyester indicating the successfulness of both methods. The 2970 cm^{-1} peak seen on the coat-

grafted fabric was also apparent on the coat-grafted and washed fabric although at a lower absorbance band. However, it does confirm the washfastness of the coat-grafted polyester fabrics. The spray-grafted and washed fabric did not show any of the characteristic pNIPAM peaks and mimicked the spectra of the untreated polyester. This result could be due to the reduced monomer solution volume used and thinness of the graft with the spray method or an indication of insufficient washfastness.

Overall, the spectra from the coat-grafted fabrics indicated successful grafting of pNIPAM onto cotton, nylon, and polyester and moderate washfastness. The differences seen between the spectra of the three fabrics likely were dependent on the wet-pickup of each type of fabric and its affinity for the monomer solution. Thus, the best grafting method is dependent on which type of fabric is desired.

4.4.2. Effects of Temperature on Wettability and Wicking

PNIPAM has been shown to change wettability with temperature. When grafted on textiles, the known wettability of the particular fabric should be altered as well. To examine the effects of the graft on the fabrics' wettability and wicking properties, the spray- and coat-grafted fabrics were evaluated with a vertical wicking test using cool ($20^{o}C$) and warm ($50^{o}C$) water. Untreated, plasma pretreated, grafted, and washed cotton, nylon, and polyester fabrics were tested. The tests were repeated four times and examined at 5, 15, and 30 minutes after immersion. The results for the coated and sprayed fabrics are shown in Figures 4.31, 4.32, and 4.33 and are reported in percentages since all of the samples were not exactly the same length.

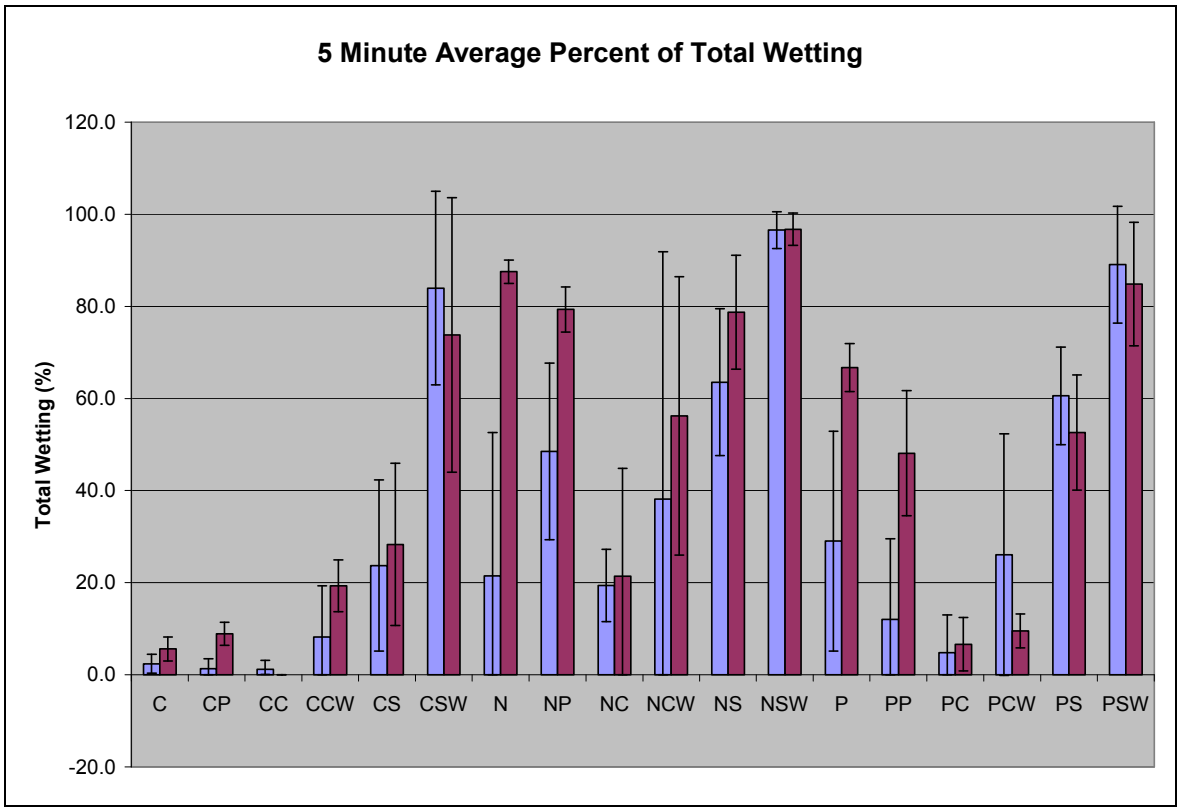


Figure 4.31: Average percent of total wetting after 5 minutes with cool water (blue) and warm water (purple).

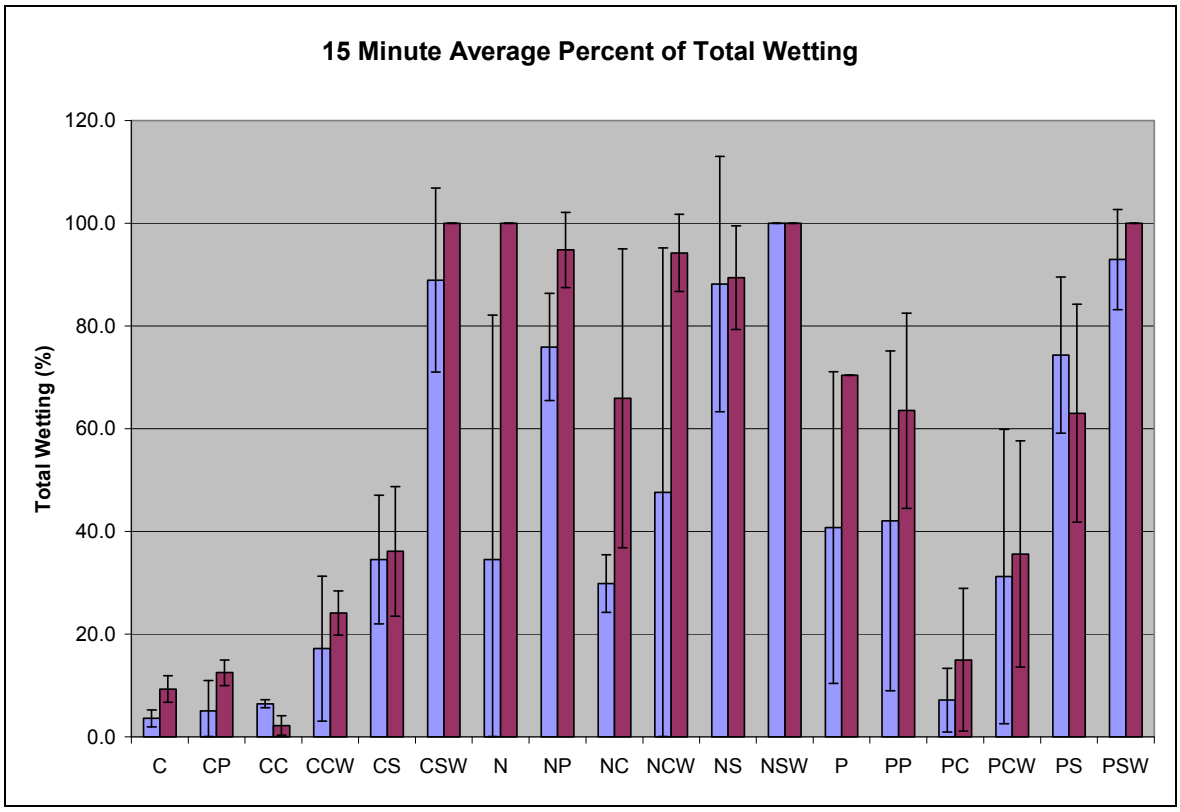


Figure 4.32: Average percent of total wetting after 15 minutes with cool water (blue) and warm water (purple).

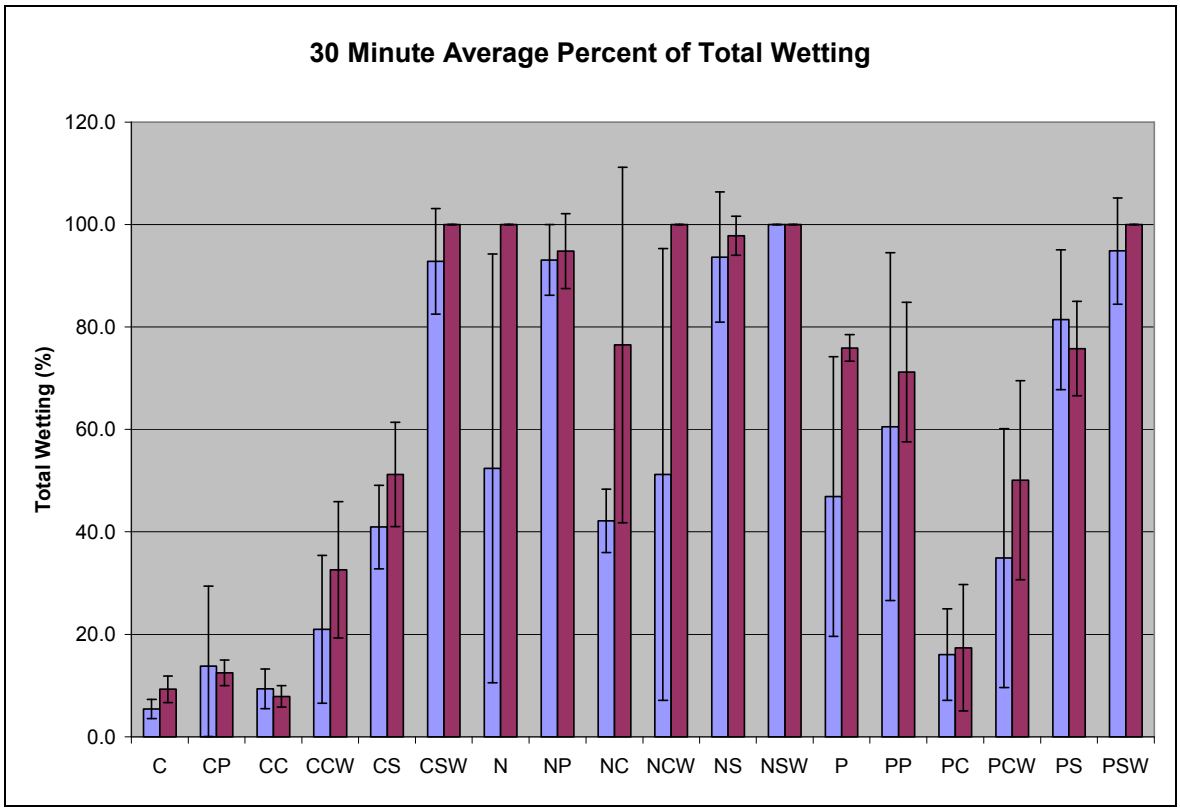


Figure 4.33: Average percent of total wetting after 30 minutes with cool water (blue) and warm water (purple).

The general trend found from these results was an increase in wettability as the temperature of the water increased. This was not expected as the hydrophobicity of pNIPAM is known to increase above 32^{o}C. The underlying causes for the results are not completely understood, and further testing and characterization needs to be performed on the fabrics. The ideal test would have had the fabrics warmed to above the LCST instead of just the water; however, this was not possible with the available resources. This limitation could have also contributed to the unanticipated results. The plasma pretreated fabrics were expected to have increased wetting due to the formation of –OH groups on the surface.[4] However, none of the plasma pretreated fabrics showed a significant increase in wettability when compared with the untreated fabrics. The grafted fabrics seem to show an

increase in wettability with the unwashed coat-grafted fabrics having the lowest wettability and the washed spray-grafted fabrics having the highest wettability. However, these washed sample results could be affected by the detergent which is thought to increase the hydrophilicity of the fabrics. Results of the testing of a second batch of spray-grafted fabrics whose results are shown in Figures 4.34, 4.35, and 4.36. The washing process seems to have a significant effect on the wettability of all the samples and results in a large increase in total wetting percent.

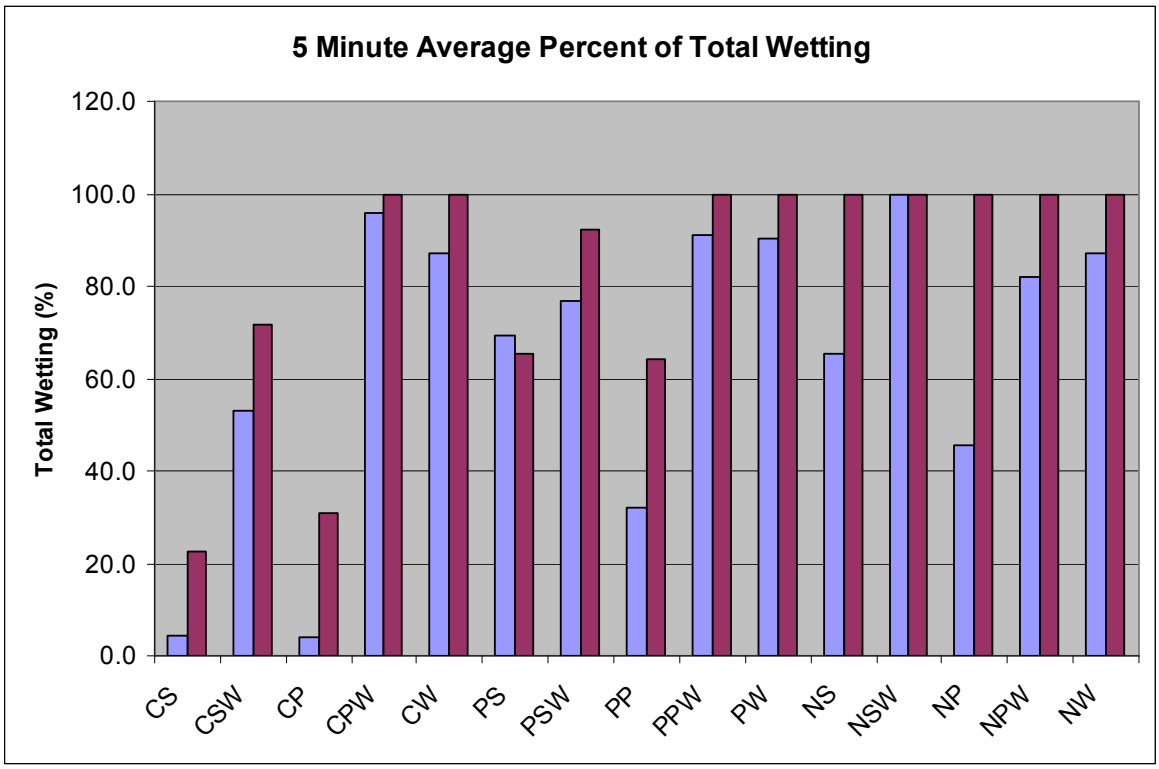


Figure 4.34: Average percent of total wetting after 5 minutes with cool water (blue) and warm water (purple).

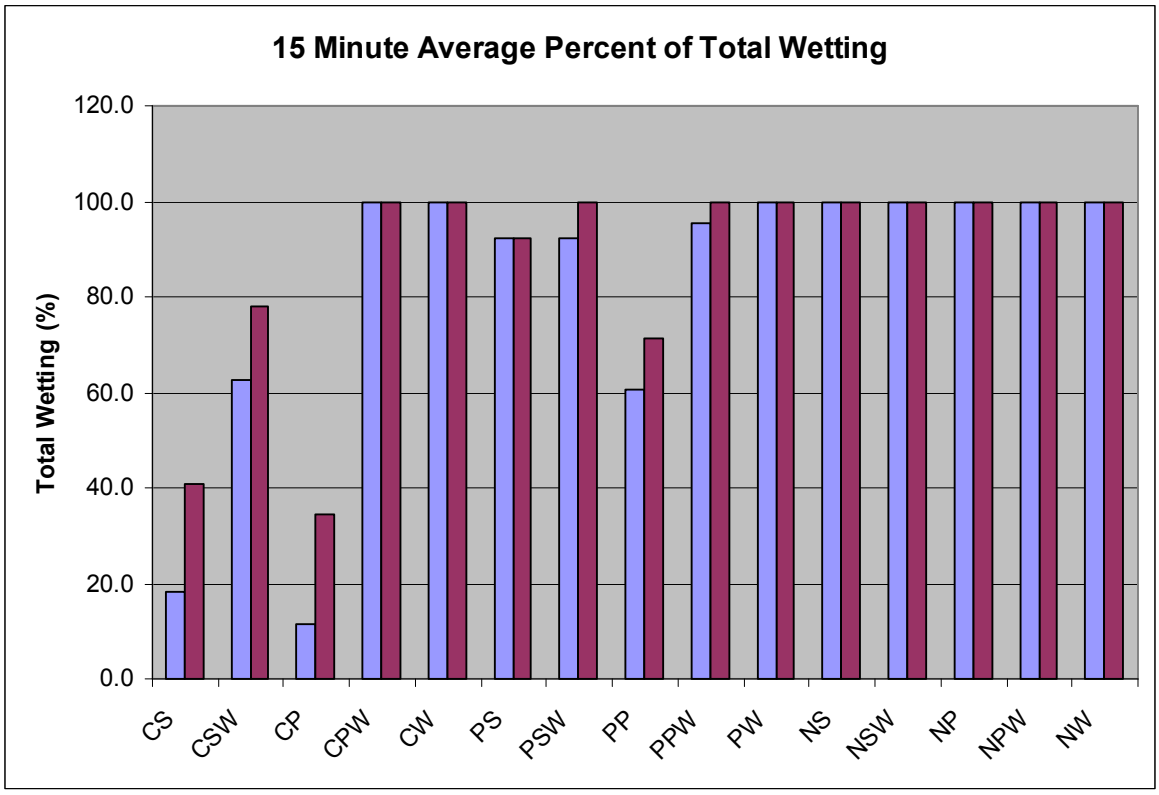


Figure 4.35: Average percent of total wetting after 15 minutes with cool water (blue) and warm water (purple).

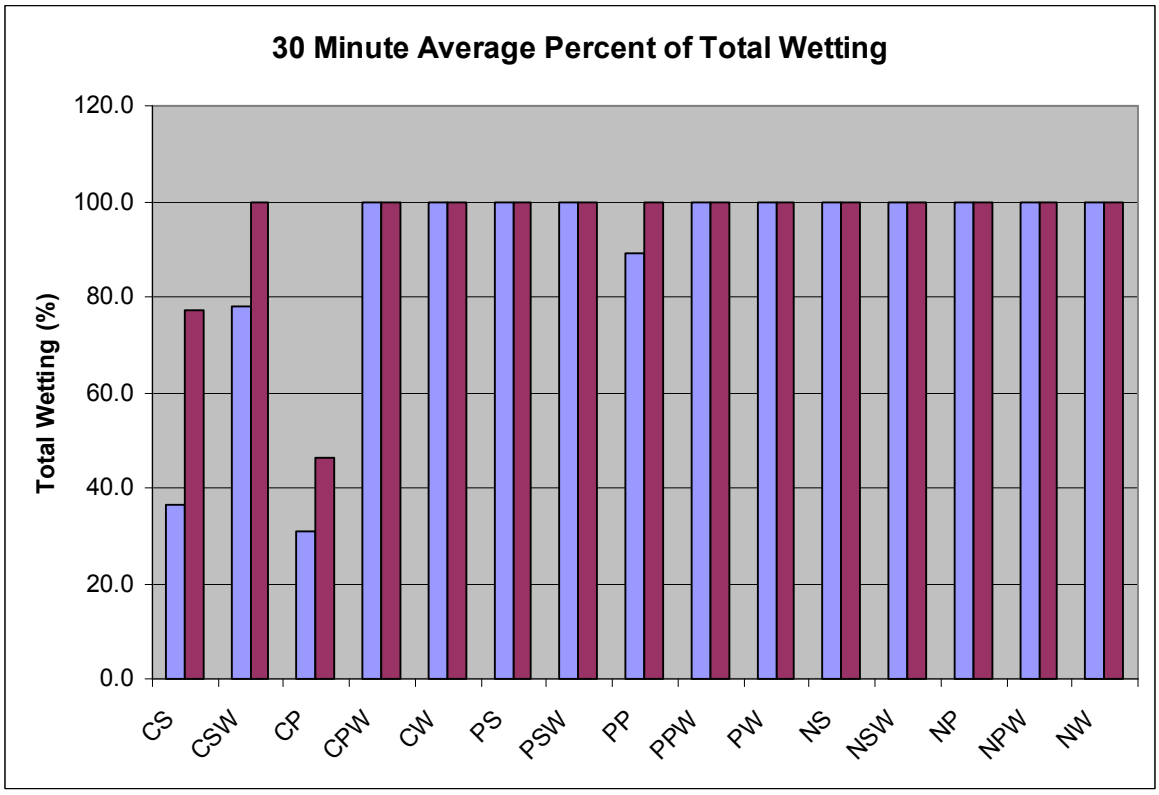


Figure 4.36: Average percent of total wetting after 30 minutes with cool water (blue) and warm water (purple).

Another interesting trend was the difference in wicking between the two different grafting methods. The only apparent difference in the methods was the amount of monomer solution applied to the fabrics. Once again, reasons for these differences cannot be explained at this point without further research. Overall, the coat-grafted fabrics had the greatest resistance to wicking, and the washed spray-grafted fabrics had the greatest wicking.

4.4.3. Graft Uniformity

After examining the surprising wettability results, evaluation of the uniformity of the graft was necessary to compare the two grafting methods and ensure washfastness of the graft. A simple dye test using an acid dye, which is known to color the amino end

groups of nylons, was used since pNIPAM is an amide.[29] This test could not be performed on the nylon fabrics since the dye colors the fabric itself. The results for the cotton fabrics are shown in Figure 4.37.

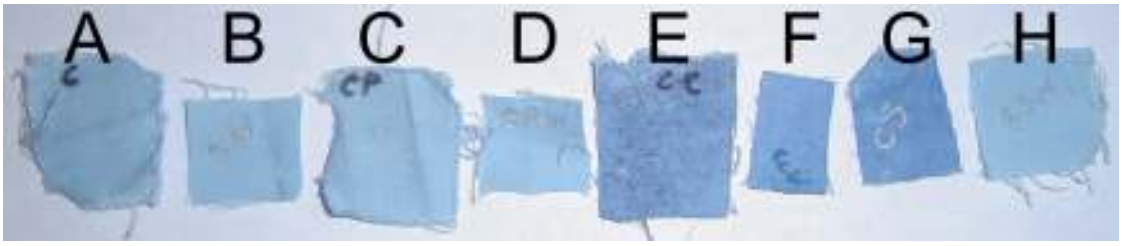


Figure 4.37: Dye test results on untreated (A), untreated and washed (B), plasma pretreated (C), plasma pretreated and washed (D), coat-grafted (E), coat-grafted and washed (F), spray-grafted (G), and spray-grafted and washed (H) cotton fabrics.

The untreated and plasma pretreated fabrics were slightly tinted after dyeing, but the coat- and spray-grafted fabrics were both dyed dark blue confirming the presence of the graft and the uniformity achieved from the grafting methods. The coat-grafted fabric also demonstrated excellent washfastness seen from the slightly lighter blue color in Figure 4.37 F. The spray-grafted fabric, however, did not display this degree of washfastness as seen in Figure 4.37 H. This was most likely due to the significant reduction in monomer solution volume applied to the spray-grafted fabrics. The washfastness results also corresponded with the findings from FTIR testing.

The results from the polyester fabrics are shown in Figure 4.38.

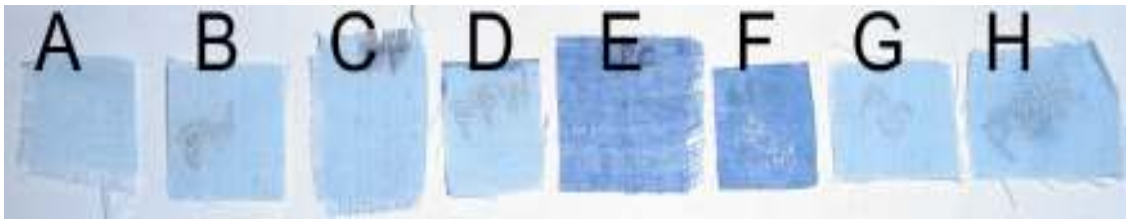


Figure 4.38: Dye test results on untreated (A), untreated and washed (B), plasma pretreated (C), plasma pretreated and washed (D), coat-grafted (E), coat-grafted and washed (F), spray-grafted (G), and spray-grafted and washed (H) polyester fabrics.

As with the cotton, the untreated and plasma pretreated fabrics were slightly tinted after dyeing. The coat-grafted fabric was dyed dark blue confirming the presence of the graft and the uniformity achieved from the grafting method. It also demonstrated excellent washfastness seen from a similar dark blue color in Figure 4.38 F. The spray-grafted fabric did not dye nearly as much as the coat-grafted fabric and showed signs of spottiness in the grafted and washed samples (Figure 4.38 G and H). This again was most likely due the reduction in monomer solution volume applied to the spray-grafted fabrics. The washfastness results also corresponded with the findings from FTIR testing.

Overall, the coat-grafted fabrics showed the best washfastness and uniform graft, and the nylon results are expected to be consistent with these findings had they been tested. Further modification of the spray-grafting method needs to be examined in order to increase graft uniformity and washfastness. Although the coat-grafted fabrics did exhibit excellent uniformity and washfastness, it has not yet been determined whether they are more useful for apparel applications than the spray-grafted fabrics; and the excessive NIPAM monomer solution volume used for the coated fabrics is less than desirable for regular use with skin-contacting textiles.

5. CONCLUSION

Thermally responsive pNIPAM was successfully grafted onto polyester film and filtration fabric. The atmospheric pressure plasma treatment provided a quick, simple means of grafting, and it sufficiently sterilized the samples for cell culture. The treatment parameters for grafted polyester films were optimized with cell culture studies with HEKs and found to be 3 minute plasma pretreatment, 5 minute plasma post-treatment, and 15.4 $\mu L/cm^2$ of the monomer solution. The optimally grafted PET film was then characterized. Contact angle measurements confirmed the phase change of the pNIPAM by showing an increase in hydrophobicity with an increase in temperature. AFM images show the surface becomes significantly rougher and more variable when placed in water as the polymer chains become hydrated and gel structure forms. The decrease in surface roughness seen with the grafted film and the SEM images confirms the graft coats the untreated film filling in the natural cavities. The graft thickness was found to be between 30 and 100 nm with AFM measurements. Cell proliferation and detachment with HFLs demonstrates the ability of the surface to be used with various cell types and the structure transformation upon lowering the temperature.

Characterization of the PET filtration fabric with contact angle measurements also confirmed the phase change and successful grafting of pNIPAM. Although FTIR and SEM did not show the graft on the fabric, they were evidence of the thin graft produced with atmospheric plasma treatment. The uniformity and further proof of grafted pNIPAM on the fabric was seen with acid dye testing. Cell culture studies with HFLs and Hep G2

cells exhibit the cellular proliferation on the grafted fabric, although no detachment was observed.

Coat- and spray-grafted woven cotton, nylon, and polyester were compared and characterized. FTIR spectra from the coat-grafted fabrics indicated successful grafting of pNIPAM onto cotton, nylon, and polyester and moderate washfastness; spectra from the spary-grafted fabrics did not confirm grafting but could have resulted from a thin graft as seen with the filtration fabric and PET film. Vertical wicking tests showed an unexpected increase in wettability with increasing water temperature. They also showed that the coat-grafted fabrics had the greatest resistance to wicking and the washed spray-grafted fabrics had the greatest wicking. The acid dye test showed that the coat-grafted fabrics had the best washfastness and uniform graft, but the type of fabric and its properties seem to have an effect on the grafting method.

6. FUTURE RECOMMENDATIONS

Although this work sufficiently optimized the plasma parameters for polyester film and gave an idea about the characteristics of the pNIPAM coating being grafted onto different surfaces, further investigation into the following areas is necessary for utilization of the responsive textiles:

- Extensive force-displacement and thickness testing of the graft in fluid at varying temperatures to obtain a better understanding of how the polymer is changing at the transition and further characterize the graft produced with atmospheric plasma;
- Cell culture studies with a microporous fabric or a membrane with small pore size to facilitate detachment and less cell growth between the filaments;
- Facilitation of cell sheet detachment from grafted film to improve release mechanism;
- Continued cell culture studies with more cell types on grafted film to enhance the practicality of using grafted surfaces for many cell sheet engineering applications;
- Optimization of plasma parameters and experimentation with different gases and configurations;
- Characterization of grafted woven textiles;
- Improvement of the spray-grafting method to achieve greater washfastness and develop a thermoresponsiveness closer to that of bulk pNIPAM.

7. REFERENCES

1. Pan, Y.V., R.A. Wesley, R. Luginbuhl, D.D. Denton, and B.D. Ratner. 2001. Plasma polymerized N-isopropylacrylamide: Synthesis and characterization of a smart thermally responsive coating. *Biomacromolecules*. 2: 32-36.
2. Kikuchi, A. and T. Okano. 2005. Nanostructured designs of biomedical materials: applications of cell sheet engineering to functional regenerative tissues and organs. *J. Contr. Rel.* 101: 69-84.
3. Lin, S., Chen, K., and L. Run-Chu. 1999. Thermal micro ATR/FT-IR spectroscopic system for quantitative study of the molecular structure of poly(N-isopropylacrylamide) in water. *Polymer*. 40: 2619-2624.
4. Kwon, O.H., Kikuchi, A., Yamato, M., Sakurai, Y., and T. Okano. 2000. Rapid cell sheet detachment from Poly(N-isopropylacrylamide)-grafted porous cell culture membranes. *J. Biomed. Mater. Res.* 50: 82-89.
5. Cheng, X., Canavan, H.E., Stein, M.J., Hull, J.R., Kweskin, S.J., Wagner, M.S., Somorjai, G.A., Castner, D.G., and B.D. Ratner. 2005. Surface chemical and mechanical properties of plasma-polymerized N-isopropylacrylamide. *Langmuir*. 21: 7833-7841.
6. Yang, J., Yamato, M., Kohno, C., Nishimoto, A., Sekine, H., Fukai, F., and T. Okano. 2005. Cell sheet engineering: Recreating tissues without biodegradable scaffolds. *Biomaterials*. 26: 6415-6422.
7. Chu, L.Y., Park, S.H., Yamaguchi, T., and S.I. Nakao. 2001. Preparation of thermo-responsive core-shell microcapsules with a porous membrane and poly(N-isopropylacrylamide) gates. *J. Memb. Sci*. 102: 27-39.
8. Wei, H., Zhang, X.Z., Zhou, Y., Cheng, S.X., and R.X. Zhuo. 2006. Self-assembled thermoresponsive micelles of poly(N-isopropylacrylamide-b-methyl-methacrylate). *Biomaterials*. 27: 2028-2034.
9. Cheng, S.X., Zhang, J.T., and R.X. Zhuo. 2003. Macroporous poly(N-isopropylacrylamide) hydrogels with fast response rates and improved protein release properties. *J. Biomed. Mater. Res. A*. 67: 96-103.
10. Kikuchi, A. and T. Okano. 2004. Temperature-responsive, polymer-modified surfaces for green chromatography. *Macromol. Symp.* 207: 217-227.
11. Kanazawa, H., Sunamoto, T., Matsushima, Y., Kikuchi, A., and T. Okano. 2000.Temperature-responsive chromatographic separation of amino acid phenylthiohydantoins using aqueous media as the mobile phase. *Anal. Chem.* 72: 5961-5966.
12. Yamada, N., Okano, T., Sakai, H., Karikusa, F., Sawasaki, Y., and Y. Sakurai. 1990. Thermoresponsive polymeric surfaces; control of attachment and detachment of cultured cells. *Makromol. Chem., Rapid Commun.* 11: 570-576.
13. Okano, T., Yamada, N., Okuhara, M., Sakai, H., and Y. Sakurai. 1995. Mechanism of cell detachment from temperature-modulated, hydrophilic-hydrophobic polymer surfaces. *Biomaterials*. 16: 297-303.

14. Yamato, M., Okuhara, M., Karikusa, F., Kikuchi, A., Sakurai, Y., and T. Okano. 1999. Signal transduction and cytoskeletal reorganization are required for cell detachment from cell culture surfaces grafted with a temperature-responsive polymer. *J. Biomed. Mater. Res.* 44: 44-52.
15. Canavan, H.E., Cheng, X., Graham, D.J., and B.D. Ratner. 2005. Cell sheet detachment affects the extracellular matrix: A surface science study comparing thermal liftoff, enzymatic, and mechanical methods. *J. Biomed. Mater. Res.* 75A: 1-13.
16. Shiroyanagi, Y., Yamato, M., Yamazaki, Y., Toma, H., and T. Okano. 2003. Transplantable urothelial cell sheets harvested noninvasively from temperature-responsive culture surfaces by reducing temperature. *Tissue Eng.* 9: 1005-1012.
17. Doorty, K.B., Golubeva, T.A., Gorelov, A.V., Rochev, Y.A., Allen, L.T., Dawson, K.A., Gallagher, W.M., and A.K. Keenan. 2003. Poly(N-isopropylacrylamide) co-polymer films as potential vehicles for delivery of an antimitotic agent to vascular smooth muscle cells. *Cardiovasc. Pathol*. 12: 105-110.
18. Von Recum, H.A., Kim, S.W., Kikuchi, A., Okuhara, M., Sakurai, Y., and T. Okano. 1998. Novel thermally reversible hydrogel as detachable cell culture substrate. *J. Biomed. Mater. Res.* 40: 631-639.
19. Nandkumar, M.A., Yamato, M., Kushida, A., Konno, C., Hirose, M., Kikuchi, A., and T. Okano. 2002. Two-dimensional cell sheet manipulation of heterotypically co-cultured lung cells utilizing temperature-responsive culture dishes results in long-term maintenance of differentiated epithelial cell functions. *Biomaterials*. 23: 1121-1130.
20. Takei, Y.G., Aoki, T., Sanui, K., Ogata, N., Sakurai, Y., and T. Okano. 1994. Dynamic contact angle measurement of temperature-responsive surface properties for poly(N-isopropylacrylamide) grafted surfaces. *Macromolecules*. 27: 6163-6166.
21. Yakushiji, T., Sakai, K., Kikuchi, A., Aoyagi, T., Sakurai, Y., and T. Okano. 1998. Graft architectural effects on thermoresponsive wettability changes of poly(N-isopropylacrylamide)-modified surfaces. *Langmuir*. 14: 4657-4662.
22. Kanazawa, H., Yamamoto, K., Matsushima, Y., Takai, N., Kikuchi, A., Sakurai, Y., and T. Okano. 1996. Temperature-responsive chromatography using poly(N-isopropylacrylamide)-modified silica. *Anal. Chem.* 68: 100-105.
23. Akiyama, Y., Kikuchi, A., Yamato, M., and T. Okano. 2004. Ultrathin poly(N-isopropylacrylamide) grafted layer on polystyrene surfaces for cell adhesion/detachment control. *Langmuir*. 20: 5506-5511.
24. Wang, Xiaoling. 2004. Responsive Biomaterial Surfaces. M.S. Thesis. North Carolina State University, Raleigh, North Carolina.
25. Matthews, S., McCord, M., and M. Bourham. 2005. Polyvinyl alcohol (PVA) desizing mechanism via atmospheric pressure plasma exposure. *J. Plasma Process. Polym.* 2: 702-708.
26. Hwang, Y.J., Matthews, S., McCord, M., and M. Bourham. 2004. Surface modification of organic polymer films treated in atmospheric plasma. *J. Electrochem. Soc.* 151: C495-C501.
27. Matzelle, T., Ivanov, D., Landwehr, D., Heinrich, L., Herkt-Bruns, Ch., Reichelt, R., and N. Kruse. 2002. Micromechanical properties of “smart” gels: Studies by scanning

force and scanning electron microscopy of PNIPAAm. *J. Phys. Chem. B*. 106: 2861-2866.
28. Shenton, M. and G. Stevens. 2001. Surface modification of polymer surfaces: atmospheric plasma versus vacuum plasma treatments. *J. Phys. D: Appl. Phys*. 34: 2761-2768.
29. Aspland, J.R. 1997. *Textile Dyeing and Coloration*. Raleigh: AATCC.
30. Lee, J.H., Lee, S.J., Khang, G., and H.B. Lee. 1999. Interaction of fibroblasts on polycarbonate membrane surfaces with different micropore size and hydrophilicity. *J. Biomater. Sci. Polym. Ed.* 10: 283-294.
31. Curtis, A. and C. Wilkinson. 1997. Topographical control of cells. *Biomaterials*. 18: 1573-1583.
32. Chan, C. 1994. *Polymer Surface Modification and Characterization*. New York: Hanser/Gardener Publications.
33. Loh, I. Plasma surface modification in biomedical applications. *AST Technical Journal*. P-2003. www.astp.com.
34. Egitto, F.D. and L.J. Matienzo. 1994. Plasma modification of polymer surfaces for adhesion improvement. *IBM J. Res. Develop.* 38: 423-439.
35. Chan, C.M. Ko, T.M., and H. Hiraoka. 1996. Polymer surface modification by plasmas and photons. *Surface Science Reports*. 24: 1-54.
36. Okano, T., Yamada, N., Sakai, H., and Y. Sakurai. 1993. A novel recovery system for cultured cells using plasma-treated polystyrene dishes grafted with poly(N-isopropylacrylamide. *J. Biomed. Mater. Res.* 27: 1243-1251.
37. Kushida, A., Masayuki, Y., Konno, C., Kikuchi, A., Sakurai, Y., and T. Okano. 1999. Decrease in culture temperature releases monolayer endothelial cell sheets together with deposited fibronectin matrix from temperature-responsive culture surfaces. *J. Biomed. Mater. Res.* 45: 355-362.
38. Kikuchi, A., Okuhara, M., Karikusa, F., Sakurai, Y., and T. Okano. 1998. Two-dimensional manipulation of confluently cultured vascular endothelial cells using temperature-responsive poly(N-isopropylacrylamide)-grafted surfaces. *J. Biomater. Sci. Polym. Ed.* 9: 1331-1348.
39. Yamato, M., Konno, C., Kushida, A., Hirose, M., Utsumi, M., Kikuchi, A., and T. Okano. 2000. Release of adsorbed fibronectin from temperature-responsive culture surfaces requires cellular activity. *Biomaterials*. 21: 981-986.
40. Shimizu, T., Yamato, M., Kikuchi, A., and T. Okano. 2001. Two-dimensional manipulation of cardiac myocyte sheets utilizing temperature-responsive culture dishes augments the pulsatile amplitude. *Tissue Eng.* 7: 141-151.
41. Shimizu, T., Yamato, M., Akutsu, T., Shibata, T., Isoi, T., Kikuchi, A., Umezu, M., and T. Okano. 2002. Electrically communicating three-dimensional cardiac tissue mimic fabricated by layered cultured cardiomyocyte sheets. *J. Biomed. Mater. Res.* 60: 110-117.
42. Shimizu, T., Yamato, M., Isoi, Y., Akutsu, T., Setomaru, T., Abe, K., Kikuchi, A., Umezu, M., and T. Okano. 2002. Fabrication of pulsatile cardiac tissue grafts using a novel 3-dimensional cell sheet manipulation technique and temperature-responsive cell culture surfaces. *Circ. Res.* 90: e40.

43. Miyagawa, S., Sawa, Y., Sakakida, S., Taketani, S., Kondoh, H., Memon, I.A., Imanishi, Y., Shimizu, T., Okano, T., and H. Matsuda. 2005. Tissue cardiomyoplasty using bioengineered contractile cardiomyocyte sheets to repair damaged myocardium: their integration with recipient myocardium. *Transplantation*. 80: 1586-1595.
44. Yamato, M., Utsumi, M., Kushida, A., Konno, C., Kikuchi, A., and T. Okano. 2001. Thermo-responsive culture dishes allow the intact harvest of multilayered keratinocytes sheets without dispase by reducing temperature. *Tissue Eng.* 7: 473-480.
45. Hasegawa, M., Yamato, M., Kikuchi, A., Okano, T., and I. Ishikawa. 2005. Human periodontal ligament cell sheets can regenerate periodontal ligament tissue in an athymic rat model. *Tissue Eng.* 11: 469-478.
46. Kanzaki, M., Yamato, M., Hatakeyama, H., Kohno, C., Yang, J., Umemoto, T., Kikuchi, A., Okano, T., and T. Onuki. 2006. Tissue engineered epithelial cell sheets for the creation of a bioartificial trachea. *Tissue Eng.* 12: 1275-1283.
47. Canavan, H.E., Cheng, X., Graham, D.J., Ratner, B.D., and D.G. Castner. 2005. Surface characterization of the extracellular matrix remaining after cell detachment from a thermoresponsive polymer. *Langmuir*. 21: 1949-1955.
48. Hsiue, G.H., Lai, J.Y., Chen, K.H., and W.M. Hsu. 2006. A novel strategy for corneal endothelial reconstruction with a bioengineered cell sheet. *Transplantation*. 81: 473-476.
49. Bohanon, T., Elender, G., Knoll, W., Koberle, P., Lee, J.S., Offenhausser, A., Ringsdorf, H., Sackmann, E., Simon, J., Tovar, G., and F.M. Winnik. 1996. Neural cell pattern formation on glass and oxidized silicon surfaces modified with poly(N-isopropylacrylamide). *J. Biomater. Sci. Polym. Ed.* 8: 19-39.
50. Liu, B. and J. Hu. 2005. The application of temperature-sensitive hydrogels to textiles: A review of Chinese and Japanese investigations. *Fibres and Textiles in Eastern Europe*. 13: 45-49.
51. Jianqin, L., Maolin, Z., and H. Hongfei. 1999. Pre-irradiation grafting of temperature sensitive hydrogel on cotton cellulose fabric. *Radiat. Phys. Chem*. 55: 55-59.
52. Yamaguchi, T., Nakao, S., and S. Kimura. 1996. Evidence and mechanisms of filling polymerization by plasma-induced graft polymerization. *J. Polym. Sci., Part A: Polym. Chem*. 34: 1203-1208.