<table>
<thead>
<tr>
<th><strong>Title</strong></th>
<th>Smart biomaterials for cell sheet engineering</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Author(s)</strong></td>
<td>Healy, Deirdre</td>
</tr>
<tr>
<td><strong>Publication Date</strong></td>
<td>2016-03-23</td>
</tr>
<tr>
<td><strong>Item record</strong></td>
<td><a href="http://hdl.handle.net/10379/5901">http://hdl.handle.net/10379/5901</a></td>
</tr>
</tbody>
</table>

Some rights reserved. For more information, please see the item record link above.

Downloaded 2019-01-02T19:15:23Z
Smart Biomaterials for Cell Sheet Engineering

by

Deirdre Healy, B.Sc.

A thesis submitted to the College of Science
National University of Ireland Galway
In partial fulfilment of the requirements for the degree of

Doctor of Philosophy
School of Chemistry
National University of Ireland Galway
March 2016

Supervisors: Dr. Yury Rochev
Prof. Peter Dockery
Dr. William Carrol

Head of School: Prof. Paul Murphy
# Table of Contents

1  Introduction .................................................................................. 1

1.1  Smart Polymers ........................................................................... 1

1.1.1  Thermoresponsive Polymers ...................................................... 1

1.1.1.1  Poly N-(Isopropylacrylamide) (pNIPAm) .............................. 5

1.2  Cold Protein Denaturation ............................................................ 5

1.3  Film Preparation Methods ............................................................. 6

1.3.1  Spin Coating ............................................................................. 6

1.3.2  Solvent Casting ......................................................................... 7

1.3.3  Electron Beam Polymerization (EBP) ........................................... 8

1.3.4  Plasma Polymerization ............................................................... 8

1.3.5  Physical Adsorption .................................................................. 9

1.4  Advantages and Disadvantages of Commonly used pNIPAm Film Preparation Methods .................................................................................. 11

1.5  Adsorption ................................................................................... 12

1.5.1  Adsorption Isotherms ............................................................... 13

1.5.1.1  Langmuir Isotherm ................................................................. 14

1.5.1.2  BET (Brunauer, Emmett and Teller) Isotherm .......................... 16

1.6  Polymer Adsorption ..................................................................... 17

1.7  Physical Adsorption of pNIPAm .................................................... 22

1.8  Protein Adsorption ....................................................................... 42

1.9  Cell Adhesion .............................................................................. 46

1.9.1  Chemical and Physical Properties Which Influence Cell Adhesion ........................................................................................................... 49

1.9.1.1  Surface Chemistry ................................................................. 50
2.3.1 Poly (NIPAm-co-NtBAm), poly (NIPAm-co-AcBzPh) and poly (NIPAm-co-NtBAm-co-AcBzPh) .................................................. 105
2.3.2 Poly (NIPAm-co-ODMA) and poly (NIPAm-co-NtBAm-co-ODMA) .................................................................................. 106

2.4 Polymer Characterization .................................................................................. 108
2.4.1 Gel Permeation Chromatography (GPC) ........................................... 108
2.4.2 Lower Critical Solution Temperature (LCST) ............................... 108

2.5 Physical Characterization ............................................................................... 109
2.5.1 Atomic Force Microscopy (AFM) ...................................................... 109
2.5.2 Roughness .......................................................................................... 111
2.5.3 Thickness-AFM Scratch Method ...................................................... 112
2.5.4 Profilometry ......................................................................................... 112
2.5.5 Contact Angle ..................................................................................... 114
2.5.6 Fourier Transform Infrared (FT-IR) Spectroscopy ....................... 119
2.5.7 X-ray photoelectron spectroscopy (XPS) ....................................... 122

2.6 Cell Culture Techniques .................................................................................. 123
2.6.1 Defrosting Cryo-Preserved Cells ...................................................... 123
2.6.2 Cryo-Preservation of Cells ............................................................... 123
2.6.3 Subculture of 3T3 Fibroblast Cells .................................................... 124
2.6.4 Preparation of Human Pulmonary Microvascular Endothelial Cell Media .............................................................................. 124
2.6.5 Subculture of HPMEC .................................................................... 125
2.6.6 Seeding Cells on Thermoresponsive Polymer Films ..................... 125

2.7 Quantitative Assessment of Cell Growth ..................................................... 126
2.7.1 Quant-iT™ PicoGreen® Assay ............................................................ 126
2.7.2 alamarBlue™ assay ........................................................................ 128
2.7.3 Trypan Blue Viability Studies ...................................................... 129

2.8 Temperature Induced Cell Detachment and Imaging Techniques ................................................................. 129

2.8.1 Temperature Controlled Cell Detachment ...................... 129
2.8.2 Scanning Electron Microscopy .......................... 130
2.8.3 Transmission Electron Microscopy .................. 135
2.8.4 Fluorescent Microscopy .......................... 138

2.9 Statistical Analysis ................................................................. 140

2.10 References ................................................................. 141

3 Nanometre scale physically adsorbed thermoresponsive films for cell culture ......................................................... 143

3.1 Introduction ......................................................................... 143

3.2 Materials and Methods .......................................................... 146

3.2.1 Materials ................................................................. 146
3.2.2 Polymer Synthesis and Characterization .................. 146
3.2.3 Film Preparation ................................................................. 147
3.2.4 Physical Characterization ................................................................. 148

3.2.4.1 Atomic Form Microscopy (AFM) .......................... 148
3.2.4.2 Thickness .................................................................. 148
3.2.4.3 Contact Angle ................................................................. 148
3.2.4.4 X-Ray Photoelectron Spectroscopy (XPS) ........... 149
3.2.4.5 LCST determination .................................................. 149

3.2.5 Cell Culture .................................................................. 149
3.2.6 Cell Detachment and Imaging ................................................................. 150
3.2.7 Cell Activity Assays ................................................................. 150
3.2.8 Transmission Electron Microscopy (TEM) ............... 150
3.2.9 Scanning Electron Microscopy (SEM) .................. 151
3.2.10 Fluorescence Microscopy ........................................... 151

3.3 Results and Discussion ................................................... 153
3.3.1 Physical Characterization ............................................ 153
3.3.2 Biocompatibility assessment ......................................... 163

3.4 Conclusions ........................................................................ 181

3.5 References .......................................................................... 183

4 Fabrication and Application of Photocrosslinked, 
Nanometer-Scale, Physically Adsorbed Hydrogels for 
Tissue Culture ........................................................................ 188

4.1 Introduction ........................................................................ 188

4.2 Materials and Methods ....................................................... 191
4.2.1 Materials ........................................................................ 191
4.2.2 Polymer Synthesis and Characterization ...................... 191
4.2.3 Film Preparation ............................................................. 192
4.2.4 Physical Characterization .............................................. 193
4.2.5 Atomic Form Microscopy (AFM) ................................. 193
4.2.6 X-Ray Photoelectron Spectroscopy (XPS) ..................... 193
4.2.7 Contact Angle ................................................................. 193
4.2.8 Thickness ............................................................ 193
4.2.9 LCST measurements ...................................................... 194
4.2.10 Cell Culture ................................................................. 194
4.2.11 Cell Detachment and Imaging ..................................... 194
4.2.12 Cell Activity Assays ..................................................... 195
4.2.13 Transmission Electron Microscopy (TEM) ................. 195
4.2.14 Scanning Electron Microscopy (SEM) ......................... 195
4.2.15 Fluorescence Microscopy ........................................... 196

4.3 Results and Discussion ....................................................... 197
4.3.1 Physical Characterization ..................................................... 197
4.3.2 Biocompatibility Assessment ................................................. 205
4.4 Conclusions .................................................................................. 221
4.5 References .................................................................................... 222

5 Development and Characterization of Poly (NIPAm-co-ODMA) and Poly (NIPAm-co-NtBAm-co-ODMA) Thermoresponsive Films and Determination of their Biocompatibility ......................................................... 225

5.1 Introduction ...................................................................................... 225
5.2 Materials and Methods .................................................................. 227
5.2.1 Materials ....................................................................................... 227
5.2.2 Polymerization ............................................................................... 227
5.2.3 Spin Coating .................................................................................... 228
5.2.4 Atomic Force Microscopy (AFM) ................................................ 228
5.2.5 Advancing Contact Angle ............................................................ 229
5.2.6 FT-IR .............................................................................................. 229
5.2.7 Profilometry .................................................................................... 229
5.2.8 Cell Culture .................................................................................... 230
5.2.9 Cell adhesion promoter coating .................................................... 230
5.2.10 Cell Assays .................................................................................... 231
5.3 Results and Discussion .................................................................. 231
5.3.1 Physical Characterization .............................................................. 231
5.3.2 Biocompatibility Assessment ....................................................... 237
5.4 Conclusion ....................................................................................... 244
5.5 References ....................................................................................... 245

6 General Conclusions ........................................................................ 249

7 Appendix I ......................................................................................... 252
Table of Figures

Figure 1.1 Phase transition of pNIPAm. A) The polymer is in its extended or water soluble phase and the temperature is below 32 °C (<LCST). B) The temperature is above 32 °C (>LCST); the polymer has adopted a globular or condensed state and is no longer water soluble. ................................................................. 3

Figure 1.2 Structure of leucine on the left and pNIPAm on the right. ...................... 4

Figure 1.3: Cartoon depicting standard plasma polymerization system [39]. ............ 9

Figure 1.4: Types of physisorption isotherms [50]. ........................................... 14

Figure 1.5: 5 × 5 μm AFM image in air of glass substrate after adsorption (10 min) of PNIPAAM from a 2 g/L solution at 50 °C. The central zone (1 × 1 μm) was scraped before the image was recorded. Vertical scale: 10 nm. Section was taken at the place indicated by the dotted line [68]. ................................................................. 23

Figure 1.6: AFM images in air of glass substrata (a, c, 2×2 μm; b, d, 5×5 μm) after adsorption (24 hrs) of PNIPAAM from a 0.02 g/L solution (a, b) and a 2 g/L solution (c, d) at 50 °C. The central zone (1 × 1 μm) of b and d was scraped before the image was recorded. Vertical scale: 5 nm (a), 10 nm (b, d), or 30 nm (c). Cross sections were taken at the places indicated by the dotted lines [68]. ................................. 24

Figure 1.7 Plots of the amount of pNIPAm on Aerosill130 (hydrophilic silica) (squares) and R-972 (circles) (hydrophobic silica) surfaces as a function of temperature [44]. ........................................................................................................ 26

Figure 1.8: DLS-measured conformational transition curves for PNIPAM chains absorbed on the surfaces of pNtBAm latex particles. The filled squares are the data measured on increasing the temperature and the empty squares are those on cooling. The empty triangles are the data measured for the sample heated first from a low to a high temperature and then kept at room temperature for about two months. The crosses are the data measured for uncoated pNtBAm latex particles. (a) Temperature dependence of the diameter for the pNtBAm latex particles coated by adsorbed
PNIPAM chains. (b) Temperature dependence of the layer thickness of the interfacial PNIPAM chains [69].

Figure 1.9: Schematic representations not to scale of the conformational changes of adsorbed pNIPAm chains at interfaces. (a) Loopy adsorption of chains at low temperatures before heating; (b) Globular conformational adopted on heating; and (c) extended brush-like conformation on cooling heated sample below the \( \theta \)-temperature [69].

Figure 1.10: Time constant of adsorption kinetics versus temperature (dashed lined). Guide for the eye; (solid line) Temperature dependence of the hydrodynamic radius of pNIPAm [71].

Figure 1.11: Relationship between the time constant and pNIPAm concentration [71].

Figure 1.12: Cartoon illustrating the different modes of pNIPAm physical adsorption above and below the LCST on gold electrodes as defined by Zhu et al [71].

Figure 1.13: Temperature dependence of the average hydrodynamic radius \(<R_h>\) of pNIPAm-co-MACA mesoglobules formed in a gradual heating process, where each data point was obtained after the temperature equilibrium was reached and the copolymer concentration is \( \sim 10^{-5} \) g/ml [72].

Figure 1.14: Temperature dependence of the hydrodynamic radius \(<R_h>\)

Figure 1.15: Retention of poly (NIPAm-co-NtBA) (60:40) (poly 64) on various substrates after adsorption at 37 °C [73].

Figure 1.16: Kinetics and phases of cell adhesion [92].

Figure 1.17: Schematic illustration depicting a) traditional cell detachment using ....
Figure 2.1: Chemical structure of a) N-isopropylacrylamide, b) N-tert-butylacrylamide, c) acrylamidebenzophenone and d) octadecyl methacrylate

Figure 2.2: Schematic of an AFM [7]

Figure 2.3: Veeco dimensions 3100 AFM (digital instruments) located in the NCBES in NUI Galway

Figure 2.4: Optical schematic of interference microscope used for measurement of a sample surface [10]

Figure 2.5: A) advancing contact angle measurements, b) receding contact angle measurements. A new boundary line is formed as the drop advances or recedes and a new contact angle is formed each time [13]

Figure 2.6: Experimental setup for axisymmetric drop shape analysis [15]

Figure 2.7: Homemade goniometer used for advancing contact angle measurements, UCD school of chemistry and chemical biology

Figure 2.8: Components of an FT-IR [16]

Figure 2.9: Cartoon showing the inside of a SEM [19]

Figure 2.10: Schematic illustrating the effect of electron bombardment on a sample. The backscattered and secondary electrons are of interest in this investigation [20]

Figure 2.11: Components of a TEM [22]

Figure 3.1: Schematic illustrating the differences in film roughness for high molecular weight (left hand side of illustration) and low molecular weight (right hand side of illustration) polymers deposited above the polymers LCST. There are two principle modes of polymer collapse. In the case of high molecular weight polymers the various chains collapse prior to the chain to globule collapse, giving rise to a mesoglobule.
Intra-chain collapse, which low molecular weights polymers undergo, results in the formation of particles that are much smaller and are dependent only on the molecular weight of the polymer. It is thought that this difference in conformation affects the amount of polymer adsorbed and also the roughness of the deposited surface.

Figure 3.2: Dashed trace - Advancing water contact angle of physically

Figure 3.3 : Percentage metabolic activity of HPMEC grown on thermoresponsive polymer films compared to HPMEC grown on the TCP controls after 4 days of incubation. Percentage metabolic activity was measured using the alamarBlue™ assay kit. The error bars refer to standard deviation where 3 separate samples of each type of substrate were used and assays were performed in triplicate. The astrix denote statistical significance.

Figure 3.4: Percentage DNA of HPMEC grown on various thermoresponsive polymer films compared to HPMEC grown on the TCP control after 4 days of incubation. The percentage DNA content of HPMEC was determined using a Quant-iT™ PicoGreen® kit. The error bars refer to the standard deviation where 3 separate samples of each type of substrate were used and assays were performed in triplicate. The astrix denote statistical significance.

Figure 3.5: Monolayer of Human Pulmonary Microvascular Cells

Figure 3.6: SEM image of a HPMEC sheet detaching from, poly (NIPAm-co-NtBAm) < LCST film. Cell detachment was initiated using temperature control prior to sample preparation for SEM viewing.

Figure 3.7: TEM image of a) overview of HPMEC grown on Thermanox™, scale bar 2 µm  b) overview of HPMEC grown on poly (NIPAm-co-NtBAm) < LCST, scale bar 2 µm  c) mitochondria in HPMEC grown on Thermanox™, scale bar 500 nm  and d) mitochondria in HPMEC grown on poly (NIPAm-co-NtBAm) < LCST, scale bar 500 nm. An incubation period of 4 days was used.
Figure 3.8: The black arrows in the TEM images highlight the following organelles observed in the HPMEC a) caveolae in HPMEC grown on Thermanox™, b) caveolae in HPMEC grown on poly (NIPAm-co-NtBAm) < LCST, c) Golgi apparatus in HPMEC grown on Thermanox™ and d) Golgi apparatus in HPMEC grown on poly (NIPAm-co-NtBAm) < LCST. The mentioned organelles are indicative of cell health. The scale bar is 500 nm in all of the above images. An incubation period of 4 days was used. .................................................................176

Figure 3.9: The black arrows in the TEM images highlight the followings organelles a) lysosome in a HPMEC grown on Thermanox™, b) lysosome in a HPMEC grown on poly (NIPAm-co-NtBAm) < LCST, c) cell junction in HPMEC grown on Thermanox™ and d) cell junction in HPMEC grown on poly (NIPAm-co-NtBAm) < LCST. The presences of the mentioned organelles are indicative of cell health and cell sheet integrity. The scale bar is 500 nm in all of the above images. An incubation period of 4 days was used.................................................................177

Figure 3.10: HPMEC grown on a) poly (NIPAm-co-NtBAm) < LCST.............178

Figure 3.11: HPMEC grown on a) uncoated Ibidi™ dish (polymer control X 40 Scale bar 56 µm, b) uncoated Ibidi™ dish (polymer control) X 100 Scale bar 22 µm, c) poly (NIPAm-co-NtBAm) < LCST (primary antibody control) X40 Scale bar 56 µm and d) poly (NIPAm-co-NtBAm) <LCST (secondary antibody control) X40 scale bar 56 µm. Red – paxillin staining, green- F-actin staining and blue- nuclear staining. The initial incubation period was 4 days after which the cells were detached and transferred to a new polymer coated substrate and an incubation period of 1 day was used. .......179

Figure 4.1: Advancing water contact angle of physically adsorbed poly (NIPAm-co-AcBzPh) < LCST .................................................................202

Figure 4.2 : Advancing water contact angle of physically adsorbed poly (NIPAm-co-NtBAm-AcBzPh) < LCST .................................................................203

Figure 4.3: Percentage metabolic activity of HPMEC grown on the prepared thermoresponsive polymer films compared to HPMEC grown on TCP controls after 4
days of incubation. Percentage metabolic activity was measured using the alamarBlue™ assay kit. The error bars refer to standard deviation where 3 separate samples of each type of substrate were used and assays were performed in triplicate. The asterisk denotes statistical significance.

Figure 4.4: Percentage DNA of HPMEC grown on various thermoresponsive polymer films compared to HPMEC grown on the TCP control after 4 days of incubation. The percentage DNA content of HPMEC was determined using a Quant-iT™ PicoGreen® kit. The error bars refer to the standard deviation where 3 separate samples of each type of substrate were used and assays were performed in triplicate. The asterisk denotes statistical significance.

Figure 4.5: Monolayer of Human Pulmonary Microvascular Cells

Figure 4.6: Monolayer of Human Pulmonary Microvascular Cells (HPMEC) growth on a) poly (NIPAm-co-AcBzPh) < LCST b) Cell detachment from poly (NIPAm-co-AcBzPh) < LCST. Seeding density of 20,000 cell/cm² and 4 days incubation. Scale bar 200 µm.

Figure 4.7: SEM image of a HPMEC cell sheet detaching from a poly (NIPAm-co-NtBAm-co-AcBzPh) < LCST film after 4 days of incubation. Cell detachment was initiated using temperature control prior to sample preparation for SEM viewing. Scale bar 200 µm.

Figure 4.8: TEM image of a) overview of HPMEC grown on poly (NIPAm-co-NtBAm-co-AcBzPh) < LCST scale bar 2 µm b) overview of HPMEC grown on poly (NIPAm-co-AcBzPh) < LCST scale bar 2 µm c) mitochondria in HPMEC grown on poly (NIPAm-co-NtBAm-co-AcBzPh) < LCST scale bar 500 nm and d) mitochondria in HPMEC grown on poly (NIPAm-co-AcBzPh) < LCST scale bar 500 nm. An incubation period of 4 days was used.

Figure 4.9: The black arrows in the above TEM images are highlighting the respective organelles in HPMEC a) lysosomes in HPMEC grown on poly (NIPAm-co-NtBAm-co-AcBzPh) < LCST, b) lysosomes in HPMEC grown on poly (NIPAm-co-AcBzPh) <
LCST, c) Golgi apparatus in HPMEC grown on poly (NIPAm-co-NtBAm-co-AcBzPh) < LCST and d) Golgi apparatus in HPMEC grown on poly (NIPAm-co-AcBzPh) < LCST. The scale bar is 500 nm in all of the above images. An incubation period of 4 days was used.

Figure 4.10: The black arrows in the above TEM images are highlighting the respective organelles: a) caveolae in a HPMEC grown on poly (NIPAm-co-NtBAm-co-AcBzPh) < LCST, b) caveolae in a HPMEC grown on poly (NIPAm-co-AcBzPh) < LCST, c) cell junction in HPMEC grown on poly (NIPAm-co-NtBAm-co-AcBzPh) < LCST and d) cell junction in HPMEC grown on poly (NIPAm-co-AcBzPh) < LCST. The scale bar is 500 nm in all of the images above. An incubation period of 4 days was used.

Figure 4.11: HPMEC grown on a) poly (NIPAm-co-NtBAm-co-AcBzPh) < LCST X 40 Scale bar 56 µm, b) poly (NIPAm-co-NtBAm-co-AcBzPh) < LCST X100 Scale bar 22 µm, c) poly (NIPAm-co-AcBzPh) < LCST X40 Scale bar 56 µm and d) poly (NIPAm-co-AcBzPh) < LCST X 100 Scale bar 22 µm. Red – paxillin staining, green- F-actin staining and blue- nuclear staining. The initial incubation period was 4 days after which the cells were detached and transferred to a new polymer coated substrate and an incubation period of 1 day was used.

Figure 5.1: Advancing water contact angle of poly (NIPAm-co-ODMA (0.5 %))......

Figure 5.2: Advancing water contact angle poly (NIPAm-co-ODMA (0.75 %))....

Figure 5.3: Advancing water contact angle poly (NIPAm-co-NtBAm-co-ODMA)......

Figure 5.4: Percentage metabolic activity of 3T3 grown on various thermoresponsive polymer films compared to 3T3 grown on the TCP (tissue culture polystyrene) control after 48 hours of incubation. The error bars refer to standard deviation where 3 separate samples of each type of substrate were used and assays were performed in triplicate. The astrix denotes statistical significance.
Figure 5.5: Percentage DNA of 3T3 grown on various thermoresponsive polymer films compared to 3T3 grown on the TCP control after 48 hours of incubation. The percentage DNA content of the 3T3 was determined using a Quant-iT™ PicoGreen® kit. The error bars refer to the standard deviation where 3 separate samples of each type of substrate were used and assays were performed in triplicate. The asterix denotes statistical significance. ..........................................................239

Figure 5.6: 3T3 grown on (a) uncoated poly (NIPAm-co-ODMA (0.75 %)), (b), collagen coated poly (NIPAm-co-ODMA (0.75 %)), (c) uncoated poly (NIPAm-co-NtBAm-co-ODMA) and (d) collagen coated poly (NIPAm-co-NtBAm-co-ODMA). Seeding density of 40,000 cell/cm^2 and an incubation period of 48 hours were used. Scale bar 500 µm. ..........................................................243
Table of Tables

Table 1.1: LCST’s of various thermoresponsive polymers [11]................................. 4

Table 1.2: Advantages and Disadvantages of commonly used pNIPAm film preparation methods ......................................................................................................................................................................................... 11

Table 1.3: Contact angle measurements of pNIPAm coatings produced by different methods ................................................................................................................................................................................... 75

Table 1.4: Thickness measurements of pNIPAm coatings produced by different methods. .......................................................................................................................................................................................... 79

Table 2.1: Substrate type used during film preparation and application .... 103

Table 2.2: PicoGreen® Standard Curve Calculations ............................................ 127

Table 3.1: Roughness measurements for the physically adsorbed films measured using AFM in tapping mode. Three samples of each surface type were analyzed in three random areas. ........................................................................................................... 153

Table 3.2: Thickness of the physically adsorbed films measured using AFM in scratch mode. Three samples of each surface type were scratched in three random areas and fifteen thickness measurements were taken from each scratch. ................. 156

Table 3.3: Advancing water contact angles for physically adsorbed films taken at 40 °C. Three samples of each surface type were measured. ....................................................... 159

Table 3.4: Relative atomic composition of physically adsorbed films compared with the stochiometrically calculated composition of the NIPAm monomer and the poly (NIPAm-co-NtBAm) monomer ........................................................................................................... 161

Table 3.5: Detachment times for HPMEC grown on various thermoresponsive polymer films. Cell detachment was initiated by lowering the temperature of the sample below
the particular polymer’s LCST. Three samples of each surface type were measured
........................................................................................................................................171

Table 4.1: Roughness measurements for the physically adsorbed films measured using AFM in tapping mode. Three samples of each surface type were analyzed in three random areas. ........................................................................................................................................197

Table 4.2: Thickness of the physically adsorbed films measured using AFM in scratch mode. Three samples were scratched in three random areas and fifteen thickness measurements were taken from each scratch. ........................................................................................................................................199

Table 4.3: Advancing water contact angles for physically adsorbed films taken at 40 °C. Three samples of each surface type were measured ........................................................................................................................................200

Table 4.4: Relative atomic composition of physically adsorbed films compared with the stochiometrically calculated composition of the NIPAm-co-AcBzPh monomer and the NIPAm-co-NtBAm-co-AcBzPh monomer ........................................................................................................................................204

Table 4.5: Detachment times for HPMEC grown on various thermoresponsive polymer films. Cell detachment was initiated by lowering the temperature of the sample below the particular polymer’s LCST. Three samples of each surface type were measured ........................................................................................................................................211

Table 5.1: Roughness measurements for the thermoresponsive polymer films measured using atomic force microscopy (AFM). Three samples of each surface type were analyzed in three random areas. ........................................................................................................................................232

Table 5.2: Thickness measurements for the thermoresponsive polymer films measured using profliometry. Three samples were scratched in three random areas and three thickness measurements were taken from each scratch ........................................................................................................................................232

Table 5.3: Measured advancing contact angle data for the thermoresponsive polymer films. Contact angles were reordered at 40 °C. Three samples of each surface type were measured. ........................................................................................................................................233
List of Abbreviations

3T3; 3 Day Transfer
AcBzPh; Acrylamidobenzophenone
AcCN; Acetonitrile
ADSA; Axisymmetric Drop Shape Analysis
AFM; Atomic Force Microcopy
AIBN; Azobisisobutyronitrile
ATP; Adenosine Triphosphate
ATR; Attenuated Total Reflectance
BAEC; Bovine Carotid Artery Endothelial Cells
BET; Brunauer, Emmett and Teller
BSA; Bovine Serum Albumin
CA; Cellulose Acetate
CAMs; Cell Adhesion Molecules
CAP; Cell Adhesion Promoter
CCD; Charge Coupled Device
CD80; Cluster of Differentiation
CLRGP; Controlled Living Radical Polymerization
CTA; Chain Transfer Agent
DLS; Dynamic Light Scattering
DMEM; Dulbecco’s Modified Eagle Medium
DMF; N,N-dimethylformamide
DMSO; Dimethyl Sulfoxide
DNA; Deoxyribonucleic Acid
DSC; Differential Scanning Calorimetry
EBP; Electron Beam Polymerization
ECM; Extracellular Matrix
EDTA; Ethylenediaminetetraacetic acid
EHEC; Ethylhydroxyethylcellulose
FAK; Focal Adhesion Kinase
FBS; Fetal Bovine Serum
FCS; Fetal Calf Serum
FEG; Field Emission Gun
FEP; Hexafluoropropylene-tetrafluoroethylene
Fg; Bovine Fibrinogen
FN; Fibronectin
FT-IR; Fourier Transform Infrared Spectroscopy
GA-FTIR; Grazing Angle Infrared Spectroscopy
GPC; Gel Permeation Chromatography
HAS; Human Serum Albumin
HBSS; Hank's Balanced Salts Solution
hMSCs; Human Mesenchymal Stem Cells
HPC; Hydroxypropylcellulose
HPM; Hydroxypropyl Methacrylate
HPMEC; Human Pulmonary Microvascular Endothelial Cells
HUVEC; Human Umbilical Vein Endothelial Cells
LCST; Lower Critical Solution Temperature
LDPE; Low Density Polyethylene
LLS; Static Light Scattering
MACA; Methacryloyl-amino-ethylene-3α, 7α, 12α-trihydroxy-5β-cholanoamide
MC3T3-E1; Murine Osteoblasts
MC; Methylcellulose
MDCK; Madin-Darby Canine Kidney Cells
MHCII; Major Histocompatibility Complex Class II
Mn; Number Average Molecular Weight
Mw; Weight Average Molecular Weight
NGS; Normal Goat Serum
NMR; Nuclear Magnetic Resonance
ODMA; Octadecyl Methacrylate
PBMEAm; Poly (N, N-bis (2-methoxyethyl) acrylamide)
PBS; Phosphate Buffered Saline
PCS; Photon Correlation Spectroscopy
PDI; Polydispersity Index
PDMA; Poly ((2-dimethylamino) ethyl methacrylate)
PEA; Poly (N-ethylacrylamide)
PEMA; Poly (N,N-ethylmethacrylamide)
PEOZ; Poly (2-ethyl-2-oxazoline)
PEPA; Poly (ethoxypropylacrylamide)
PEPM; Polyethylpyrrolidone methacrylate
PET; Polyethyleneetherphalate
PIPOZ; Poly (2-isopropyl-2-oxazoline)
PLGA; Poly (lactic-co-glycolic acid)
PLLA; Poly (L-lactic acid)
PMBA; Poly (butyl methacrylate)
PMPAm; Poly (N-(3-methoxypropyl) acrylamide)
PNIPAm; Poly (N-isopropylacrylamide)
PNIPAm; Poly N-(isopropylacrylamide)
PNPAm; Poly (N-n-propylacrylamide)
PNtBAm; Poly N-tertbutylacrylamide
PPO; Poly (propylene oxide)
PS; Polystyrene
PVCL; Poly (N-vinylcaprolactam)
PVME; Poly (vinyl methyl ether)
QCM-D; Quartz Crystal Microbalance with Dissipation
Ra; Arithmetic Mean
RAFT; Reversible Addition Fragmentation Chain Transfer Polymerization
RAW 264.7; Mouse Macrophage
RER; Rough Endoplasmic Reticulum
R_h; Hydrodynamic Radius
RI; Refractive Index
RMS; Route Mean Square
SAMs; Self-assembled Monolayers
SEM; Scanning Electron Microscopy
SI-ATRP; Surface Atom Transfer Radical Polymerization
SMC; Smooth Muscle Cell
SPM; Scanning Probe Microscopy
TCP; Tissue Culture Polystyrene
TE Buffer; “T ten E one buffer” Tris (Trisaminomethane)-EDTA buffer
TEM; Transmission Electron Microscopy
TEOS; Tetraethyl Orthosilicate
TGA; Thermal Gravimetric Analysis
T_g; Glass Transition Temperature
TMSPM; 3-(trimethoxysilyl) propyl methacrylate
UCST; Upper Critical Solution Temperature
XPS; X-ray Photoelectron Spectroscopy
ΔR_ω(θ); Excess Rayleigh Ratio
Declaration

The work in this thesis is based on the research carried out at the National Centre for Biomedical Engineering (NCBES), School of Chemistry and School of Anatomy, National University of Ireland Galway.

I, Deirdre Healy, hereby certify that this thesis has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a degree or qualification.
Acknowledgements

I’m extremely fortunate to have a large number of people to thank here and I wouldn’t have made it through this without all of you. I would like to thank my supervisors, Dr Yury Rochev, Prof Peter Dockery and Dr William Carrol for their support and guidance over the course of my PhD. It was a privilege to work with all of you. I’d like to thank the Irish research Council and the College of Science for their financial help over the course of my PhD.

To the past and present members of the biomaterials group, Jennifer, Fan, Rongbing, Olga, Nina, Karl and Reyhannah I would like to thank you for your friendship, advice and for helping me get to grips with cell culture when I was starting out. Dr Alexander Gorelov I’d like to thank you for reading my manuscripts, synthesizing polymer and answering my numerous questions throughout the years. Dr Eadaoin Timmins a big thank you for always helping me with all of my microscopy queries, your support has made all the difference. Dr Maria Nash I feel as if you should get credit for being another supervisor, thank you for reading pretty much everything I’ve written since I started my PhD, your advice, support and friendship have been invaluable. Dr Kerry Thompson you’ve taught me so much over the past few years, thank you. Pierce Lalor I’d like to thank you for sharing your knowledge of microscopy with me. I’d like to say a huge thank you to the all of the staff in the school of chemistry, school of anatomy, NCBES and Biosciences. To my students Julia, Awatif and Sarah thank you for just being you and for giving me the opportunity to mentor you.

To my parents Lourda and Timothy and my siblings Michael and Fiona thank you for always believing in me, for giving me a soft place to land when things got tough, and most importantly for the love and hugs over the years. To my other family, Nora, Cecelia, Ciara, Amy, Sinead, Niamh and Karla thank you for picking me up and dusting me off and for the unconditional love and support. I would like to thank the staff of the university health unit, the university hospital and all of my medical team; you people are absolutely amazing. I would also like to thank you whomever you are for taking the time to read this.
Abstract

The current state of art in cell detachment involves the use of digestive enzymes, such as trypsin, or mechanical disaggregation; both of these methods cleave cell-cell junctions yielding either individual cells or clusters of cells. Temperature mediated cell detachment can be used to detach cell sheets with intact cell-cell junctions and synthesized extracellular matrix. These cell sheets can be used for a variety of purposes depending on the cell type which has been cultured. This body of work focuses on the use of poly N-(isopropylacrylamide) (pNIPAm) and pNIPAm copolymers as temperature sensitive substrates for cell culture. pNIPAm undergoes a hydrophilic to hydrophobic transition at 32°C. The temperature at which this transition takes place is known as the lower critical solution temperature or LCST. Cells can be cultured on pNIPAm films above the LCST, when the polymer is in its hydrophobic state. Once the desired level of cell growth has been reached the cells can be detached by lowering the temperature below the polymers LCST. This reduction in temperature causes the polymer to dissolve if the polymer layer is not bound to the underlayer substrate or swell if the polymer is bound to the underlayer substrate.

Numerous methods have been used to produce pNIPAm films for cell culture and these include: solvent casting, spin coating, spray coating, dip coating, plasma polymerization, electron beam polymerization, surface initiated atom transfer radical polymerization (SI-ATRP) and reversible addition fragmentation chain transfer (RAFT). This body of work will mainly focus on physical adsorption as a method of film formation. It has been shown that physical adsorption can be used to produce polymer films but the cell culture potential of these films has not been investigated. Physical adsorption is an attractive method of film formation as it is methodologically straightforward, it is a technique which is already used in cell culture to produce coatings of cell adhesion promoter, it can be used to coat surfaces with complicated geometric profiles and it can be used to create nano-meter thick polymer films. It is believed that there is a thickness threshold above which pNIPAm films will no longer be conducive to cell adhesion and proliferation; as a result films thicker...
than 30 nm are thought to be unsuitable as cell culture substrates. While there are some exceptions to this thickness threshold such as plasma polymerized pNIPAm film and spin coated films of pNIPAm homopolymer, the ability to produce nanometre thick films via physical adsorption is advantageous.

Four different polymers were used in this investigation, commercially sourced pNIPAm which has the advantage of polymerization circumvention. Poly (NIPAm-co-N-tertbutylacrylamide (NtBAm)) is a more hydrophobic copolymer of pNIPAm which possess an LCST of 16°C, it is thought that more hydrophobic pNIPAm copolymers perform better in terms of cell growth and this hypothesis will be investigated here. Poly (NIPAm-co-NtBAm)’s lower LCST minimizes the risk of pre-mature cell detachment during routine cell observation. Poly (NIPAm-co-acrylamide benzophenone (AcBzPh)) is a UV sensitive pNIPAm copolymer, which is capable of forming covalent bonds to the underlayer substrate upon exposure to UV light; this means that upon temperature reduction the polymer film will simply swell rather than dissolving. The final polymer is poly (NIPAm-co-NtBAm-co-AcBzPh) which possesses the advantages of both poly (NIPAm-co-NtBAm) and poly (NIPAm-co-AcBzPh). Polymer films were formed both above and below the chosen polymers LCST in order to determine whether or not the temperature induced changes in the polymers’ conformation and hydrophobicity affected the films physical and chemical properties and their ability to successfully host cells. In total, 8 types of films were prepared (4 different polymers physically adsorbed both above and below the polymers’ LCSTs).

The physically adsorbed films were characterized using atomic force microscopy (AFM) (roughness and thickness), x-ray photoelectron spectroscopy (XPS) (percentage composition), and advancing contact angle (surface wettability). Gel permeation chromatography (GPC) was used to determine the molecular mass of the polymers. Human pulmonary microvascular endothelial cells (HPMEC) were seeded on the prepared films and cell viability was assessed qualitatively using light microscopy and quantitatively using cell viability assays. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used to investigate the surface of the cell sheets and cellular junctions respectively. Fluorescence microscopy was used to assess the re-adhesion
potential of cells and cell sheets grown and detached from the physically adsorbed thermoresponsive polymer films.

This investigation showed that it is possible to produce thermoresponsive surfaces capable of both cell adhesion and detachment using physical adsorption. HPMEC grew better on the polymer films produced below the chosen polymers LCST; these films were smoother, thinner and more hydrophilic than those prepared above the polymers LCST. SEM and TEM analysis showed that the HPMEC grown and detached from the thermoresponsive polymer films prepared below the LCST were comparable to HPMEC grown on the tissue culture polystyrene (TCP) controls. Fluorescence microscopy showed that the HPMEC which had been detached from the thermoresponsive polymer films were capable of forming focal adhesions on new thermoresponsive polymer substrates.

The second portion of this investigation focused on the use of a novel class of thermoresponsive polymer; the poly (NIPAm-co-octadecyl methacrylate (ODMA)) copolymers. The introduction of the ODMA monomer should prevent polymer dissolution upon temperature reduction below the LCST; instead the polymer will simply swell. This swelling will enable cell detachment. This is advantageous as it will enable the production of thermoresponsive polymer films via physical deposition methods, such as solvent casting and spin coating which swell rather than dissolve upon temperature reduction. It is possible to produce thermoresponsive polymer films, which are bound to the underlayer substrate using physical deposition but this adds an addition step to film preparation as a specialized polymer such as poly (NIPAm-co-AcBzPh) which requires UV exposure in order to form a covenant bond to the underlayer substrate must be used. The use of poly (NIPAm-co-ODMA) copolymers should remove the need for this additional step. Three different polymers were used in this investigation poly (NIPAm-co-ODMA (0.5%)), poly (NIPAm-co-ODMA (0.75%)) and poly (NIPAm-co-ODMA-co-NtBAm). Spin coating was used as the film deposition method, as it is a simple film preparation method which is capable of producing uniform nanometre thick polymer films reproducibly.
The polymer films were characterized using the following techniques; AFM (roughness), profilometry (thickness) and advancing contact angle (surface wettability). 3T3 cells were seeded on the thermoresponsive polymer films and cell viability was assessed quantitatively using cell viability assays and qualitatively using light microscopy. FT-IR was used to confirm successful film formation.

The nano-meter thick thermoresponsive polymer films formed from these polymers via spin coating proved unable to facilitate cell adhesion and proliferation without the addition of a cell adhesion promoter layer. After the addition of a cell adhesion promoter layer the poly (NIPAm-co-ODMA (0.75%)) films proved to be capable of supporting cell adhesion and detachment. Cell viability assays confirmed that the cells grown on the collagen coated poly (NIPAm-co-ODMA (0.75%)) films were comparable to those grown on the TCP controls.
Dedication

I would like to dedicate this work to my family.
1 INTRODUCTION

1.1 SMART POLYMERS

Smart materials have been studied across a variety of disciplines due to their incredible ability to adapt to changes in their environment. This study focuses on the use of smart polymers in order to create cell culture surfaces capable of facilitating cell sheet adhesion and detachment. The majority of polymers display a linear response to stimuli. Smart polymers are unique in that they display a non-linear and rapid response to stimuli [1, 2]. Smart polymers undergo a strong conformational change in response to a change in their environment [3]. The stimulus for this change varies depending on the polymer, e.g. pH, temperature, electric and magnetic fields. Smart polymers have a wide range of applications which include the following; biocatalysts, bio-separation, cell culture, biomimetic actuators, glucose sensors, gene delivery systems and drug delivery [4].

1.1.1 Thermoresponsive Polymers

Thermoresponsive polymers display reversible hydrophobic to hydrophilic transitions in response to a change in temperature. Similar to other smart polymers no change is observed until a critical point is reached. Polymers which display increased solubility in response to an increase in temperature possess an upper critical solution temperature or UCST [5]. There is a second class of polymer whose solubility improves as the temperature is decreased and these polymers possess a lower critical solution temperature or LCST. This body of work will focus on the second class of polymers those which possess an LCST. The critical point for each polymer is solvent and polymer specific and this investigation will focus on polymers which display an LCST in an aqueous environment. The phase transitions that thermoresponsive polymers undergo are due to a change in the balance of the hydrophobic and hydrophilic forces that the polymer experiences [6]. Below the polymers LCST polymer solvent interactions are favored. As the temperature increases intra-polymer and inter-
polymer interactions are favored and the polymer undergoes a transition from a hydrophilic Flory coil to a hydrophobic globule (Figure 1.1) [7, 8].

\[ \Delta G_{\text{mix}} = \Delta H_{\text{mix}} - T\Delta S_{\text{mix}} \] (1)

\( \Delta G_{\text{mix}} \) = The Gibbs free energy of mixing

\( \Delta H_{\text{mix}} \) = The enthalpy of mixing

\( \Delta S_{\text{mix}} \) = The entropy of mixing

\( T \) = Temperature of mixing (Kelvin)

In order for a process to be spontaneous the Gibbs free energy must be negative. Below the LCST the polymer exhibits a large negative \( \Delta H_{\text{mix}} \) due to hydrogen bonding between the polymer and the surrounding water molecules. The polymer is highly ordered in this state which gives rise to a large negative \( \Delta S_{\text{mix}} \) but the large negative \( \Delta H_{\text{mix}} \) means that below the LCST mixing is favored. As the temperature increases the entropic term becomes more dominant until eventually spontaneous demixing takes place [9, 10]. A number of factors can influence the LCST of thermoresponsive polymers for example; the addition of a hydrophobic monomer will decrease the LCST while the introduction of a hydrophilic monomer will increase the LCST. The presence or absence of salt and surfactants, pH, molecular weight and polymer concentration will also affect the LCST [9].
Figure 1.1 Phase transition of pNIPAm. A) The polymer is in its extended or water soluble phase and the temperature is below 32 °C (<LCST). B) The temperature is above 32 °C (>LCST); the polymer has adopted a globular or condensed state and is no longer water soluble.

There are a number of thermoresponsive polymers which possess a LCST; table 1.1 below contains a list of the most commonly investigated polymers. Poly (N-vinylcaprolactam (PVCL) (LCST 25-35 °C), Poly (vinyl methyl ether) (PVME) (LCST 33.8 °C), Poly (N-isopropylacrylamide) (pNIPAm) (LCST 32 °C), Poly (ethoxypropylacrylamide) (PEPA) (LCST ~32 °C) all possess LCST’s close to physiological temperature but pNIPAm is the most widely utilized of the above mentioned polymers in biomedical applications. pNIPAm is the most widely utilized of the above mentioned polymer for a variety of reasons; it copolymerizes easily which enables researchers to adjust the LCST depending on the choice of copolymer, it is non-cytotoxic, its phase transition is close to physiological temperature and it possesses a sharp phase transition. The pNIPAm unit is an isomer of poly-leucine (Figure 1.2) and it is thought that this structural similarity may be the reason why pNIPAm is non-cytotoxic.
Figure 1.2 Structure of leucine on the left and pNIPAm on the right.

Table 1.1: LCST’s of various thermoresponsive polymers [11].

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Name</th>
<th>LCST(°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNIPAm</td>
<td>Poly(N-isopropylacrylamide)</td>
<td>32</td>
</tr>
<tr>
<td>PVCL</td>
<td>Poly(N-vinylcaprolactam)</td>
<td>25-35</td>
</tr>
<tr>
<td>PPO</td>
<td>Poly(propylene oxide)</td>
<td>10-20</td>
</tr>
<tr>
<td>PVME</td>
<td>Poly(vinyl methyl ether)</td>
<td>33.8</td>
</tr>
<tr>
<td>MC</td>
<td>Methylcellulose</td>
<td>60-80</td>
</tr>
<tr>
<td>EHEC</td>
<td>Ethyl(hydroxyethyl)cellulose</td>
<td>65</td>
</tr>
<tr>
<td>PDMA</td>
<td>Poly((2-dimethylamino)ethyl methacrylate)</td>
<td>50</td>
</tr>
<tr>
<td>PEOZ</td>
<td>Poly(2-ethyl-2-oxazoline)</td>
<td>~62</td>
</tr>
<tr>
<td>PIPOZ</td>
<td>Poly(2-isopropyl-2-oxazoline)</td>
<td>~36</td>
</tr>
<tr>
<td>PEA</td>
<td>Poly(N-ethylacrylamide)</td>
<td>82</td>
</tr>
<tr>
<td>PEMA</td>
<td>Poly(N,N-ethylmethylacrylamide)</td>
<td>70</td>
</tr>
<tr>
<td>PNPAm</td>
<td>Poly(N-n-propylacrylamide)</td>
<td>25</td>
</tr>
<tr>
<td>PBMEAm</td>
<td>Poly(N,N-bis(2-methoxyethyl) acrylamide)</td>
<td>49</td>
</tr>
<tr>
<td>PMPAm</td>
<td>Poly(N-(3-methoxypropyl)acrylamide)</td>
<td>&gt;60</td>
</tr>
<tr>
<td>PEPA</td>
<td>Poly(ethoxypropylacrylamide)</td>
<td>~32</td>
</tr>
</tbody>
</table>
1.1.1.1 POLY N-(ISOPROPYLACRYLAMIDE) (PNIPAM)

This body of work will describe the use of pNIPAm and its copolymers as cell culture substrates. pNIPAm and its copolymers are the most extensively studied thermoresponsive polymers. pNIPAm consists of a hydrocarbon backbone, a hydrophilic amide group and a hydrophobic isopropyl group (Figure 1.2). When the temperature is decreased, in aqueous conditions pNIPAm undergoes a sharp phase transition from hydrophobic to hydrophilic at 32 °C (Figure 1.1) [12, 13]. Below the LCST pNIPAm is water soluble and it exists in a relaxed coil conformation (Flory coils) due to hydrogen bonding between the hydrophilic groups in the polymer and the surrounding water molecules [10, 14, 15]. A cage like structure is formed around the isopropyl group by water molecules and this increases polymer solubility [15]. As the temperature is increased the hydrogen bonds between the water molecules and the polymers carboxyl and amide groups break [16, 17]. The previously bound water molecules are extruded and the polymer collapses into a hydrophobic globular conformation [18-20].

1.2 COLD PROTEIN DENATURATION

Cold protein denaturation is similar to the LCST observed in thermoresponsive polymers; both are driven by the hydrophobic effect. A short description of cold protein denaturation is provided below.

Denaturation is the loss of a protein’s tertiary structure. A loss of tertiary structure results in a loss of protein function. Cold protein denaturation is thought to occur for nearly all proteins. Intrinsically disordered proteins which have been shown to be resistant to heat denaturation are also thought to be resistant to cold denaturation. The interactions between the side chains or “R” groups of the amino acids in a proteins primary structure are responsible for a
proteins tertiary structure [21]. Protein denaturation at low temperatures is due to low enthalpy values and this is why cold denaturation is an exothermic process as opposed to heat denaturation which is an endothermic process. Globular proteins undergo cold denaturation because of their large positive heat capacity of unfolding. The hydrophobic effect and the hydration of non-polar amino acids in globular proteins are responsible for the large positive heat capacity of unfolding. As the hydrophobic effect is experienced by all globular proteins it is thought that cold denaturation will be a general phenomenon of globular proteins [22]. For the majority of material interactions, strength between atoms increases as the thermal energy decreases; this is not the case for hydrophobic interactions. As the temperature decreases the strength of hydrophobic interactions decreases, this unusual behavior is due to the interplay between enthalpy and entropy [23]. When the temperature decreases the destabilizing effect of enthalpy increase at the same time as the stabilizing effect of entropy increases, as the temperature decreases the water surrounding the outside of the protein becomes more ordered as the number of hydrogen bonds that are formed increases. The destabilizing effect of enthalpy increases faster than the stabilizing effect of entropy and this is why hydrophobic interactions weaken as the temperature decreases leading to cold denaturation [24].

1.3 FILM PREPARATION METHODS

There are a wide range of techniques which can be used to prepare pNIPAm films, each with its own advantages and disadvantages. A brief review of the each technique is given below.

1.3.1 Spin Coating

Spin coating is widely used in the semiconductor industry in order to create thin films of photoresists on silicon wafers [25]. Spin coating is comprised of four steps; deposition, spin-up, spin-off and evaporation [26, 27]. During the deposition stage solution is deposited onto the slowly spinning substrate. Centripetal force causes the solution to spread on the substrate. No loss of solution is experienced at this stage as the substrate and the solution are co-
rotating and the system is balanced. The spin speed then accelerates rapidly resulting in a loss of solution as the system is no longer balanced. Balance is eventually regained after the volume of solution has lessened to the point where co-rotation is once again possible [28]. A more gradual thinning of the polymer film takes place in the spin off stage. Edge effects begin to form at this point as the centrifugal force is pushing the solution towards the edge of the substrate. A viscous drag force is acting on the solution and this causes a region of thicker film to develop at the edge of the substrate. Solution viscosity, surface tension, and spinning velocity all contribute to the viscous drag force. The solution forms droplets at the edge of the substrate due to surface tension. These droplets may remain on the edge giving rise to the edge bead effect. If the coating solution possesses linear Newtonian viscosity and if coating conditions are optimal it is possible to avoid the edge bead effect. Circular substrates are preferred for spin coating as the edge bead effect is more pronounced on non-circular substrates. The edge bead may be removed by either dissolving it in a suitable solvent or it may be cut off. The edge bead effect does not hamper cell adhesion or detachment from thermoresponsive spin coated surfaces [27, 29]. The final step in spin coating is evaporation. In this step the volatile solvent evaporates leaving behind a solid film of the polymer of interest [27]. The film must be dried in a suitable manner in order to ensure that all of the residual solvent has been removed. Proper drying protocol ensures that the films attain stability [30]. Depending on the spin speed, concentration and choice of solvent, spin coating can be used to prepare pNIPAm films ranging in thickness from 13 nm up to 3000 nm in thickness. Solution concentrations ranging from 0.3 to 20 % w/v of pNIPAm in ethanol have been successfully used to fabricate spin coated films.

1.3.2 Solvent Casting

Solvent casting can be used to produce micrometre thick films. The main advantage of solvent casting is its simplicity. This film deposition method only requires materials which can be found in nearly every laboratory. The production of a solvent cast film requires the following: a suitable substrate, polymer, volatile solvent, a micro-pipette which can be used to spread the polymer solution on the substrate, a dessicator and a vacuum oven for drying the films. When the volatile solvent evaporates it leaves behind a polymer film [31]. Film
thickness is dependent on the volume of solution dispensed and on the concentration and density of the polymer solution.

During the evaporation process voids may form in the film, polymer can fill these voids but if the concentration of polymer is too low divots or holes will be present in the film. Consequently there is a thickness limitation on the formation of solvent cast films [32, 33].

1.3.3 Electron Beam Polymerization (EBP)

During EBP a high energy electron beam is used to polymerize monomers that have been spread on the chosen substrate. The electron beam irradiation of the initiator results in the cleavage of the chemical bonds which gives rise to free radicals. These free radicals initiate the polymerization of the monomer [34]. The advantages of EBP are the high degree of control and ability to perform site specific polymerization [35]. Film thickness can be controlled by varying the energy of the electron beam and the concentration of monomer [36]. It is possible to produce a vapor phase coating use EBP; this is beneficial if the polymer of interest cannot be deposited via one of the wet coating methods [37].

1.3.4 Plasma Polymerization

Plasma polymerization is a one-step solvent free film deposition method. Plasma energy is used to polymerize monomers. The monomers fragment due to exposure to the high energy plasma. The monomers recombine but they may undergo rearrangement and this rearrangement may result in a loss of polymer function [38]. This possible loss of polymer function is the main disadvantage of plasma polymerization [39]. Careful regulation of pressure, temperature, monomer flow rate and reactivity and discharge power will increase the odds of maintaining structural integrity from monomer to polymer [40]. An example of the standard plasma polymerization setup can be seen in Figure 1.3 below.
Studies have shown that it is possible to produce pNIPAm coatings which retain their LCST using plasma polymerization [41, 42]. It has been noted that there is a difference in the swelling ratio of plasma polymerized pNIPAm films and pNIPAm films produced via other deposition methods. Plasma polymerized films are suitable substrates for cell adhesion and detachment [16, 41, 42].

1.3.5 Physical Adsorption

The attractive forces between the adsorbate (polymer solution) and the adsorbent (chosen substrate) are what lead to the formation of a physically adsorbed film. In order to prepare a physically adsorbed film, a solution of the polymer of interest is prepared and this solution is then deposited on the chosen substrate [43]. A period of incubation then follows after which any remaining solution is removed from the substrate. The resultant film is then dried in order
to remove any residual solvent. It is possible to form multi-layer systems using physical adsorption but the layers adsorbed above the initial monolayer are only weakly bound and they may desorb easily. Physical adsorption can be used to prepare ultrathin (nm thick) films. This method of film preparation possesses several advantages; it is a simple coating method which is already utilized in cell culture and it is possible to coat samples with non-planar geometries. The main disadvantage of physical adsorption is the lack of reproducibility due to the lack of control of the shear stress when extracting polymer solution or rinsing the polymer film. Tanashi et al. [44] studied the adsorption of pNIPAm on both hydrophobic and hydrophilic substrates. They observed that adsorption was greater on the hydrophobic substrates, though adsorption on hydrophilic substrates is possible [44]. Investigations into the adsorption of pNIPAm have shown that adsorption is greater above the polymers LCST [45]. Above the LCST polymer-solvent interactions are no longer favored and polymer-polymer interactions dominate resulting in increased adsorption above the LCST [46].
1.4 ADVANTAGES AND DISADVANTAGES OF COMMONLY USED PNIPAM FILM PREPARATION METHODS

Table 1.2: Advantages and Disadvantages of commonly used pNIPAm film preparation methods

<table>
<thead>
<tr>
<th>Method of Film Formation</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spin Coating</td>
<td>Produces uniform films</td>
<td>Only suitable for planar substrates</td>
</tr>
<tr>
<td></td>
<td>Can be used to create nm thick polymer films</td>
<td>Only one side of the substrate can be coated</td>
</tr>
<tr>
<td></td>
<td>Ease of preparation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Good control over polymer surface properties</td>
<td></td>
</tr>
<tr>
<td>Solvent casting</td>
<td>Simple technique</td>
<td>Only suitable for planar substrates</td>
</tr>
<tr>
<td></td>
<td>No investment in equipment necessary</td>
<td>Limitation in terms of thickness</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lengthily drying times depending on film thickness and solvent used</td>
</tr>
<tr>
<td>Electron Beam</td>
<td>Site specific polymerization is possible.</td>
<td>Restrictions in terms of substrate material and geometry.</td>
</tr>
<tr>
<td></td>
<td>Produces nm thick films in a highly controlled manner</td>
<td>It is difficult to control the density, macromolecular morphologies and molecular weight of the</td>
</tr>
<tr>
<td></td>
<td>Film is covalently bound to the substrate</td>
<td></td>
</tr>
</tbody>
</table>
As is evident from table 1.2 all of the discussed methods of thermoresponsive polymer film preparation possess their own advantages and disadvantages. When selecting a particular film preparation method it is important to consider the type of film produced (grafted or non-grafted) and the accessibility and practicality of each technique. The two film preparation methods used in this investigation are physical adsorption and spin coating. Physical adsorption was selected as it is an operationally simple method of producing polymer coating which is already utilized in cell culture. Spin coating can be used to produce uniform nanometer thick thermoresponsive polymer films rapidly.

### 1.5 ADSORPTION

Adsorption may be classified as either physical (physisorption) or chemical (chemisorption). The enthalpy change associated with physical adsorption is typically of the order of 20 kJ mol\(^{-1}\). The interaction between the adsorbate and the substrate in physical adsorption is due to Van der Waals interaction and as a result physical adsorption is also referred to as Van der Waals adsorption [47, 48]. During physisorption molecules retain their primary structure as the energy of physisorption is not large enough to break the bonds between the atoms. The
enthalpy of chemisorption is of the order of 200 kJ mol\(^{-1}\) and the molecules adsorb by forming chemical bonds with the surface of the substrate. During chemisorption molecules may break apart in order to satisfy the valencies of surface atoms [47, 48]. Chemisorption usually yields monolayer coverage but it is possible to form multilayer systems using physisorption.

1.5.1 Adsorption Isotherms

Adsorption isotherms are graphs showing the change in fractional surface coverage with the change in pressure. Figure 1.4 shows the IUPAC physisorption isotherms. Fractional surface coverage, (theta) \(\theta\) is defined as the

\[
\theta = \frac{\text{number of adsorption sites occupied}}{\text{number of adsorption sites available}}
\]

Isotherms are based on a number of assumptions. The list below contains the assumptions upon which the Langmuir isotherm is based;

1. Adsorption cannot proceed past monolayer coverage
2. All adsorption sites are equivalent and the surface is uniform
3. There are no interactions between the adsorbed molecules and as such the ability of a molecule to adsorb at a particular site is not governed by the occupation of neighboring sites.

In this body of work will discuss the Langmuir and Brunauer, Emmett and Teller (BET) isotherms as these are most applicable to the physisorption of pNIPAm and pNIPAm derivatives [48, 49].
1.5.1.1 LANGMUIR ISOTHERM

The Langmuir isotherm can be used to describe either associative or dissociative adsorption to monolayer coverage. The Langmuir isotherm is only valid at low temperatures [47, 48, 51, 52]. The application of the above mentioned assumptions results in the formation of the following equations for associative adsorption:

\[
\theta = \frac{K_p}{1+K_p} \quad \text{and} \quad K = \frac{k_a}{k_d}
\]  

(3)

Where \( k_a \) and \( k_d \) are the rate constants of adsorption and desorption respectively, \( P \) is pressure and \( K \) is the equilibrium constant. The rate at which a
molecule strikes the surface is proportional to the partial pressure and the number of unoccupied sites on the surface; therefore the rate of adsorption \( r_{ad} \) can be written as:

\[
r_{ad} = k_aN(1 - \theta)p
\]  

(4)

The rate of desorption \( r_d \) is directly related to the number of sites currently occupied by that molecule on the surface and it can be written as:

\[
r_d = k_dN\theta
\]  

(5)

When the system is at equilibrium the rate of adsorption and the rate of desorption are equal

\[
k_aN(1 - \theta)p = k_dN\theta
\]  

(6)

The Ns cancel giving:

\[
K_p(1 - \theta) = \theta
\]  

(7)

The Langmuir isotherm may be modified in the following manner in order to accurately describe dissociative adsorption. During dissociative adsorption the rate of adsorption is no longer just proportional to the partial pressure; it is influenced by the probability that both atoms will find adsorption sites on the surface

\[
r_{ad} = k_a p(N(1 - \theta))^2
\]  

(8)
The rate of desorption is proportional to the rate at which atoms strike the surface

\[ r_d = k_d (N\theta)^2 \]  \hspace{1cm} (9)

At equilibrium the rate of adsorption and desorption are again equal and following equation is formed;

\[ k_a p (N(1 - \theta))^2 = k_d (N\theta)^2 \]  \hspace{1cm} (10)

Cancelling the Ns, using \( K = k_a/k_d \) and taking the square root of both sides of the equation yields the following expression which describes dissociative adsorption;

\[ \left( K_p \right)^{1/2} (1 - \theta) = \theta \]  \hspace{1cm} (11)

### 1.5.1.2 BET (BRUNAUER, EMMETT AND TELLER) ISOTHERM

The Brunauer, Emmett and Teller (BET) isotherm is used to describe adsorption that proceeds beyond monolayer formation. The initially adsorbed monolayer acts as a substrate upon which additional layers may be adsorbed. Multi-layer adsorption can be observed in a physically adsorbed system \([47, 48, 52, 53]\). Due to the formation of multi-layers the BET isotherm does not level off as pressure is increased.

\[ \frac{V}{V_{\text{mon}}} = \frac{CZ}{(1 - z)(1 - (1 - c)z)} \]  \hspace{1cm} (12)

\[ z = \frac{p}{p^*} \]  \hspace{1cm} (13)
$p^*$ = vapor pressure of the bulk liquid

$p = $ pressure

$V_{\text{mon}} = $ volume necessary for monolayer coverage

$c = $ constant

When $c >> 1$ the BET isotherm simplifies to the following;

$$\frac{V}{V_{\text{mon}}} = \frac{1}{1-z}$$

(14)

### 1.6 POLYMER ADSORPTION

Polymer can adsorb strongly on a surface even if the individual monomers do not, this is due to the large aggregate surface free energy that the polymer as a whole possess. Consequently polymer desorption is often unfavourable due to the strength of adsorption. When polymer adsorbs from dilute solution it has been shown that film thickness is of the same order of magnitude as the individual polymer chains radius of gyration [54]. Polymer adsorption can be divided into a number of stages;

- Diffusion of the polymer to the adsorbate surface and adsorption onto the bare surface.
- As more polymer molecules are adsorbed conformational rearrangements take place in the surface layer to accommodate further adsorption.
- Rearrangements in the outer most layer of polymer takes place as a result of the exchange between bound polymer and the free polymer in solution.

Polymers can adsorb in a number of configurations similar to protein adsorption. Consequently the number of interaction points between the polymer and the
adsorbent surface can vary depending on the specific conformation. Polymers can adsorb as loops, trains or in condensed globular conformations. This variation in polymer conformation is responsible for the slow rate of adsorption equilibrium. The adsorbed polymers may desorb depending on the adsorption affinity of another polymer conformation. This process may continue for hours or days before steady state equilibrium is reached.

Several parameters must be used to define the state of an adsorbed polymer. These parameters include thickness, number of interaction points and the horizontal spread of the polymer which is given by its average radius. One of the simplest models for describing polymer adsorption is given below and it was derived from the mass action approach i.e.

$$N_2 + N_{s1} = N_{s2} + N_1$$  \hspace{1cm} (15)

$N_1 = $ solvent in solution

$N_2 = $ solute in solution

$N_{s1} = $ adsorbed solvent

$N_{s2} = $ adsorbed solute

The term $v$ which describes the molecules of solvent displaced per polymer molecule is introduced and this gives rise to $(N_{s1})^v$.

$$\frac{\theta}{v(1 - \theta)^v} = bC_2$$  \hspace{1cm} (16)

$C_2$ is the concentration of solute following adsorption. $\theta$ is the fraction of surface sites occupied, $b = K/a_1$, $a_1$ is the solvent activity in solution.

The equilibrium constant for the process is

$$K = \frac{N_{s2}}{N_{s1}a_2}$$  \hspace{1cm} (17)
a_1 and a_2 are the solvent and solute activities in solution and N_s^1 and N_s^2 are the mole fractions which describe the activities in the adsorbed layer.

This project focused on dilute solutions and a_1 is thus constant and b=K/a_1 and N_s^1 + N_s^2 = 1 thus

\[ N_s^2 = \frac{ba_2}{1 + ba_2} \]  \hspace{1cm} (18)

\[ \theta = \frac{ba_2}{1 + ba_2} \]  \hspace{1cm} (19)

\[ \theta = \frac{n_s^2}{n^s} \]  \hspace{1cm} (20)

As n_s^2=N_s^2n^s where n^s is the number of moles of adsorption sites per gram.

There are numerous more complicated models for polymer adsorption including the random walks approaches developed by Rubin and Dimarzio and Simha, statistical approaches developed by deGennes, Everett and Frisch. In light of the numerous sophisticated models it is interesting to note that the majority of polymer adsorption data fits the simple Langmuir equation [54]. Polymer adsorption may increase as the temperature increases and numerous researchers have speculated that polymer adsorption is entropy driven rather than enthalpy driven. As the polymer losses entropy upon adsorption the increase in entropy must be coming from the solvent molecules desorbing from the surface [54, 55].

Polymer adsorption may be irreversible. The amount of polymer adsorbed is determined by allowing the solution of polymer to reach equilibrium and then calculating the reduction in solution concentration of the polymer. Adsorption may be defined as irreversible if the supernatant polymer solution is removed once equilibrium has been reached and then pure solvent is added and no polymer desorption then takes place. There is evidence which shows that if a better solvent or a strongly competitive polymer is introduced then desorption
will take place rapidly. The work of Granick et al. [54] supports this theory. Granick et al. [54] studied the adsorption of deuterio (d) polystyrene (PS) (PS-d) and protio PS (PS-h) in cyclohexane. The substrate used was oxidized silicon. PS-h ($M_w = 575,000 \text{ g/mol}$) was first incubated with the oxidized silicon and diffusion to the surface took place in less 30 minutes. The PS-h solution was then replaced with PS-d solution ($M_w = 550,000 \text{ g/mol}$). The same concentration was used for both polymer solutions 1 mg/ml. The adsorption of PS-d is slightly favoured over PS-h. The temperature was kept at $30^\circ \text{C}$ throughout the experiment. Granick et al. [54] noticed that desorption of PS-h was followed by the adsorption of PS-d. PS-d adsorbed more rapidly than PS-h desorbed supporting the theory that polymers can penetrate or worm their way into a previously adsorbed layer. The rate of PS-h desorption was thus the rate determining step. Granick et al. [54] also varied the incubation time of the PS-h solution with the oxidized silicon from 0-40 hours and they found that the displacement time increased with increasing incubation or residence time. Desorption or displacement time for PS-h was also effected by the solution concentration. The solution concentration was reduced from 1mg/ml to 0.1 mg/ml and the displacement time decreased from 23 hours to 7.6 hours. Granick et al. [54] proposed that the reduction in $t_{off}$ or displacement time with increased incubation time is due to the rearrangement of the polymer chains in the direction of conformational equilibrium. As mentioned earlier adsorption kinetics can be divided into diffusion of polymer chains from the bulk solution to the adsorbant surface (this took place rapidly here (within 30 minutes)), adsorption and conformational equilibrium. During conformational equilibrium the polymer chains may become more tightly bound to the adsorbant surface as the number of interaction points may increase; if the attraction between the polymer and the surface increases it will take more time to remove the adsorbed polymer molecules. The time required for conformational equilibrium increases with molecular weight. [56]
The following points summarize the large volume of literature regarding polymer adsorption:

- Polymer desorption when the polymer solution has been replaced with pure solvent is very slow or it does not take place and it is insensitive to temperature. This is because the activation energy required to remove all of the polymer segments from the surface at once is exceedingly large [57, 58].

- Polymer exchange will take place between adsorbed polymer and polymer in solution. Adsorbed polymer may be displaced by polymer molecules of larger molecular weight. This exchange takes place as the polymer segments detach piece by piece rather than all at once and consequently the energy barrier isn’t as large. The time scale required for the exchange is of the order of hours or days [59, 60].

- A part of the adsorbed polymer layer may exchange with another section of the adsorbed layer due to lateral diffusion. This means that some areas of the adsorbed layer are more tightly bound than others [61, 62].

- If adsorbed chains are very dense it is possible for them to form a glassy layer near the surface. This glassy layer may be responsible for the slow rate of conformational equilibrium compared to diffusion [63, 64].

- Adsorption may proceed faster in good solvent but there are several studies which state that the opposite is true [65-67].
1.7 PHYSICAL ADSORPTION OF PNIPAM

This section will summarize the current literature pertaining to the adsorption of pNIPAm.

Callewaert et al. [45] studied the adsorption of pNIPAm at two different concentrations 0.02 g/L and 2 g/L and at two different temperatures 25 °C and 50 °C. The physically adsorbed films were formed by adding 1.8 ml of pNIPAm (M_w 4×10^4 g/mol) aqueous solution to well plates containing glass slides. After an incubation period at either 25 °C or 50 °C, 1.6 ml of solution was removed and replaced with 1.6 ml of MilliQ water at the appropriate temperature. The MilliQ water was allowed to incubate with the sample for 5 minutes. After a 24 hr incubation period the 0.02 g/L solution produced smooth featureless films. An AFM scrapping test in conjunction with XPS proved that a physically adsorbed film had been formed. Callewaert et al. [68] used XPS to calculate film thickness and the 0.02 g/L solution at 25 °C produced a 1nm thick film with an adsorbed amount of pNIPAm equal to 1.1 mg/m^2. Adsorption of 2 g/L solution at the same temperature and incubation time produced a 2 nm thick film. The increased concentration is thought to affect the adsorption conformation of the polymer. At low concentration the polymer adsorbed in loops or trains with numerous adsorption points. At high concentration the polymer chains stand normal to the substrate and there are fewer interaction points. A glass cover slip was incubated for 10 minutes at 50 °C with the 2 g/L solution; AFM scrapping tests showed that the thickness of the adsorbed layer is of the order of 2 to 3 nm (Figure 1.5).
Figure 1.5: 5 × 5 μm AFM image in air of glass substrate after adsorption (10 min) of PNIPAAM from a 2 g/L solution at 50 °C. The central zone (1 × 1 μm) was scraped before the image was recorded. Vertical scale: 10 nm. Section was taken at the place indicated by the dotted line [68]. This figure has been removed due to copyright restrictions and can be viewed in the reference given here.

XPS analysis was carried out and the film formed after 10 minutes was comparable to the film formed after 24 hours of incubation; this proved that the adsorption process is quiet rapid. AFM scrapping tests showed that the films formed above the LCST are more cohesive than those formed below the LCST (Figure 1.6). Scrapping tests performed on the films produced below the LCST simply disturbed the polymer film but above the LCST scrapping peeled off the polymer layer.
Figure 1.6: AFM images in air of glass substrata (a, c, 2 × 2 μm; b, d, 5 × 5 μm) after adsorption (24 hrs) of PNIPAAm from a 0.02 g/L solution (a, b) and a 2 g/L solution (c, d) at 50 °C. The central zone (1 × 1 μm) of b and d was scraped before the image was recorded. Vertical scale: 5 nm (a), 10 nm (b, d), or 30 nm (c). Cross sections were taken at the places indicated by the dotted lines [68]. This figure has been removed due to copyright restrictions and can be viewed in the reference given here.
Photon correlation spectroscopy (PCS) analysis proved that pNIPAm coils of 30 nm formed at 25 °C in the 2 g/L solution. AFM analysis of the 2 g/L films formed at 25 °C showed the presence of sporadic globular particles of approximately 5 nm in height on the top of the adsorbed film. A single pNIPAm molecule with a mass of $4\times10^4$ g/mol will possess a diameter of 4.8 nm when dry; this led Callewaert et al. [68] to the conclusion that the globules were individual molecules of pNIPAm. Above the LCST the 0.02 g/L films possessed smooth areas and areas covered with globules with a height of approximately 5 nm. Below the LCST the 0.02 g/L films were featureless. Callewaert et al. [68] offer no explanation for the 15-30 nm height aggregates observed on the 2 g/L films formed at 50 °C [68].

Tanahashi et al. [44] studied the physical adsorption of pNIPAm on silica surfaces. Both hydrophobic and hydrophilic silica were used in order to determine whether or not substrate wettability influences pNIPAm adsorption. Adsorption temperatures ranging from 10 to 30 °C were investigated. Silica (0.1 g) was incubated with 20 ml of 0.2 g/100 ml solution of pNIPAm prepared in MilliQ water. An incubation period of 24 hours was used. Tanahashi et al. [44] found that the amount of pNIPAm adsorbed was temperature dependent. Above 19 °C the amount of pNIPAm adsorbed on the hydrophobic silica was greater than that on the hydrophilic silica. This was the case for all temperatures investigated between 19 °C and 30 °C. The amount of pNIPAm absorbed increased with increasing temperature on both the hydrophilic and hydrophobic silica (Figure 1.7). A larger amount of pNIPAm was adsorbed on the hydrophilic silica at 15 °C. Tanahashi et al. [44] suggested that this was due to hydrogen bonding between the silanol groups in silica and the carbonyl groups in pNIPAm. The water cage surrounding the isopropyl group in pNIPAm is disrupted as the temperature increases; this means that as the temperature increases the isopropyl groups will be capable of interacting with the siloxane groups on the silica surface. The number of interaction sites is larger on the hydrophobic silica and this is why a larger amount of pNIPAm is adsorbed on the hydrophobic silica [44].
Zhu et al. [69] studied the adsorption of pNIPAm on pntBAm latex particles at temperatures ranging from 5 °C to 60 °C. An incubation period of 24 hours was used. Dynamic light scattering (DLS) was used to determine the size of the latex particles during the experiment. The thickness of the pNIPAm layer was determined by subtracting the thickness of the latex particles and dividing by 2. Zhu et al. [69] found that below the LCST pNIPAm adsorbed in a loopy conformation with a large number of interaction points. As the temperature increased to the LCST and above the number of interaction points decreased and pNIPAm transitioned from loopily adsorbed to a condensed globular formation. Zhu et al. [69] then decreased the temperature below the LCST and they observed a globual to extended conformation transition. This globual to extended conformation presents itself as an increase in pNIPAm thickness as the temperature was decreased below the LCST. The thickness of the extended
conformation is greater than the initial film thickness below the LCST. **Figure 1.8** contains the DLS data showing the temperature dependence of pNIPAm layer thickness on the pNtBAm latex particles [69].

Figure 1.8: DLS-measured conformational transition curves for PNIPAM chains absorbed on the surfaces of pNtBAm latex particles. The filled squares are the data measured on increasing the temperature and the empty squares are those on cooling. The empty triangles are the data measured for the sample heated first from a low to a high temperature and then kept at room temperature for about two months. The crosses are the data measured for uncoated pNtBAm latex particles. (a) Temperature dependence of the diameter for the pNtBAm latex particles
coated by adsorbed PNIPAM chains. (b) Temperature dependence of the layer thickness of the interfacial PNIPAM chains [69]. This figure has been removed due to copyright restrictions and can be viewed in the reference given here.

The extended conformation is unstable and it transitions to the loopily adsorbed state below the LCST. Figure 1.9 provides an illustration of the loopy, extended and globular conformation of adsorbed pNIPAm.
Wu et al. [70] studied the physical absorption of pNIPAm using gold coated electrodes and a quartz crystal microbalance with dissipation (QCM-D). The mass and polydispersity index of pNIPAm used in this investigation are as follows 20,000 g/mol and 1.17. They proved that the adsorption of pNIPAm is different above and below the polymers LCST and this difference is related to the polymers change in conformation. The different polymer conformations
interact with the substrate differently resulting in unique adsorption isotherms. Wu et al. [70] measured the changes in the frequency and dissipation and then plotted them against concentration in order to create adsorption isotherms. Their results show that the below the LCST pNIPAm can only form a single layer of polymer in the concentration range (0-160 ppm) and temperature range (18-36 °C) investigated. Above the LCST pNIPAm formed much thicker multilayer systems and film thickness was shown to increase with increasing temperature. The change in frequency is proportional to the mass of absorbed pNIPAm. Wu et al. [70] measured the Δf and ΔD at the third harmonic as this gives a better signal to noise ratio than measurements conducted using the fundamental frequency of the quartz crystal. Below the LCST pNIPAm adsorption reached a plateau at a concentration of 20 ppm. Above the LCST at concentrations of 50 ppm and greater the adsorption isotherm began to increase rapidly with concentration, proving that multiple layers of pNIPAm were forming above the LCST. The changes in dissipation corroborated the changes in frequency. Frequency undergoes a negative shift due to adsorption while dissipation experiences a positive shift. The adsorption kinetics for pNIPAm in the collapsed and extended coil states differ as the rate of diffusion differs [70].

Zhu et al. [71] also studied the physisorption of pNIPAm using QCM-D. The mass and polydispersity index of pNIPAm used in this investigation are as follows 20,000 g/mol and 1.17 respectively. Concentrations in the range of 0-160 ppm of pNIPAm where used at temperatures ranging from 18-36 °C. Incubation times in the range of 0-9,000 seconds were used. They found that below the LCST the time constant for adsorption increased exponentially with increasing concentration and temperature. Above the LCST temperature and concentration did not appear to affect the adsorption time constant (Figures 1.10 and 1.11).
Figure 1.10: Time constant of adsorption kinetics versus temperature (dashed line). Guide for the eye; (solid line) Temperature dependence of the hydrodynamic radius of pNIPAm [71]. This figure has been removed due to copyright restrictions and can be viewed in the reference given here.
Similar to Wu et al. [70] they observed that below the LCST pNIPAm was capable of forming only a single layer of polymer. Above the LCST multiple layers were formed on the gold electrode. Zhu et al. [71] postulated that the differences in adsorption behaviour were due to differences in the interactions between the polymer and the substrate above and below the LCST and differences in molecular size. The radius of gyration of pNIPAm is approximately 6 to 8 times smaller above the LCST than below the LCST. The adsorption of pNIPAm can be divided into a number of stages; diffusion of the polymer in solution, attachment onto the substrate and conformational relaxation and
adsorption. The differences in the radius of gyration of pNIPAm above and below the LCST will result in significant differences in the diffusion of pNIPAm and consequently the adsorption kinetics will be drastically different. The chemical potential of the adsorbed pNIPAm layer decreases as pNIPAm changes from its swollen to its collapsed state, at the same time the chemical potential of pNIPAm in solution increases; this favours the formation of pNIPAm multilayers above the LCST (Figure 1.12 illustrates the difference in adsorption above and below the LCST of pNIPAm as proposed by Zhu et al.) [71].
Figure 1.12: Cartoon illustrating the different modes of pNIPAm physical adsorption above and below the LCST on gold electrodes as defined by Zhu et al [71]. This figure has been removed due to copyright restrictions and can be viewed in the reference given here.
Zhu et al. [71] fitted the adsorption of pNIPAm to a Langmuir adsorption isotherm. This means that the time dependence of molecular transport and attachment to the substrate surface can be described together by an averaged adsorption rate. The equations below describe the balance between the rates of adsorption and desorption and surface coverage ($\theta$);

$$\frac{d\theta}{dt} = k_a(1 - \theta)c - kd\theta$$  \hspace{1cm} (21)

$k_a$ and $k_d$ are the adsorption and desorption constants respectively and $c$ is the concentration of pNIPAm. Zhu et al. [71] assumed that $k_a$ and $k_d$ were independent of surface coverage and then they integrated the equation above to give the time dependence of surface coverage.

$$\theta = \theta_{eq} + (\theta_i - \theta_{eq})e^{-\frac{t}{\tau}}$$  \hspace{1cm} (22)

$t$ is the time constant and $\tau = \frac{1}{k_a c + k_d c}$ and $\theta_{eq} = \frac{c}{c + (\frac{k_d}{k_a})}$, $\theta_{eq}$ is the equilibrium coverage at concentration $c$ and $\theta_i$ is the initial coverage. The adsorption time for pNIPAm in its collapsed state is insensitive to concentration. If we take the Stokes-Einstein relationship

$$R_h = \frac{k_B T}{6\pi\eta D}$$  \hspace{1cm} (23)

$k_B$, $\eta$ and $T$ are the Boltzmann constant, the solution viscosity and the absolute temperature respectively. The average hydrodynamic radius of a polymer ($R_h$) is therefore proportional to the translational diffusion coefficient; $D$. Translational diffusion affects the rate of adsorption. If a polymer possesses a large hydrodynamic radius it will consequently possess a large translational diffusion coefficient. Above the LCST it is thought that mechanisms other than translational diffusion dominate the adsorption kinetics and consequently the concentration of the polymer solution no longer determines the adsorption
kinetics. In other words there is always polymer near the surface to be adsorbed, thus it makes sense that the adsorption time constant for pNIPAm above the LCST is not sensitive to concentration. Zhu et al. [71] found that their adsorption isotherms for the physisorption of pNIPAm suggested that the physisorption of pNIPAm proceeds via densification rather than thickness growth. This means that more and more surface sites are occupied as a larger quantity of pNIPAm is adsorbed, thus the pNIPAm molecules will undergo surface rearrangement as more and more surface sites are occupied [71].

Wu et al. [72] synthesized a range of pNIPAm copolymers with varying percentages of methacryloyl-amino-ethylene-3α, 7α, 12α-trihydroxy-5β-cholanoamide (MACA). They used DLS to study the changes in the hydrodynamic radii ($R_h$) of the polymer chains over a range of temperatures (10-50 °C). Wu et al. [72] found that as the percentage of MACA increased the $R_h$ of the polymer decreased (Figure 1.13). This was surprising as it was thought that hydrophilic interactions stabilized polymer globules and consequently as the percentage of hydrophobic monomer increased it was thought that $R_h$ would increase and the stability of the globule would decrease. The opposite proved true as the mesoglobules formed from the copolymers with increasing MACA were stable over months. This stability is due to the fact that increased MACA promotes both inter-chain association and intra-chain contraction. The pNIPAm-co-MACA mesoglobules are stabilized by both the concentration of hydrophilic monomer on the outside of the polymer and the hydrophobic association taking place inside the mesoglobules. The viscoelastic effect results in the formation of very stable mesoglobules as the entanglement time ($\tau_e$) between colliding mesoglobules is longer than the collision or interaction time ($\tau_c$). This means that the mesoglobules act as non-adhesive glass balls [72].
Figure 1.13: Temperature dependence of the average hydrodynamic radius \(<R_h>\) of pNIPAm-co-MACA mesoglobules formed in a gradual heating process, where each data point was obtained after the temperature equilibrium was reached and the copolymer concentration is ~\(10^{-5}\) g/ml [72]. This figure has been removed due to copyright restrictions and can be viewed in the reference given here.

Gao et al. [43] studied the adsorption of pNIPAm on polystyrene latex particles over a range of temperatures (20-40 °C). The values for the weight average molecular weight \((M_w)\), the polydispersity index (PDI) and the average hydrodynamic radius of the pNIPAm used in this investigation, are \(8.11 \times 10^5\) g/mol, 1.8 and 30 nm respectively. The pNIPAm/particle weight ratio was varied within the range of 0.2-3.0. Gao et al. [43] state that adsorption equilibrium was reached within minutes of mixing the pNIPAm solution and the latex particles. Static laser light scattering (LLS) and DLS were used to measure the changes in the hydrodynamic radius \((R_h)\) and the excess Rayleigh ratio \((\Delta R_{vv}(\theta))\). The \(\Delta R_{vv}\) \((\theta)\) is the Rayleigh ratio difference between the nanoparticles with and without
adsorbed pNIPAm. The pNIPAm/PS latex particle mixture was heated from 20 to 40 °C and then cooled from 40 to 20 °C. Gao et al. [43] observed three distinct stages of $R_h$ and $\Delta R_{vv}(\theta)$ behaviour upon heating and cooling (Figure 1.14).

Figure 1.14: Temperature dependence of the hydrodynamic radius $<R_h>$ and the excess Rayleigh ratio $\Delta R_{vv}(\theta)$ [43]. This figure has been removed due to copyright restrictions and can be viewed in the reference given here.
During the heating process, the first stage is from 20-29 °C and $R_h$ decreases slightly as temperature increases and $\Delta R_{\text{vv}}(\theta)$ remains constant. The adsorbed pNIPAm chains are shrinking/dehydrating and no additional pNIPAm chains are adsorbed. In the second stage, from 29-32.5 °C $R_h$ increases slightly while $\Delta R_{\text{vv}}(\theta)$ increases dramatically. This proves that further pNIPAm chains are adsorbed on the surface. In this temperature range the adsorbed pNIPAm chains will shrink reducing the $R_h$ but enough additional pNIPAm is adsorbed to result in a slight increase. In the third stage from 32.5-40 °C $\Delta R_{\text{vv}}(\theta)$ is constant as the adsorption of pNIPAm on the surface of the PS latex particles has reached saturation level. A slight decrease in $R_h$ is observed up until 35 °C at which point the $R_h$ becomes constant. The cooling process can be divided into the same three stages. The values observed for $R_h$ and $\Delta R_{\text{vv}}(\theta)$ are reversible, this proves that the adsorbed pNIPAm is capable of reversibly shrinking and swelling depending on temperature. Gao et al. [43] noted that the coil to globule transition for adsorbed pNIPAm took place at 29 °C, three degrees lower that the recorded LCST for pNIPAm in solution [43].

Miura et al. [73] investigated the adsorption of a poly (NIPAm-co-NtBAm) copolymer (60:40) on a range of different substrates; hexafluoropropylene-tetrafluoroethylene (FEP), low density polyethylene (LDPE), polystyrene (PS), polyethyleneterphalate (PET) and cellulose acetate (CA).

The adsorptions of poly (NIPAm-co-NtBAm) solutions in the following concentration range were investigated 10-200 µg/ml on FEP. Phosphate buffered saline (PBS) was used as the solvent. Adsorption was carried out at either 37 °C or 0 °C. The FEP samples were incubated with the polymer solutions for 2 hours after which the samples were rinsed with deionized and distilled water for 5 minutes. The samples were the dried overnight in a vacuum oven. Poly (NIPAm-co-NtBAm) films were formed on the other samples in a similar manner, only the 100 µg/ml solution was used. In order to investigate polymer retention, samples were rinsed with 0 °C deionized and distilled water following an incubation period of 2 hours at either 0 °C or 37 °C. Percentage nitrogen content was determined by XPS and the difference in percentage nitrogen content before and after adsorption was used to determine film thickness. A larger amount of polymer was adsorbed on FEP up until 50 µ/ml at
37 °C than at 0 °C. At concentrations greater than 50 µg/ml, an increase in polymer adsorption did not take place indicating that adsorption equilibrium had been reached for polymer adsorption on FEP at 37 °C. A gradual increase in the amount of polymer adsorbed was observed for concentrations greater than 50 µg/ml at 0 °C, indicating that equilibrium had not been attained. Over all less polymer is still adsorbed on the 0 °C incubated films than on the 37 °C incubated films. Miura et al. [73] state that at 37 °C the FEP surface is almost completely covered by a 50 Å thick film of poly (NIPAm-co-NtBAm). Below the LCST a smaller quantity of polymer is adsorbed in order to reduce the interfacial energy between the FEP surface and water. In this investigation the temperature was raised from 0 °C to 37 °C for incubation at 37 °C. Consequently the polymer which had adsorbed on the surface would collapse or shrink and this would create more available adsorption sites. At 37 °C the adsorption of poly (NIPAm-co-NtBAm) is driven by the hydrophobic effect as the substrate and polymer must at least partially dehydrate before adsorption can take place. This means that a larger quantity of polymer adsorbs above the LCST. Miura et al. [73] noticed that when the FEP coated substrates were rinsed with 37 °C deionized and distilled water that 100 % of the adsorbed polymer remained on the surface. When the samples were rinsed with 0 °C deionized and distilled water 80 % of the adsorbed polymer was removed from the surface. Polymer adsorption and retention were investigated on the four other substrates and adsorption and polymer retention at 37 °C was found to be independent of substrate type and wettability (Figure 1.15).
Figure 1.15: Retention of poly (NIPAm-co-NtBAm) (60:40) (poly 64) on various substrates after adsorption at 37 °C [73]. This figure has been removed due to copyright restrictions and can be viewed in the reference given here.

Substrate wettability seems to influence polymer adsorption at 0 °C and polymer retention when the sample is rinsed with water below the polymer’s LCST. Polymer adsorption decreases dramatically when the contact angle of the substrate drops below 60°. Miura et al. [73] suggested that this was due to an increase in the interaction between water molecules and the substrate as the main driving force for the adsorption of poly (NIPAm-co-NtBAm) is the release of bound water molecules and the subsequent hydrophobic interactions. PET possessed the best polymer retention rate after a 0 °C rinse. Miura et al. [73] proposed that the 0 °C retention rate reflected the strength of the combined polar and dispersive interactions and hydrogen bonding. PET has a contact
angle of 71.3° and Miura et al. [73] state that it possesses the right combination of polar and dispersive groups allowing it to maximize adsorption [73].

pNIPAm adsorption can take place on either hydrophilic or hydrophobic substrates. A larger quantity of pNIPAm is adsorbed on hydrophobic substrates as at least partial dehydration must take place before polymer adsorption can take place and dehydration of a hydrophobic surface is favoured over a hydrophilic surface. The quantity of pNIPAm adsorbed is temperature dependent and the quantity increases as temperature increases until an equilibrium state has been reached. Adsorption temperature influences film thickness and stiffness, this is due to the conformational changes pNIPAm undergoes above and below the LCST. Adsorption of pNIPAm appears to take place rapidly. Multi-layer systems can be formed when adsorption is carried out above the LCST. Adsorbed pNIPAm is capable of reversibly swelling and deswelling.

1.8 PROTEIN ADSORPTION

Adherent cells do not interact with the biomaterial of interest directly; instead they interact with an adsorbed protein layer. A layer of proteins are immediately adsorbed onto a biomaterial upon insertion either in vivo or in vitro. These proteins come from either blood or interstitial fluid in vivo or from serum in vitro. This study deals with in vitro cell-biomaterials interactions and consequently the focus will be on in vitro protein adsorption. Adherent cells must adhere to a suitable substrate in order to survive. Cell adhesion is necessary for cell proliferation and maintenance of cell morphology. Cells interact with adsorbed serum proteins such as fibronectin and vitronectin through integrins. When antibodies are introduced which prevent cell-integrin interactions cell adhesion does not take place. Different integrins are expressed depending on the composition of the adsorbed protein layer; this may account for the differences in cell adhesion on different materials. Due to the pivotal role adsorbed proteins play in cell adhesion and biocompatibility it is necessary to first discuss the factors which influence protein adsorption and protein adsorption kinetics before
discussing cell adhesion. The composition of the adsorbed protein layer plays a vital role in regulating cell behaviour. If the necessary proteins are present in the correct quantities and configuration cell adhesion will take place, if this isn’t the case the biomaterial in question will prove unsuitable for cell adhesion and proliferation [74, 75].

Proteins adsorption takes place rapidly such that cells do not interact with the bare biomaterial. Horbertt and Schway proved that adsorption of serum proteins takes place in ten seconds [76]. In order for protein adsorption to take place a number of entropic and enthalpic changes must first take place. The entropic and enthalpic changes necessary for protein adsorption were defined by Norde and Haynes and they are as follows [77, 78]:

- Dehydration of the protein and adsorbent surface.
- Redistribution of charged groups
- Conformational changes in the protein.

It has been proven that protein adsorption increases with increasing concentration and incubation time until the protein layer has approached monolayer coverage. The rate of protein adsorption then decreases as it becomes dependent on protein surface affinity and the number of available adsorption sites [78-80]. Competitive protein adsorption is seen in any mixed solution of proteins such as serum and blood. Adsorption in this case is dependent on surface affinity and the number if binding sites. Initially the adsorbed layer will consist of faster diffusing proteins but over time these faster diffusing proteins will be displaced by proteins which have a higher surface affinity; this phenomenon is known as the Vroman effect. The Vroman effect is more pronounced on hydrophilic surfaces than on hydrophobic surfaces as proteins do not bind as tightly to hydrophilic surfaces. The exact composition of an adsorbed protein layer is dependent on the rate of adsorption and desorption of competing proteins [81-83].
There are a number of factors which will either promote or inhibit protein adsorption. The effect of surface wettability, charge interaction and conformational changes on protein adsorption will be discussed briefly. Surface wettability is perhaps the most widely studied surface property in terms of protein adsorption and cell adhesion. As discussed earlier for protein adsorption to take place, both the protein and the adsorbent surface must undergo partial dehydration at least. Partial or full dehydration is thermodynamically favourable for hydrophobic surfaces, as this causes an increase in entropy. The removal of water from hydrophilic surfaces is conversely thermodynamically unfavourable. This is why less protein is adsorbed on surfaces with a high affinity for water.

Protein adsorption can take place on hydrophilic surfaces due to protein conformational changes and possible charge interactions. While hydrophobic surfaces generally adsorb greater quantities of protein it is important to remember that there are exceptions and in some studies hydrophobic and hydrophilic surfaces have been shown to adsorb similar quantities of protein [79, 84, 85].

Charge interactions can play an important role in protein adsorption. It is logical to assume that opposite charges will result in greater quantities of protein being adsorbed. This may not always be the case when dealing with aqueous solutions as water molecules may shield the surface charge, the surface charge may be countered by the presence of ions and solution pH will also affect protein adsorption. The pH of the solution will affect the proteins net charge; if the pH is above the isoelectric point the protein will be negatively charged. If the pH is below the isoelectric point the protein will have a net positive charge. An electrical double layer may form at the charged surface due to the presence of ions such as Cl⁻, Ca²⁺, Na⁺ and K⁺ [74, 77].

Protein conformational change is the main driving force for protein adsorption on seemingly unfavourable surfaces such as hydrophilic surfaces or those which possess like charges. Protein conformational change increases the entropy of the system, for example if an alpha helix is replaced by a random coil in the proteins secondary structures this will increase bond mobility and entropy. Conformational changes can increase the strength and the number of contacts between the biomaterial and the adsorbed protein. A protein’s stability and
flexibility will influence its adsorption kinetics. Highly flexible proteins can form numerous non-covalent interactions in a short period of time where as more compact proteins will take longer to interact with the adsorbent surface. It has been shown that protein conformational change takes place in nearly all protein adsorption cases. Proteins are not completely denatured upon adsorption; in fact proteins can retain a large percentage of their secondary structure upon adsorption. Proteins can regain their native structure upon desorption though their ability to do so is dependent on the adsorbent surfaces hydrophobicity and the proteins own stabilizing forces [77, 79, 86].

Protein function is determined by its conformation and consequently it is possible to engineer surfaces which will adsorb proteins in specific conformations and thus create surfaces with specific functions. Changes in protein conformation on adsorption may be the reason why cell adhesion is better on slightly hydrophilic surfaces even though protein adsorption is greater on hydrophobic surfaces. When fibronectin is adsorbed on hydrophobic surfaces the resultant conformational changes reduce its cell adhesion function. This reduction in cell adhesion function is not seen in fibronectin adsorbed on hydrophilic surfaces. During cell proliferation adsorbed fibronectin is rearranged and thus the irreversibility of protein adsorption may also contribute to the differences in cell adhesion on hydrophilic and hydrophobic surfaces as adsorbed proteins on hydrophobic surfaces will be bound much tighter than those adsorbed on hydrophilic surfaces.[86-88]

Roach et al. [89] used grazing angle infrared spectroscopy (GA-FTIR) and quartz crystal microbalance (QCM) to study the effect of surface chemistry on the adsorption of bovine serum albumin (BSA) and bovine fibrinogen (Fg). One surface was modified with OH groups and the other was modified with CH₃ groups. The contact angle of the OH modified surface is 48° and 94° for the CH₃ modified surface. Roach et al. [89] found that BSA adsorbed much faster on the CH₃ terminated surface than on the OH terminated surface. The adsorption data for both polymers was fitted to a Langmuir adsorption isotherm and the binding constant K was calculated for both proteins on both surfaces. Both BSA and Fg have a higher affinity for the CH₃ terminated surface. GA-FTIR showed that BSA adsorbed on the more hydrophobic surface had undergone deformation. Fg also
appears to undergo deformation upon adsorption on the CH$_3$ terminated surface. Greater quantities of BSA and Fg were adsorbed on the OH terminated surface. Roach et al. [90] proposed that this is due to deformation caused by adsorption on the hydrophobic surface which results in lower surface saturation values. Roach et al. [90] also stated that while adsorption of both proteins takes place rapidly (approximately 50 seconds) Fg undergoes surface rearrangement after initial adsorption. This multi stage adsorption profile for Fg was only observed at high concentrations. The greater quantity of Fg absorbed on the OH terminated surface may be due to the fact that the protein isn't bound as tightly and as a result it is able to undergo surface rearrangement at high concentrations (>0.5 mg/mL) creating more available adsorption sites. BSA appears to undergo a single step adsorption process regardless of concentration. Both proteins lose some of their alpha helical character upon adsorption on the CH$_3$ terminated surfaces [89, 90].

Protein adsorption is a complex balance between the rate of adsorption and desorption which affects a number of important cellular process. Surface wettability, charge interactions, conformational changes and surface chemistry can all affect the composition of the adsorbed protein layer.

1.9 CELL ADHESION

Cells interacting with a biomaterial either in vivo or in vitro do not interact with the bare surface of the biomaterial; rather the cells interact with an adsorbed layer of proteins. These proteins are adsorbed either from the blood or interstitial fluid in vivo or from cell culture media in vitro [91]. The process of cell adhesion can be divided into stages. The first stage involves the formation of short term interaction such as Van der Waals forces, ionic interactions etc. The second stage involves ligand integrin binding. The third stage involves the interaction of cell membrane proteins, cytoskeletal proteins, cytoskeletal rearrangement, and receptor clustering and mechanical pre-stress. The fourth and final stage involves ECM synthesis [92]. The interaction of these proteins induces signal transduction which regulates gene expression. The following events must take
place in order for cells to adhere. First proteins adsorb onto the surface of the biomaterial either *in vivo* or *in vitro*. Then the cells adhere to the surface, start spreading and begin to express integrins and cytoskeletal proteins. The cytoskeleton then reorganizes, the integrins begin to form groups and the cells start to actively spread. Cells generate mechanical force at the site of adhesion by contracting their actomyosin cytoskeleton. This contraction of the actomyosin cytoskeleton causes pre-stress. Adherent cells maintain their cytoskeleton in a state of pre-stress in order to maintain their morphology. Cells then synthesize ECM proteins creating a cell/matrix/biomaterial interface [92, 93]. **Figure 1.16** summarises the phases of cell adhesion.
There is some debate among cell adhesion researchers regarding the division of cell adhesion into stages. Garcia and Gallent studied the adhesion of murine osteoblasts (MC3T3-E1) and they concluded that adhesion consists of two stages; the stick and the grip stage. The stick stage consists of the initial integrin-ligand binding event and the grip stage consists of cell spreading, receptor clustering, and focal adhesion assembly [94]. Murphy-Ullrich proposed that cell adhesion can be divided into three stages, attachment, spreading and focal adhesion and stress fibre formation. The Murphy-Ullrich model of cell adhesion is based on fibroblast adhesion [95]. Anselme et al. [96] studied the adhesion of primary human bone derived cells. They concluded that cell adhesion consists of two stage; short term and long term adhesion. Short term
adhesion takes place in the first 24 hrs after the cells have been seeded on the biomaterial. After 24 hrs the cells will have spread and the cytoskeleton will have organized but synthesis of ECM proteins will not have taken place. During long term adhesion (up to 21 days) the cells will synthesis ECM proteins and develop cell-cell contacts [96, 97].

As is evident from the examples provided above the exact mechanism of cell adhesion is dependent on cell phenotype. Blood cells adhere in a transient fashion on endothelial cells and as such their first interactions are generally cell/cell interactions mediated by selectins or cadherins. Connective tissue cells interact first via integrins which regulate cell-ECM interactions. Another factor which can influence cell adhesion is the origin of the cells. Many cell lines used in biomaterials research are derived from tumours. Tumour cells proliferate rapidly but their adhesion mechanisms and ability to synthesis ECM are dramatically different than those found in healthy cells [98].

### 1.9.1 Chemical and Physical Properties Which Influence Cell Adhesion

Cell adhesion is vital for anchorage dependent cells. The strength and extent of cell adhesion will determine whether or not the cells in question will proliferate or undergo apoptosis. If cells adhere too strongly to a substrate they will be unable to undergo the migration and reorganization necessary for cell proliferation. Consequently a balance must be achieved between adhesion strength and cell motility if a substrate is to be biocompatible [93]. Cell adhesion is influenced by the chemical and physical properties of the chosen substrate. This review will focus on the influence of the following physical and chemical properties:

- Surface Chemistry
- Wettability
- Electrical Charge
- Roughness
- Substrate Stiffness
The chemistry of the substrate surface influences the wettability and zeta potential of the material. There is some debate as to which functional groups are the most suitable for promoting cell adhesion. Oxygen containing functional groups have been shown to increase substrate hydrophilicity and surface free energy and consequently it is thought that oxygen containing groups enhance cell adhesion [92]. Cell adhesion is mediated by the adsorbed protein layer which is spontaneously adsorbed from either serum or biological fluids. Proteins adsorb strongly on hydrophobic materials and this frequently results in denaturing of the protein. If a protein is adsorbed in a conformation which is unsuitable for cell adhesion the substrate in question will not be biocompatible regardless of the quantity of protein adsorbed. If a material is too hydrophilic the protein layer will not adsorb strongly enough to promote cell adhesion. A balance between strong adsorption and maintenance of suitable protein conformation must be met if a material is to prove suitable for cell adhesion [99].

Human osteoblast like MG 63 cells were seeded on a number of nanocrystalline diamond surfaces with a range of contact angles. When the MG 63 cells were seeded on the oxygen terminated surfaces with contact angles in the range of 20-35° the cells exhibited spontaneous cell detachment due to the weak adhesion between the substrate and the adsorbed protein layer. The MG 63 cells exhibited no cell loss after 7 days on the H terminated surface with contact angles in the range of 85-90°. An extremely hydrophilic nanostructured diamond surface with a contact angle of 2° resisted the adhesion of mesenchymal stem cells. A more hydrophobic nanostructured surface with a contact angle of 86° proved more suitable for MSC adhesion and proliferation [92, 99].

The presence of oxygen containing groups such as carboxyl, ester and carbonyl groups has been shown to increase the adsorption of adhesion promoting
proteins such as collagen, fibronectin and vitronectin; while inhibiting the adsorption of non-cell adhesive proteins such as albumin. The adsorption of Oregon green 488 labelled collagen IV was studied on polyethylene samples and it was observed that collagen IV adsorption was significantly greater on the samples with oxygen containing functional groups. As mentioned earlier the conformation of the adsorbed protein is more important than the quantity of protein adsorbed. A large amount of adsorbed protein has been shown to be disadvantageous in some instances as the inter-protein interactions which take place inhibit cell adhesion [100]. The adsorption of hen egg lysosome, insulin dimer and ribonuclease A were studied on hydrophobic self-assembled monolayers possessing CH₃ and CF₃ functional groups. The same proteins were adsorbed on hydrophilic surfaces possessing OH and CONH₂ groups. Larger quantities of protein were adsorbed on the hydrophobic surfaces but the larger concentration of protein resulted in inter-protein interactions. All of the proteins adsorbed on the hydrophobic surfaces underwent unfolding to some extent. The hydrophilic surfaces possessed lower concentrations of adsorbed protein with no significant inter-protein interactions and the proteins were adsorbed in their native state. Corroborating results were attained for fibronectin adsorption in another study utilizing self-assembled monolayers. 3T3 cells were seeded on the adsorbed fibronectin layer and cell adhesion was greatest on the OH and COOH functionalized surfaces rather than on the NH₂ and CH₃ terminated surfaces. The difference in fibroblast adhesion across the different surfaces is due to the changes in the conformation of the adsorbed fibronectin [93, 100].

\[1.9.1.2 \text{WETTABILITY}\]

Surface free energy or wettability also influences cell adhesion. Numerous attempts have been made to determine the exact surface free energy or contact angle that a biomaterial must possess in order for cell adhesion to take place. Surface free energy is defined as the excess energy at the surface compared to the bulk. Substrate wettability influences protein adsorption as discussed earlier in section 1.8.

Schakenraad et al. [101] studied the spreading of human fibroblasts on different polymer and glass substrates. They found that fibroblast adhesion and
spreading was greater on surfaces with surface energies of 57 mJ/m² [102]. They also investigated the effect of serum proteins on fibroblast spreading and found that cells adhered and spread equally well on all of the investigated substrates in the presence of serum. Osteoblastic cell adhesion and spreading was shown to be greater on substrates with contact angles less than 60° [101]. Keselowsky et al. [100] demonstrated that osteoblast gene expression and mineralization was better on self-assembled monolayers (SAMs) with OH and NH₂ groups than on SAMs with CH₃ terminal groups [100]. Lee et al. [103] studied cell adhesion on polyethylene substrates that had been treated with corona discharge. They found that endothelial and neural cell adhesion was better on moderately hydrophilic surfaces than on more hydrophobic surfaces [103]. Lim et al. [104] proved that the adhesion and proliferation of human fetal osteoblasts was better on more hydrophilic substrates [104]. Zelzer et al. [105] seeded fibroblasts on plasma polymerized surface with a gradient of contact angles. The adhesion and proliferation of fibroblasts was greater on the section with a contact angle of 60°. Cell density decreased as the contact angle increased above 60° [105]. Hydrophilic surfaces adsorb less protein than hydrophobic surfaces but the proteins undergo fewer conformational changes on hydrophilic surfaces. Consequently it is thought that adhesive proteins will retain their active conformation better on hydrophilic surfaces and this may be why cells adhesion is better on more hydrophilic surfaces.

1.9.1.3 ELECTRICAL CHARGE

The electrical charge of a surface also affects the extent and strength of cell adhesion. Cell membranes and cell adhesion promoting proteins are negatively charged and consequently cell adhesion is generally better on positively charged surfaces [106]. Stromal cells were seeded on positively charged and negatively charged 2-hydroxyethyl methacrylate hydrogels. Stromal cell adhesion and proliferation were better on the positively charged surfaces. 3T3 fibroblasts, human embryonic kidney cells and smooth muscle cells all adhered better on positively charged polytetrafluorethylene surfaces. As is the case with surface wettability it is the conformation of the adsorbed protein layer rather than thickness of said protein layer which determines whether or not electrically charged surfaces are cell adhesive [107]. Liu et al. [108] studied the adsorption
of osteopontin on self-assembled monolayers of alkane thiols terminated with either negatively charged COOH groups or positively charged NH₂ groups. They found that the quantity of protein adsorbed on both surfaces was equivalent but the number of bovine aortic endothelial cells was greater on the positively charged NH₂ terminated surfaces. Liu et al. [108] findings suggest that the positively charged surface allows osteopontin to adsorb in a conformation which is more favourable for cell adhesion [108]. Negatively charged surfaces have been shown to reduce cell-material adhesion and cell-cell adhesion between endothelial cells and platelets. Sulphonate groups are another example of a negatively charged functional group which can suppress cell adhesion. Sulphonate groups have been used to produce blood repulsive surfaces suitable for anti-coagulation [109].

1.9.1.4 SUBSTRATE STIFFNESS

Cell adhesion is regulated by the interaction of integrins with ECM proteins secreted by the cell. Inside the cell integrins interact with focal adhesions which are complex multiprotein structures. Focal adhesions connect the actin cytoskeleton and myosin stress fibres to the ECM. Tension forces are exerted via the stress fibres which are connected to the focal adhesions. The magnitude of the tension forces experienced by the cell is dependent on the elasticity or rigidity of the substrate the cells have adhered to. In vivo cells are exposed to a wide range of elastic moduli depending on the tissue type e.g. 0.1-1 kPa for neural tissue, 8-17 kPa for muscle and 25-40 kPa for bone and cartilage. Substrate stiffness plays a vital role in the modulation of several cell functions including motility, ECM synthesis, proliferation and differentiation [106].

Cell adhesion on extremely soft substrates is similar to cell adhesion on very hydrophilic substrates. Highly hydrophilic substrates prevent proteins from adsorbing strongly on the substrate surface. These proteins then detach from the surface and cell adhesion does not take place. In the case of soft substrates the adsorbed proteins also detach from the substrate as they are unable to withstand the fractional forces exerted on them by cells. Substrate rigidity influences the formation of cell adhesion complexes, cell spreading, cytoskeletal
assembly and cell differentiation. Engler et al. [110] seeded rat vascular smooth muscle cells on polyacrylamide gels covalently bound with collagen. The different gels possessed the same chemical composition but different rigidities. The smooth muscle cells were unable to form adhesion complexes on the soft polyacrylamide gels (elastic modulus $E=1$ kPa) and as a result cells did not adhere and proliferate. The down regulation of focal adhesion structural proteins talin, paxillin and focal adhesion kinase was observed for Madin-Darby canine kidney (MDCK) cells cultured on soft collagen gels. Polyacrylamide gels with an elastic modulus of 8 kPa were shown to promote the formation of focal adhesion complexes, actin cytoskeletal and consequently cell proliferation. A cell’s response to therapeutic drugs is also influenced by the substrate’s stiffness. MSCs which were cultured on substrates with elastic moduli in the range of 10-30 kPa were more sensitive to mytomycin C which is an anti-proliferative drug than MSCs which were cultured on stiffer substrates such as glass. Cell proliferation was impeded on highly rigid substrates as the cells adhered too strongly and consequently they were unable to undergo the reorganization required for proliferation [111].

Substrate stiffness can induce durotaxis. Durotaxis is observed in several cell types including epithelial and fibroblasts cells. Durotaxis is a phenomenon whereby cells migrate from softer regions of a substrate to more rigid regions. Durotaxis involves the reorganisation of the stress fibres and focal adhesions. AFM analysis of fibroblasts has shown that the cell modulus is influenced by the substrate modulus. Fibroblasts are capable of altering their internal stiffness to match that of their substrates. This modulation of cell stiffness may play a role in directing the migration of neighbouring cells and in wound repair [112].

It is well known that substrate properties can influence ECM protein synthesis. Adherent cells which have been seeded on rigid substrates synthesis greater quantities of fibronectin and collagen type I and III. Cell proliferation is also influenced by substrate stiffness though the exact effect on proliferation is dependent on cell type and function. Myoblasts in culture will fuse into myotubes irrespective of substrate stiffness but they will only striate on substrates with an elastic modulus similar to normal muscle. Embryonic cardiomyocytes cultured on a substrate with an elastic modulus equal to developing myocardium possess
actomyosin striation and 1Hz beating. When embryonic cardiomyocytes are cultured on a more rigid substrate they stop beating and they lack striated myofibrils. Proteome analysis of the embryonic cardiomyocytes showed that there was a distinct difference in the conformation of cytoskeletal proteins (filamin, vimentin and myosin) across the two surface types. Substrate stiffness can also be used to trigger the differentiation of mesenchymal stem cells (MSCs). MSCs which are cultured on substrates with an elastic modulus of muscle are myogenic, substrates mimicking the elasticity of the brain are neurogenic and substrates which mimic the elasticity of bone are osteogenic. As is clear from the examples discussed above substrate stiffness plays an important role in many cellular processes [113].

1.9.1.5 ROUGHNESS

The effect of surface topography or roughness is dependent on the cell phenotype. This is because the effect of surface topography is directly related to cell size/scale. Human osteosarcoma cells were seeded on titanium surfaces with hemispherical cavities measuring 10, 30 and 100 µm in diameter. Human osteosarcoma cells are approximately 30 µm in diameter [98]. The cells adhered and divided in the 30 µm cavities but the cells possessed a rounded morphology. The cells in the smaller and larger cavities displayed a flatter and spread morphology. Large cells such as human primary bone cells (50 µm) react differently than smaller cells such as platelets (2 µm) and monocytes (10 µm); this is due to the size difference of the cells biological elements which are interacting with the biomaterials surface. Essentially surface topography effects protein adsorption which in turn influences cell adhesion. Cells can sense surface topography via their focal adhesions and cytoskeleton. There is a threshold for a cells response to surface topography and it appears as if this threshold is close to the cells size [106].

The effect surface roughness has on cell adhesion depends on the scale of the surface roughness. In this review we will consider the effect of four different classifications of surface roughness; macro-roughness, micro-roughness, submicron-roughness and nano-roughness. Macro-roughness consists of surface roughness’s that are visible to the naked human eye. Micro-roughness consists of roughness values between 1 to 100 µm, submicron-roughness is
from 100 nm to 1 µm and nano-roughness describes any surface roughness less than 100 nm. Each classification will influence cell adhesion in a different manner. Macro-roughness is generally favourable in terms of cell adhesion as the adhering cells are able to spread/adhere between the surface irregularities. In some cases macro-roughness has been shown to improve cell adhesion and spreading on medical implants [106].

There is some debate as to whether or not micro-roughness promotes or inhibits cell adhesion. Adherent cells used in the majority of biomaterials studies typically possess diameters in the range of 10-50 µm when in suspension. Upon adhesion these cells flatten and spread and consequently their surface area will be in the range of micro-roughness. Due to the correlation between micro-roughness and cell area, micro-roughness will have an appreciable effect on cell adhesion. As previously mentioned some studies have reported a positive effect for micro-roughness. MG 63 cells were seeded on titanium micro-rough surfaces. The MG 63 cells on the micro-rough surfaces exhibited increased alkaline phosphatase activity, osteocalcin, prostaglandin E₂ and transforming growth factor. Rat osteoblasts seeded on titanium dental implants exhibited increased spreading on the micro-rough surfaces. Micro-topography was shown to induce the expression of genes encoding osteocalcin, alkaline phosphatase and Runx 2 in human embryonic palatal mesenchymal cells. The micro-rough surface also reduced the activity of osteoclasts and consequently the resorption of bone. There are an equal number of studies describing the negative effect of micro-roughness on cell adhesion. Kim et al. [114] studied the effect of micro-roughness on MG 63 cell seeded on Ti-6Al-4V. They found that the number of MG 63 cells on the micro-rough surface was significantly greater than on the submicron-rough surface. Also the MG 63 cells on the rougher substrate possessed an irregular morphology with numerous elongated processes. In contrast the cells on the smoother substrate displayed the typical MG 63 morphology. Cell proliferation was also slower on the rougher substrate. Micro-rough surfaces have been shown to inhibit the formation of focal adhesion plaques and cell proliferation. Osteoblasts grown on titanium surfaces with micro-roughness exhibited diminished alkaline phosphatase activity and proliferation [106, 115].
Similar to micro-roughness submicron-roughness may either enhance or inhibit cell adhesion and proliferation. Zhao *et al.* [116] reported decreased quantities of MG 63 cells on titanium surfaces with submicron-roughness compared to substrates with nano-roughness. The MG 63 cells on the submicron-rough sample synthesized more prostaglandin and transforming growth factor β₁ and osteocalcin. Prostaglandin and transforming growth factor β₁ promote osteoblastic activity and osteocalcin is a marker of osteogenic cell differentiation [116]. Grausova *et al.* [117] seeded MG 63 cells and bovine pulmonary artery endothelial cells on submicron-rough diamond layers. They found that endothelial cell adhesion was reduced on the submicron-rough substrate but MG 63 cell adhesion was unaffected [117].

The lack of a standard method for defining surface roughness may be the reason for this apparent contradiction. Surface roughness is commonly reported as either route mean square (RMS) or arithmetic average (Ra) but neither of these measurements provides information on the on the shape or spacing of the surface irregularities. All of these factors will influence cell adhesion and spreading. Consequently it can be difficult to compare roughness measurements from different research groups.

Nanoscale roughness generally has a positive effect on cell adhesion, viability, proliferation and differentiation. Nano-roughness is thought to facilitate the adsorption of cell adhesion promoting proteins in a conformation that is suitable for cell adhesion. Nano-roughness is similar to the nano-architecture of the ECM and consequently it can enhance cell adhesion. Smooth muscle cells and rat vascular endothelial cells were seeded on poly (lactic-co-glycolic acid) (PLGA) substrates. Cell proliferation and adhesion was better on the nano-rough surfaces compared to flat PLGA [106, 118].

As is clear from the discussion above cell adhesion is a complex process. It is thought that the presence of oxygen containing groups will enhance cell adhesion; these groups facilitate the adsorption of cell adhesion promoting proteins in a suitable conformation for cell adhesion. Hydrophobic surfaces have been shown to adsorb greater quantities of protein but this does not always result in improved cell adhesion, as it is the conformation of the adsorbed
protein which is important. Cell adhesion promoting proteins undergo fewer conformational changes upon adsorption on hydrophilic surfaces and as a result they retain their active conformation and hydrophilic surfaces perform better in terms of cell adhesion. Cell adhesion is greater on positively charged surfaces rather than on negatively charged surfaces; this is again due to the conformation in which cell adhesion promoting proteins are adsorbed. Cells will not adhere and proliferate on substrates which are too soft as cell adhesion promoting proteins will detach from surfaces which are extremely soft. A cells preference for substrate stiffness is dependent on cell type. The effect of roughness is dependent on cell type though macro and nano scale roughness are thought to be beneficial for the majority of cell types. The effect of micro and sub-micro roughness are more controversial with some studies stating that they support cell adhesion and others that they inhibit cell adhesion. In order for a material to be suitable for cell culture a balance must be achieved between adhesion strength and the cells ability to undergo the conformational rearrangements and movement necessary for proliferation.

When developing a biomaterial it is important to consider the intended application as this will determine the properties said biomaterial should possess. It is critical that the thermoresponsive polymer films discussed in this body of work are capable of promoting cell adhesion and proliferation. In some cases the opposite is desirable i.e. in the case of anti-fouling surfaces. Understanding the physical and chemical factors which influence cell adhesion is vital in order to be able to develop a biomaterial with the appropriate properties.

The majority of cells are anchorage dependent and they must adhere to a suitable surface in order to proliferate. Anchorage dependent cells will initially adopt a rounded morphology in cell culture media. If a substrate is biocompatible the cells will rearrange their cytoskeleton and adhere to the surface [111]. Consequently the properties of the chosen biomaterial will influence cell morphology along with cell density. At low seeding densities 3T3 fibroblasts adopt multipolar fibroblast morphology; as cell density approaches confluence 3T3 cells take on a more epithelial or cobble stone morphology. Human lung and kidney fibroblasts are multi or bipolar at low seeding density but at high seeding density they are bipolar only [119]. It is vital that
comparisons between cells grown on different samples; are only carried out when the cells are at the same stage of growth or possess the same cell density. In the case of adherent cells; studies have shown that the larger the number of cell that have adhered after 24 hours of culture the larger the number of cells after 21 days of culture. Anchorage dependent cells need to adhere to a suitable substrate in order to proliferate [120]. Cell morphology can be used as an indicator of cell health [121]. If a substrate is unsuitable the cells become granulated and develop vacuoles. Successful cell adhesion is vital for normal cell junction development and proper cell function.

1.10 CELL ADHESION MOLECULES (CAM)

Plasma proteins are necessary for cell adhesion. A biomaterial must be capable of adsorbing both proteins present in the cell culture media and those produced by the cells. Cell surface receptors mediate the adhesion of cells through protein interactions. Cell adhesion molecules (CAMs) are an important class of molecule located on the surface of animal cells [122]. CAMs play a vital role in cell-cell and cell-ECM interactions. CAMs ensure that cells combine in the correct proportions in order to produce multicellular organs which carry out the desired function [123]. CAMs can also act as chemical and mechanical sensors. CAMs can bind homophilicly or heterophilicly based on their interaction with CAMs in neighboring cells. Homophilic CAMs only interact with the same type of CAM. Heterophilic CAMs can interact with different types of CAMs [124]. The transmembrane proteins responsible for these interactions can be divided into four categories

- Integrins
- Selectins
- Immunoglobulins
- Cadherins

Integrins link the cytoskeleton and the ECM and they are responsible for sending biochemical signals across the plasma membranes. The cells cytoskeleton rearranges in response to these signals. This allows the cell to
remodel or migrate in response to stimuli. Integrins are involved in gene expression, cell adhesion, the regulation of cell growth and cell shape. Integrins can also bind to immunoglobulin superfamily members [125].

Selectins mediate cell-cell adhesions in the bloodstream. They are carbohydrate binding proteins (lectins). In vertebrates one of the main roles selectins carry out is regulating the movement of white blood cells. Selectins allow white blood cells to bind to the endothelium facilitating the movement of white blood cells from the bloodstream to tissue. There are three classes of selectin; L, P and E. L-selectins bind to lectin domains on white blood cells, P selectins bind to platelets and E-selectins bind to the endothelium. Selectins work in tandem with integrins in binding blood cells to the endothelium [126].

Immunoglobulins are involved in the differentiation and morphogenesis of nerve, muscle and glial cells. These CAMs also facilitate cell surface recognition and they promote leukocyte adhesion and extravasation in vascular endothelial cells. Immunoglobulins also play a vital role in trapping white blood cells at sites of inflammation [127].

Cadherins are involved in the formation of adherens junctions. There are several subclasses of cadherin. Cadherins play an important role in cell sorting or cellular segregation due to their ability to form highly selective homophilic interactions. Cadherins mainly interact with their homologous analogues on adjacent cells e.g. E-cadherins which are found on epithelial cells will interact with other E-cadherins. Cadherins require Ca$^{2+}$ ions in order to function [98, 124].

### 1.11 CELL-CELL JUNCTIONS

Cell junctions play a vital role in communication between cells in a multicellular organism. The different types of cell junction also play a role in determining the shape and strength of a cell along with the cellular composition in a multicellular organ [98]. Cell junctions are classified according to their function and the four different types of cell junctions are;
• Anchoring junctions which are connected to the cytoskeletal filaments in a cell. This type of junction includes both cell-cell and cell-ECM adhesions.

• Channel forming junctions include gap junctions in animals and plasmodesmata in plants. This class of junction connects the cytoplasm of neighboring cells.

• Occluding junctions include tight junctions, found in vertebrates and septate junctions, found in invertebrates. Occluding junctions are responsible for sealing the spaces between cells creating an impermeable or semipermeable barrier.

• Signal relaying junctions include immunological and chemical synapses, transmembrane ligand receptors and cell-cell signaling contacts. Signal relaying junctions facilitate the transmittance of signals from one cell to another at areas of cell-cell contact.

A brief description of anchoring, channel forming and occluding junctions will be given below as these are the types of junctions that are of interest in this body of work.

Adherens junctions are a type of anchoring junction that connect actin filaments in one cell to actin filaments in an adjacent cell [128]. Desmosomes connect intermediate filaments in one cell to those in a neighboring cell. Hemidesmosomes connect the ECM to intermediate filaments in the cell. The final type of anchoring junction is the actin linked cell matrix adhesions. As the name suggests this type of junction connects the ECM to actin filaments located in the cell [129]. Adherens junctions play a vital role in cell motility as they allow cells to contract their actin cytoskeleton in a coordinated fashion. Desmosomes provide mechanical strength [130].

Epithelial cell sheets act as barriers; they separate the fluid in tissues on their basal side from the fluid on their apical side [122, 129]. Occluding junctions act as seals preventing molecules and proteins from diffusing from one side to another. The main family of proteins found in occluding junctions is claudins. Different claudins are expressed in different tissues resulting in different
permeabilities e.g. claudin type 1 is located in the epidermal layer of the skin [130].

Gap junctions allow connected cells to share inorganic ions and small molecules (<900 Daltons) [131]. They are vital in coordinating the activity of electrically active cells. Gap junctions couple cells chemically and electrically [132]. The permeability of gap junctions can be regulated. Gap junctions are composed of two different families of channel forming proteins; connexins (found in vertebrates) and innexins (found in invertebrates). Connexins combine in groups of six to form a connexion or hemi-channel. Gap junctions in vertebrates are composed of many different connexions acting together to form a molecular sieve. Different tissues express different connexions and these combine to form gap junctions with different permeabilities [98].

1.12 MOTIVATION FOR THE DEVELOPMENT OF THERMORESPONSIVE CELL CULTURE SURFACES

Traditionally adherent cells in cell culture have been detached either by mechanical scrapping with a silicon spatula or via proteolytic enzymes such as trypsin. Detachment via mechanical scrapping yields clumps of cell surrounded by a crystalline matrix. Enzymatic digestion gives individual rounded cells. Neither of these techniques is suitable for the recovery of intact cell sheets. Both mechanical scrapping and enzymatic digestion have been shown to reduce cell functionality and disrupt cell membranes [133]. Trypsin has been shown to alter the proteomics of mammalian cells. Huang et al. [134] observed that exposure to trypsin resulted in the upregulation of apoptotic associated proteins and the down regulation of growth related proteins [134]. Cellular glutathione was reduced by approximately 95% in murine keratinocytes that were exposed to trypsin. Glutathione loss has a detrimental effect on apoptotic and cytotoxic activities. Reiners et al. [135] showed that this loss of glutathione was only temporary and that normal cellular levels of glutathione were replenished within 24 hours [135]. This temporary reduction in cellular glutathione may invalidate any cytotoxicity studies carried out during the period of glutathione reduction. Mechanical disaggregation has been shown to result in a loss of intracellular DNA. The crystalline matrix which surrounds cells detached via mechanical
scraping is the intra-cellular contents leaking out of the cell via the damaged cell membranes [136].

Cells and cell sheets detached via temperature control from thermoresponsive polymer surfaces do not experience the damage associated with either mechanical scraping or enzymatic digestion. Canavan et al. [133] proved that temperature mediated cell detachment could be used to detach intact cell sheets and the associated extracellular matrix (ECM). This study showed that both mechanical scraping and enzymatic digestion were detrimental to cell function and the secreted ECM. Enzymatic digestion proved less invasive than mechanical scraping [133]. Another study by Canavan et al. [137] proved that residual lipid fragments were left behind on cell culture substrates, following cell detachment via mechanical scraping [137]. This supports the argument that mechanical scraping can damage cell membranes. A comparison of mesenchymal stem cells (MSCs) detached via proteolytic digestion and temperature controlled detachment from pNIPAm copolymer films proved that MSCs detached from the pNIPAm copolymer films possessed better differentiation, cell viability and larger colonies than those detached via proteolytic digestion [138]. Von Recum et al. [139] observed that retinal pigment epithelial cells which had been detached via trypsinisation suffered from a loss of function resulting in impaired retinoid metabolism upon repeated subculturing. When the retinal pigment epithelial cells were subcultured using temperature mediated cell detachment no loss of function was observed [139].

Traditional cell detachment methods result in individual cells or clumps of cells with damaged cell membranes and ECM; none of which is attractive from a tissue engineering point of view [140]. It is possible to detach intact cell sheets with the associated ECM using thermoresponsive cell culture substrates (Figure 1.17). These cell sheets are suitable for transplantation or they can be stacked or combined in 2D/3D tissue engineering. pNIPAm's ability to change from a hydrophilic substance below the LCST to a hydrophobic substance above the LCST has led to its popularity in the field of cell culture. Adherent cells will adhere and spread on the pNIPAm coated surfaces above the LCST when pNIPAm is in its condensed hydrophobic state. Once the desired level of cell detachment has been achieved cell detachment is facilitated simply by lowering
the temperature below the polymers LCST. Below the LCST the pNIPAm coating will swell and possibly dissolve depending on whether or not it is covalently bound to the underlayer substrate. This swelling and possible dissolution will result in cell detachment. There are some drawbacks to this seemingly simple and attractive method of cell detachment. Cell adhesion and proliferation is poor on bulk or thick pNIPAm coatings. Several strategies have been developed in order to increase the bio-adhesion of pNIPAm. A detailed review of these attempts is given in this section [141, 142].

Figure 1.17: Schematic illustration depicting a) traditional cell detachment using either a proteolytic enzyme or mechanical scrapping b) cell adhesion and detachment from a thermoresponsive polymer film [141]. This figure has been removed due to copyright restrictions and can be viewed in the reference given here.
1.13 HISTORY OF CELL ADHESION AND DETACHMENT FROM THERMORESPONSIVE POLYMER FILMS

1.13.1 Grafted Films

Grafted films are bound to the underlayer substrate and upon temperature reduction the polymer layer will simply swell rather than dissolving. This swelling facilitates the detachment of cell and cell sheets. The lack of dissolution is advantageous from a tissue engineering point as there is no free polymer in contact with the harvested cell sheet.

The use of electron beam polymerization (EBP) in order to create pNIPAm coatings was pioneered by Yamada et al.[143]. EBP has been further developed by the work of Okano et al.[144]. Okano et al. [144] have grafted ultra-thin (nm) thick layers of pNIPAm onto tissue culture polystyrene (TCP) in order to create suitable thermoresponsive surfaces for cell adhesion and detachment [144-148]. There is a thickness threshold when working with EBP polymer films. Films thicker than 30 nm are unsuitable for cell adhesion and proliferation. Okano et al. [148] found that films with sub 30 nm thicknesses were capable of both cell adhesion and detachment. A wide range of cell types have been successfully grown on EBP films including; corneal epithelial cells, cardiomyocytes, epidermal keratinocytes, oral mucosal epithelial cells and skeletal myoblasts [148-154]. It is possible to produce intact cell sheets using this method of cell detachment and there has been some success in stacking the resultant cell sheets. Depending on the cell type used these cell sheets can be utilized in a variety of clinical applications including; repair of esophageal ulcerations, dilated cardiomyopathy, plastic reconstructive and regenerative surgery and ophthalmology. Sheets of myoblasts have been transplanted in human patients; this resulted in a significant improvement in the patients’ cardiac function [143, 151, 155]. Bare EBP pNIPAm is not a suitable substrate for all cell types. A coating of cell adhesion promoter (CAP) such as fibronectin is necessary in order to achieve cell proliferation when using primary cells. This fibronectin coating impaired cell detachment and a coating of serum was necessary in
order for cell detachment to proceed [156]. Sumide et al. [157] reported that a layer of collagen type IV was necessary in order to achieve a monolayer of human corneal endothelial cells. It is possible to buy EBP pNIPAm dishes for cell culture use [157]. Of all of the coating techniques available for the production of thermoresponsive polymer surfaces for cell culture, EBP is the most extensively researched.

Pan et al. [41] proved that it was possible to graft pNIPAm onto a range of substrates using plasma polymerization [41]. Canavan et al. [158] continued Pan’s work and they successfully grafted pNIPAm onto TCP [158]. Plasma polymerization can cause monomer fragmentation which results in a loss of the polymers thermoresponsive properties. Bullet et al. [40] conducted an investigation in order to determine the ideal power and temperature conditions necessary to produce polymer films which retained their intrinsic properties [40]. They found that high temperature and high power produced the most stable films. As the plasma power was increased the nitrogen and oxygen content of the film decreased and consequently monomer integrity was lost. High operating temperatures resulted in a loss of chemical functionality. In order to produce pNIPAm coatings which possess the ability to swell and de-swell reversibly a balance between moderate power and low temperature must be maintained. XPS analysis has confirmed that if optimized conditions are used, it is possible to produce pNIPAm coatings without fragmentation or loss of functionality [41, 133]. Bovine carotid artery endothelial cells (BAEC) have been grown on plasma polymerized pNIPAm surfaces. The BAEC detached completely within 2 hrs of temperature reduction [158, 159]. Retinal tissue cells have also been successfully grown on pNIPAm surfaces prepared via plasma polymerization [160, 161]. Successful cell growth on plasma polymerized surfaces appears to be thickness independent.

Controlled living radical polymerization (CLRP) techniques were developed in recent years in order to graft pNIPAm with specific thickness/density for particular applications. The previously discussed grafting techniques EBP and plasma polymerization may suffer from batch to batch inconsistencies and it is hoped that CLRP will eliminate this problem, yielding highly reproducible thermoresponsive films with the desired properties every time [147, 162]. Linhui
et al. [17] used surface atom transfer radical polymerization (SI-ATRP) to prepare pNIPAm films of varying thicknesses. HepG2 and HeLa cells were seeded on these films and they observed that brush thickness was a determining factor in successful cell adhesion and detachment. Cells grew on surfaces with brush thicknesses in the range of 20-45 nm and temperature mediated cell detachment was also successful. Surface hydrophilicity increased as brush thickness increased and Linhui et al. [17] noted that brushes of 200 nm thickness possessed highly hydrophilic contact angles (10°) above the LCST [17]. Highly hydrophilic surfaces are not conducive to protein adsorption and this would have a knock on effect in terms of cell adhesion. Thinner brushes are generally less hydrophilic as the polymer molecules are more tightly packed and this prevents a high level of water penetration. Mizutani et al. [147] reported a similar thickness dependence for pNIPAm films produced by SI-ATRP [147]. Xu et al. [163] experienced the opposite effect; they observed that 3T3 cell adhesion increased as brush thickness increased [163]. This apparent anomaly may be due to the difference in the underlayer substrate used. Xue et al. [164] and Yu et al. [165] studied protein adsorption on pNIPAm brushes prepared via SI-ATRP and they observed a similar juxtaposition. Bovine serum albumin (BSA) adsorption as a function of molecular weight and brush density was studied by Xue et al.[164]. BSA adsorption was greater on the surfaces with lower brush density and molecular weight. They used the primary, secondary and tertiary protein adsorption theory developed by Halperin and Norde to explain their observations [166, 167]. Primary protein absorption is adsorption directly onto the underlayer substrate, adsorption on the outer brush surface is classified as secondary adsorption and tertiary adsorption requires inter brush penetration. Surfaces with high brush densities would inhibit primary and tertiary adsorption due to the tight packing of the polymer chains. Xue et al. [164] proposed that hydration of the outer brush surface would inhibit secondary adsorption thus rendering the surfaces unsuitable for cell culture. Contact angle measurements were taken above the LCST for surfaces with different molecular weights and densities and no significant difference in surface wettability was observed. Consequently secondary protein adsorption must be inhibited by something other than surface wettability. Yu et al. [165] investigated the adsorption of human serum albumin (HSA) on pNIPAm surfaces prepared via
SI-ATRP [165]. They observed that HSA adsorption was greater on surfaces above the LCST irrespective of brush thickness. Surfaces with thicknesses less than 15 nm only displayed a marginal increase in HSA adsorption above the LCST. In the sub-15 nm range the largest amount of HSA adsorption was observed on the 2.5 nm thick substrate. This may be due to influence of the silicon underlayer substrate. Yu et al. [165] proposed that the sub 15nm films may be suitable anti-fouling surfaces. This is surprising as these films possess contact angles in the range of 65-71° and anti-fouling surfaces are generally highly hydrophilic in order to prevent protein adsorption. Yu et al. [165] observed a 300% increase in HSA adsorption on the 38 nm thick films above the LCST compared to HSA adsorption on the same film below the LCST. HSA adsorption is likely to proceed via secondary protein adsorption due to its size. Yu et al. [165] suggested that the underlayer substrate may restrict conformational collapse in the shorter brush regime. As brush thickness increases the underlayer substrate will no longer have such a large influence and this would result in a different hydration profile for the thicker film. Further experimentation with more samples in the thicker brush regime is required in order to either prove or disprove this theory.

Reversible addition fragmentation chain transfer polymerization (RAFT) is another CLRP technique which can be used to prepare grafted pNIPAm surfaces with precise thickness/density. RAFT polymerization uses a chain transfer agent (CTA) which determines the growth of the polymer chains. If the ratio of monomer to CTA is carefully controlled it is possible to produce grafted pNIPAm surfaces with the desired thickness/density. If the amount of CTA to monomer is increased the graft length decreases [168].

Takahashi et al. [169] found that graft density could be controlled by using initiator immobilized surfaces of predetermined densities. Takahashi et al. [169] noted that cell adhesion was poor on surfaces with high graft densities but subsequently cell detachment was much faster on surfaces with high graft densities due to the ease of hydration. While cell adhesion was greater on low graft density surfaces large quantities of adhered cells failed to detach upon temperature reduction. A balance must be achieved in order for both cell adhesion and detachment to be possible. BAEC were seeded on RAFT pNIPAm
films with a range of chain lengths and densities. Cells on the lower graft density films were confluent within 48 hours but an incubation time of 5 days was required for the BAEC to become confluent on the high graft density surfaces. Cell sheet detachment was observed within 30 minutes of cold treatment on the high graft density surfaces. Cell detachment was impeded on the low graft density surfaces, with some cells requiring 24 hours for successful detachment. Static contact angle measurements were taken and these values were compared to contact angle measurements for EBP films. A clear difference in contact angle measurements above and below the LCST was observed for the EBP films. Only a negligible difference in contact angle was recorded for the RAFT films. This suggests that changes in the surface wettability are not solely responsible for cell adhesion and detachment. It is possible that some conformational change takes place which effects pNIPAm’s ability to bind with various biomolecules.

It is possible to use RAFT to create surfaces with specific terminal functionality as the living macro CTA group may be retained during polymerization. Takahashi et al. [169] prepared pNIPAm surfaces with a range of different end groups [169]. Smooth muscle cells (SMC) were seeded on the prepared surfaces and they found that carboxylation of the thermoresponsive surfaces increased cell adhesion. This functionalization of the polymer surfaces meant that high graft density surfaces capable for cell adhesion and detachment could be produced. This study suggests that it may be possible to develop surfaces specifically optimized for the growth of particular cell types.

1.13.2 Non-grafted films

Takezawa et al. [170] reported on one of the first attempts to improve the cell adhesion of non-grafted pNIPAm coatings. Solvent casting was used to prepare films of pNIPAm and pNIPAm conjugated with collagen. Human dermal fibroblasts were seeded on both film types. Cell adhesion was poor on the pNIPAm only films but the human dermal fibroblasts reached confluence on the pNIPAm-collagen films. Cells were successfully detached from the pNIPAm-collagen films upon temperature reduction [170]. Rollason et al. [171] postulated
that bulk pNIPAm films were unsuitable cell culture substrates due to their high level of hydration. Rollason et al. [171] synthesized a pNIPAm-co-N-tert butylacrylamide (NtBAm) copolymer in order to try and improve the bioadhesiveness of pNIPAm. The copolymer they developed was 70% pNIPAm and 30% NtBAm and it possessed an LCST of 8°C. Solvent casting was used to produce the copolymer films and normal rat kidney cells were seeded on the films. Cell growth was significantly less on the copolymer films compared to either the glass or TCP controls. Complete cell detachment was observed within 2 hrs of temperature reduction [171]. Selezneva et al. [172] produced a series of pNIPAm: NtBAm copolymers with a range of compositions; 100:0, 85:15, 65:35 and 50:50. They noted that L929 fibroblast grew successfully on all of the solvent cast copolymer films but cell growth was poor on the pNIPAm homopolymer film. Cell detachment was possible for all of the copolymer films but as the percentage of NtBAm increased so did the cell detachment times [172]. Moran et al. [173] reported on the use of a CAP layer in order to increase the bio-adhesion of thick pNIPAm solvent cast films, 3T3 cells and human umbilical vein endothelial cells (HUVEC) were seeded on uncoated pNIPAm films, TCP controls and pNIPAm films coated with collagen, fibronectin and laminin. Cell growth on the CAP coated films was comparable to the TCP control. Cell detachment rates were not affected by the addition of the CAP layer [13, 173]. A chitosan –pNIPAm copolymer was synthesized by Chen et al. [174] and films were prepared via solvent casting. 3T3 fibroblasts grew to confluence and detached from the copolymer films [174]. Shimizu et al. [175] treated solvent cast pNIPAm films with high intensity plasma. The treated films exhibited increased cell adhesion compared to the untreated films. Shimizu et al. [175] proposed that the plasma treatment increased the number of oxygen containing groups on the films surface and this subsequently increased cell adhesion. The plasma treated portion of the film lost its thermoresponsive properties. As a result a thin layer of polymer was left attached to the cell sheet after the bulk of the polymer had dissolved. The recovered murine skeletal muscle cells contracted upon exposure to an electrical pulse and thus the cells appeared to be fully functional. Nash et al. [176] used solvent casting to produce pNIPAm films of varying thicknesses (1-4 µm). 3T3 fibroblasts and hMSCs were seeded on the prepared films. The pNIPAm films were not coated with a layer of cell
adhesion promoter prior to cell seeding. As film thickness increased cell adhesion and proliferation decreased. Cell viability assays confirmed that cells grown on the 1μm thick solvent cast films were comparable to those grown on the TCP control. Intact cell sheets were recovered within 15 minutes of temperature reduction for the 3T3 fibroblasts and 30 minutes for the hMSCs. The detached hMSCs were reseeded and detached from new 1μm thick pNIPAm films three times. Flow cytometry was used to examine cell surface markers on the hMSCs. Multi passaging of the hMSCs using temperature mediated cell detachment did not result in the differentiation of the hMSCs [176]. Fan et al. [177] used spin coating and solvent casting to prepare pNIPAm and poly (NIPAm-co-NtBAm) films ranging in thickness from 100 nm to 4 μm. AFM was used to determine the thickness of the polymer films. The spin coated films possessed thicknesses of 100 nm and 1 μm and the solvent cast films possessed the following thicknesses; 1, 2 and 4 μm. RAW 264.7 cells were seeded on the prepared films. RAW 264.7 cell adhesion and proliferation was observed on the solvent cast pNIPAm films and on both solvent cast and spin coated poly (NIPAm-co-NtBAm) films. The spin coated pNIPAm films proved to be unsuitable cell culture substrates for the RAW 264.7 cells. Cell viability assays showed that RAW 264.7 cells grown on poly (NIPAm-co-NtBAm) films were comparable to those grown on the TCP control. As the thickness of the solvent cast pNIPAm films increased, cell adhesion decreased. This may be due to the increased hydration observed for the thicker pNIPAm films. The increased hydrophilicity would hamper protein adsorption and this would have a negative effect on cell adhesion. IL-1β production was lower in the RAW 264.7 cells grown on the poly (NIPAm-co-NtBAm) films. Cluster of Differentiation 80 (CD80) and major histocompatibility complex class II (MHCII) levels were measured in the detached cells using flow cytometry. CD80 levels were lower in cells detached from the poly (NIPAm-co-NtBAm) films. Low levels of MHCII were observed in cells detached from solvent cast pNIPAm and poly (NIPAm-co-NtBAm) and spin coated poly (NIPAm-co-NtBAm) films [177].

Nash et al. [178] developed a spin coating system to prepare pNIPAm coating for cell culture using commercially available (Sigma Aldrich) pNIPAm. Their objective was to make thermoresponsive surfaces more accessible by avoiding
the expense and expertise associated with grafting techniques as well as the need to synthesize polymer in house. The main disadvantage of their system is that the polymer layer will dissolve upon temperature reduction. Studies have shown that there is no cytotoxicity associated with pNIPAm. Nevertheless it is still better to avoid having free polymer in contact with cells. A wide range of cell types were grown on these films including; human mesenchymal stem cells (hMSCs), human keratinocytes and 3T3 fibroblasts. Cells grew well on a wide range of film thicknesses and consequently there does not appear to be a thickness dependency associated with this deposition method [178]. Nast et al. [179] also studied cell growth on poly (NIPAm-co-ethylpyrrolidone methacrylate (EPM)) films. EPM is more hydrophilic than NIPAm and consequently the addition of EPM will increase the LCST, bringing it closer to physiological temperature. This is advantageous as this opens up the possibility of in vivo thermoresponsive polymer applications. Films were prepared via spin coating and 3T3 fibroblasts grew to confluence on both the copolymer films and the TCP controls. Cell detachment was observed on the copolymer films within 70 minutes of cold treatment [179]. A poly (NIPAm-co-acrylamidobenzophenone (AcBzPh)) copolymer was developed in order to overcome the dissolution problem discussed earlier. Upon exposure to UV light the 1 % of AcBzPh ensures that the polymer layer is covalently bound to the TCP underlayer substrate. A thickness threshold was observed with 3T3 cells growing and detaching successfully from sub 30 nm films. Films thicker than 30 nm were not suitable for cell adhesion [180]. Reed et al. [181] reported on the co-deposition of pNIPAm with a sol gel using spin coating. The tetraethyl orthosilicate (TEOS) was prepared in a solvent mixture of H₂O-EtOH-HCl. pNIPAm was dissolved in HCl. The pNIPAm solution was introduced to the TEOS and the mixture was deposited onto a glass coverslip at 2000 RPM for 60 seconds. Reed et al. [181] seeded BAEC on the films and the cells reached confluence after four days on all of the films irrespective of their composition. Cell detachment was not observed on either the glass coverslip control or on the 100 % TEOS samples. The BAECs detached from the pNIPAm containing samples in clumps or as single cells, this limits the usefulness of this technique from a tissue engineering point of view. Reed et al. [181] postulated that the poor cell detachment was due to the non-uniform surface topography. Yang et al. [182] prepared a pNIPAm
terpolymer poly (NIPAm-co-hydroxypropyl methacrylate-co-3(trimethoxysilyl) propyl methacrylate (NIPAm-co-HPM-co-TMSPM)). This terpolymer is capable of crosslinking to a substrate via temperature annealing. Once again an optimal thickness range for cell growth was noted. HEK293 cells and HeLa cells proliferated on films within the 3 to 13 nm thickness range. Cell detachment upon temperature reduction was possible within this thickness range [182]. Nakayama et al. [183] used spin coating to prepare nanometer thick films of poly (butyl methacrylate) PMBA--b-PNIPAm. The block polymer was dissolved in a mixture of AcCN/DMF (5:1 v/v) and a range of concentrations were prepared (0.1, 0.3 and 0.5 wt. %). Polymer films were formed on silicon wafers for physical characterization and on TCP dishes for cell culture. Poly (PBMA-b-PNIPAm) was used as Nakayama et al. [183] hoped that the PBMA would prevent or lessen polymer dissolution upon temperature reduction. The polymer films were immersed in water and the temperature was reduced from 37 °C to 4 °C. Negligible changes in pNIPAm concentration were observed upon temperature reduction. Ellipsometry was used to determine film thickness and film thickness ranged from 7.0 to 23.3 nm. AFM analysis proved that the polymer films were smooth (roughness less than 2 nm for the 7.0 nm thick film). Static contact angle measurements were taken at 20 °C and 37 °C and a negligible difference in contact angle above and below the LCST was observed. Nakayama et al. [183] proposed that the lack of change in surface wettability was due to the nanophase separated structures formed on the silicon wafers. The pNIPAm domains are surrounded by PBMA domains and thus it is possible that this is masking the change in wettability. Bovine aortic endothelial cells (BAECs) were seeded on the polymer films and on the TCP controls. As polymer film thickness increased cell adhesion and proliferation decreased. BAECs seeded on the 23.3 nm thick films possessed a doubling time of 61 hrs compared to the 31 hrs doubling time observed on bare TCP and the 7.0 and 15.4 nm thick films. Upon temperature reduction to 20 °C BAECs detached from the 15.4 and 23.3 nm thick films within 30 minutes. An incubation time in excess of 20 hrs was required for complete cell detachment from the 7 nm thick film. Fibronectin (FN) adsorption was investigated using rhodamine labelled FN. FN adsorption decreased as film thickness increased and larger amounts of FN were adsorbed at 37 °C than at 20 °C on the polymer films. Bare TCP adsorbed
the same amount of FN regardless of temperature and it adsorbed more FN than any of the polymer films. Nakayama et al. [183] suggested that the differences in FN adsorption were due to increased polymer chain hydration as polymer film thickness increased. This increased hydration would prevent FN and other vital cell adhesion mediating proteins from adsorbing and consequently this is why cell adhesion and proliferation decreased as film thickness increased. It is thought that the conformational change of pNIPAm affects protein adsorption/desorption which in turn triggers cell detachment. The thinnest polymer film (7.0 nm) possessed a larger cell surface affinity than the other polymer films and consequently cell detachment was exceedingly slow. The 15.4 nm thick is the most promising in terms of both cell adhesion and detachment [183].

The previously discussed methods of thermoresponsive cell detachment give rise to planar cell sheets. While these cell sheets are certainly useful it would be more beneficial to be able to produce pNIPAm coatings which could be used to produce cell sheets mimicking their native structure in vivo. Matsuda et al. [184] produced a pNIPAm gelatin copolymer which was then cast into the inside of a glass capillary tube. The resultant thermoresponsive film was 54 μm thick [184]. BAECs were seeded on the thermoresponsive coating via a syringe. The capillary tube was then immersed in a container of cell culture media and incubated for four days. Cell detachment was facilitated by the injection of culture media via a syringe after the capillary tube had been allowed to reach room temperature. The copolymer coating was not covalently bound to the underlayer substrate and it dissolved upon temperature reduction. Considering the thickness of the coating a considerable amount of polymer may have been adsorbed by the cells. No information on the possible effects of this was provided. Duarte et al. [185] developed a 3D thermoresponsive poly D, L lactic acid construct loaded with pNIPAm in super critical CO₂. This 3D cell culture construct was developed in order to increase the cell culture surface compared to a 2D cell culture surface. L929 fibroblasts were seeded in the 3D construct and cell adhesion and detachment was successful. The recovered cells were comparable to the control cells in terms of cell viability [185].
1.14 FACTORS WHICH EFFECT CELL ADHESION AND DETACHMENT FROM THERMORESPONSIVE POLYMER FILMS

1.14.1 Surface Wettability

It is thought that a biomolecule’s ability to support cell adhesion is largely dependent on surface wettability. Contact angles are widely reported in the literature as a result. A summary of contact angles reported for surfaces prepared using a range of polymer deposition methods is given below in Table 1.3.

Table 1.3: Contact angle measurements of pNIPAm coatings produced by different methods

<table>
<thead>
<tr>
<th>pNIPAm deposition method</th>
<th>T&gt;LCST</th>
<th>T&lt;LCST</th>
<th>Cell Adhesion</th>
<th>Cell Detachment Time (hr.)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBP (0.8 µg/cm²)</td>
<td>46.3° *</td>
<td>35.9° *</td>
<td>Yes</td>
<td>1</td>
<td>[186]</td>
</tr>
<tr>
<td>pNIPAm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EBP (1.35 µg/cm²)</td>
<td>42.2° *</td>
<td>31.7° *</td>
<td>No</td>
<td>n/a</td>
<td>[186]</td>
</tr>
<tr>
<td>pNIPAm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EBP (1.4 µg/cm²)</td>
<td>77.9° +</td>
<td>65.2° +</td>
<td>Yes</td>
<td>Detachment occurred, time not given</td>
<td>[187]</td>
</tr>
<tr>
<td>pNIPAm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EBP (2.9 µg/cm²)</td>
<td>69.5° +</td>
<td>60.0° +</td>
<td>No</td>
<td>n/a</td>
<td>[187]</td>
</tr>
<tr>
<td>pNIPAm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spin coating</td>
<td>45.94° ^</td>
<td>n/a</td>
<td>Yes</td>
<td>0.2</td>
<td>[188]</td>
</tr>
<tr>
<td>pNIPAm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Method</td>
<td>pNIPAm coating temperature (°C)</td>
<td>pNIPAm adsorption temperature (°C)</td>
<td>Detachment occurred</td>
<td>Time (h)</td>
<td>Source</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>---------------------------------</td>
<td>-----------------------------------</td>
<td>---------------------</td>
<td>----------</td>
<td>--------</td>
</tr>
<tr>
<td>Spin coating (5 mg/ml)</td>
<td>51°+</td>
<td>42.5°+</td>
<td>Yes</td>
<td></td>
<td>[189]</td>
</tr>
<tr>
<td>wet</td>
<td></td>
<td></td>
<td>Detachment occurred, time not given</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma polymerization (1 W)</td>
<td>40°*</td>
<td>34°*</td>
<td>Yes</td>
<td></td>
<td>[190]</td>
</tr>
<tr>
<td>pNIPAm</td>
<td></td>
<td></td>
<td>Detachment occurred, time not given</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UV crosslinked pNIPAm-co-CCMS</td>
<td>87.8°l</td>
<td>37.2°l</td>
<td>Yes</td>
<td>0.5</td>
<td>[191]</td>
</tr>
<tr>
<td>(0.7%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UV crosslinked pNIPAm-co-CCMS</td>
<td>92.6°l</td>
<td>40.5°l</td>
<td>Yes</td>
<td>0.5</td>
<td>[191]</td>
</tr>
<tr>
<td>(1.2%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sol-gel (0.35%)pNIPAm/TEOS</td>
<td>50°a</td>
<td>33°a</td>
<td>Yes</td>
<td>2 (75.3% detached)</td>
<td>[192]</td>
</tr>
<tr>
<td>Sol-gel (0.3%) pNIPAm/TEOS</td>
<td>48°a</td>
<td>44°a</td>
<td>Yes</td>
<td>2 (39.3% detached)</td>
<td>[192]</td>
</tr>
<tr>
<td>Solvent cast oxygen plasma treated pNIPAm (60 W)</td>
<td>19.2°+</td>
<td>n/a</td>
<td>Yes</td>
<td>Detachment occurred, time not given</td>
<td>[193]</td>
</tr>
<tr>
<td>ATRP (PNIPAm-b-PS)</td>
<td>79°+</td>
<td>62.1°+</td>
<td>Yes</td>
<td>24</td>
<td>[165]</td>
</tr>
<tr>
<td>ATRP (2.1±0.2 µg/cm²)</td>
<td>45°+</td>
<td>n/a</td>
<td>Yes</td>
<td>2</td>
<td>[194]</td>
</tr>
<tr>
<td>ATRP (5.2 ±0.3 µg/cm²)</td>
<td>43.94°+</td>
<td>37.81°+</td>
<td>Yes</td>
<td>1</td>
<td>[147]</td>
</tr>
<tr>
<td>pNIPAm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATRP (8.2 ± 0.2 µg/cm³)</td>
<td>35.9°+</td>
<td>29.5°+</td>
<td>No</td>
<td>n/a</td>
<td>[147]</td>
</tr>
<tr>
<td>pNIPAm</td>
<td>59°+</td>
<td>n/a</td>
<td>Yes</td>
<td>Detachment occurred, time not given</td>
<td>[164]</td>
</tr>
<tr>
<td>ATRP pNIPAm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Contact Angle</td>
<td>Static Contact Angle</td>
<td>Cell Detachment</td>
<td>Cell Detachment</td>
<td>Reference</td>
</tr>
<tr>
<td>--------------------------</td>
<td>---------------</td>
<td>----------------------</td>
<td>-----------------</td>
<td>-----------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ATRP 1% PEGMA-pNIPAm</td>
<td>49° +</td>
<td>n/a</td>
<td>Yes **</td>
<td>0.16</td>
<td>[163]</td>
</tr>
<tr>
<td>ATRP (PEGMA-b-PNIPAm)</td>
<td>59° +</td>
<td>n/a</td>
<td>Yes</td>
<td>0.66</td>
<td>[163]</td>
</tr>
<tr>
<td>ATRP (0.90±0.18 µg/cm²) pNIPAm</td>
<td>75.52° +</td>
<td>72.52° +</td>
<td>Yes</td>
<td>2</td>
<td>[195]</td>
</tr>
<tr>
<td>ATRP pNIPAm (8 mm)</td>
<td>93° +</td>
<td>65° +</td>
<td>Yes</td>
<td>1</td>
<td>[17]</td>
</tr>
<tr>
<td>RAFT (COOH-PNIPAm)</td>
<td>68.8° +</td>
<td>n/a</td>
<td>Yes</td>
<td>0.5</td>
<td>[196]</td>
</tr>
<tr>
<td>RAFT(pNIPAm) 0.25D0.04</td>
<td>45.57° +</td>
<td>41.4° +</td>
<td>Yes (20% after 6hrs)</td>
<td>0.16</td>
<td>[168]</td>
</tr>
<tr>
<td>RAFT (pNIPAm) 1.0D0.04</td>
<td>54.5° +</td>
<td>55.24° +</td>
<td>Yes (50% after 6hrs)</td>
<td>0.5</td>
<td>[168]</td>
</tr>
<tr>
<td>UV crosslinked</td>
<td>70° ^</td>
<td>n/a</td>
<td>Yes</td>
<td>0.16</td>
<td>[180]</td>
</tr>
<tr>
<td>Poly (NIPAm-co-AcBzPh) (13nm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UV crosslinked Poly (NIPAm-co-AcBzPh) (188nm)</td>
<td>70° ^</td>
<td>n/a</td>
<td>No</td>
<td>n/a</td>
<td>[180]</td>
</tr>
</tbody>
</table>

* = captive air bubble, += static contact angle, ^= advancing contact angle **= cell growth less than control! =dynamic Wilhelmy plate, a= inverted bubble contact angle

Except in the case of thermoresponsive polymer films prepared by RAFT there is a marked difference in the contact angle behavior above and below the polymers LCST. Cell detachment from RAFT films is thought to be driven by conformation changes which occur in the polymer film rather than hydration dynamics. As is evident from the data shown in table 1.3 above there is a large disparity in terms of reported contact angles for successful cell adhesion and detachment. This may be a result of the range of methods available for measuring contact angle; advancing/captive air bubble/sessile/static/Wilhelmy plate. Xu et al. [163] and Mizutani et al. [147] observed contradictory behavior in terms of cell adhesion and thickness for their SI-ARTP prepared pNIPAm films
[147, 163]. When we examine the contact angles for these surfaces we see that Xu et al. recorded a contact angle of 59° for a 30 nm thick film and Mizutani et al. [147] observed a contact angle of 44° for a film of similar thickness. Interestingly cell adhesion and spreading was possible on the more hydrophobic surface but not on the more hydrophilic one. Both of the above mentioned contact angles were measured using the same technique; static contact angle. Mizutani et al. [147] recorded a contact angle of 46° for their thinner polymer films (thickness of 11 nm). Cell adhesion and proliferation was observed on the thinner films. The marginal difference in contact angle of the thinner cell adhesive film and the thicker non-adhesive film highlights the fact that surface wettability alone is not a clear indicator as to whether or not a surface will be suitable for cell culture.

1.14.2 Thickness

Thicknes measurements are widely reported in thermoresponsive polymer literature, as there appears to be a thickness threshold for successful cell adhesion and detachment. Plasma polymerized thermoresponsive polymer films and spin coated films prepared from pNIPAm sourced from Sigma Aldrich appear to be acceptations to this thickness threshold. CLRP techniques were developed in order to try and accurately control the thickness of polymer films. Table 1.4 below summarizes some of the thickness measurements available for thermoresponsive polymer films prepared via a number of different methods.
Table 1.4: Thickness measurements of pNIPAm coatings produced by different methods.

<table>
<thead>
<tr>
<th>pNIPAm deposition method</th>
<th>Thickness&lt;LCST (Dry nm)</th>
<th>Thickness&gt;LCST (After Immersion nm)</th>
<th>Thickness&lt;LCST (After Immersion nm)</th>
<th>Cell Adhesion</th>
<th>Cell Detachment Time (hr.)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBP (0.8 µg/cm²) pNIPAm</td>
<td>3.3*</td>
<td>6.0*</td>
<td>8.0*</td>
<td>Yes</td>
<td>1</td>
<td>[186]</td>
</tr>
<tr>
<td>EBP (1.35 µg/cm²) pNIPAm</td>
<td>8.8*</td>
<td>12.9*</td>
<td>25.4*</td>
<td>No</td>
<td>n/a</td>
<td>[186]</td>
</tr>
<tr>
<td>EBP (1.4 µg/cm²) pNIPAm</td>
<td>15.5*</td>
<td>n/a</td>
<td>n/a</td>
<td>Yes</td>
<td>Detachment occurred, time not given</td>
<td>[187]</td>
</tr>
<tr>
<td>EBP (2.9 µg/cm²) pNIPAm</td>
<td>29.3*</td>
<td>n/a</td>
<td>n/a</td>
<td>No</td>
<td>n/a</td>
<td>[187]</td>
</tr>
<tr>
<td>Spin coating pNIPAm</td>
<td>106*</td>
<td>n/a</td>
<td>n/a</td>
<td>Yes</td>
<td>0.2</td>
<td>[188]</td>
</tr>
<tr>
<td>Spin coating (5 mg/ml) cycle 1 pNIPAm</td>
<td>14+</td>
<td>18+</td>
<td>67+</td>
<td>Yes</td>
<td>Detachment occurred, time not given</td>
<td>[189]</td>
</tr>
<tr>
<td>Spin coating (5 mg/ml) cycle 2 pNIPAm</td>
<td>n/a</td>
<td>22+</td>
<td>47+</td>
<td>Yes</td>
<td>Detachment occurred, time not given</td>
<td>[189]</td>
</tr>
<tr>
<td>Process</td>
<td>pNIPAm</td>
<td>Plasma polymerization (1 W) sample1</td>
<td>UV crosslinked 0.3% Poly (NIPAm-co-AcBzPh)</td>
<td>ATRP (pNIPAm-b-PS)</td>
<td>ATRP (2.1±0.2 µg/cm²)</td>
<td>ATRP (5.2 ±0.3 µg/cm²)</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>--------</td>
<td>-----------------------------------</td>
<td>---------------------------------------------</td>
<td>------------------</td>
<td>-----------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>Spin coating 5 mg/ml</td>
<td>14+</td>
<td>n/a</td>
<td>30+</td>
<td>Yes</td>
<td>Detachment occurred, time not given</td>
<td></td>
</tr>
<tr>
<td>cycle 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pNIPAm</td>
<td>n/a</td>
<td>42*</td>
<td>55*</td>
<td>n/a</td>
<td>n/a</td>
<td>[189]</td>
</tr>
<tr>
<td>Plasma polymerization (1 W) sample1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[190]</td>
</tr>
<tr>
<td>UV crosslinked 0.3% Poly (NIPAm-co-AcBzPh)</td>
<td>13.1*</td>
<td>n/a</td>
<td>n/a</td>
<td>Yes</td>
<td>0.16</td>
<td>[197]</td>
</tr>
<tr>
<td>ATRP (pNIPAm-b-PS)</td>
<td>44.+</td>
<td>n/a</td>
<td>n/a</td>
<td>Yes</td>
<td>24</td>
<td>[165]</td>
</tr>
<tr>
<td>ATRP (2.1±0.2 µg/cm²)</td>
<td>10.9+</td>
<td>n/a</td>
<td>n/a</td>
<td>Yes</td>
<td>2</td>
<td>[194]</td>
</tr>
<tr>
<td>ATRP (5.2 ±0.3 µg/cm²)</td>
<td>30.4+</td>
<td>n/a</td>
<td>n/a</td>
<td>Yes</td>
<td>1</td>
<td>[147]</td>
</tr>
<tr>
<td>pNIPAm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[147]</td>
</tr>
<tr>
<td>ATRP (8.2 ± 0.2 µg/cm²)</td>
<td>64.7+</td>
<td>n/a</td>
<td>n/a</td>
<td>No</td>
<td>n/a</td>
<td>[147]</td>
</tr>
<tr>
<td>pNIPAm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[147]</td>
</tr>
<tr>
<td>ATRP pNIPAm</td>
<td>30+</td>
<td>n/a</td>
<td>n/a</td>
<td>Yes</td>
<td>Detachment occurred, time not given</td>
<td></td>
</tr>
<tr>
<td>ATRP 1% PEGMA-pNIPAm</td>
<td>33+</td>
<td>n/a</td>
<td>n/a</td>
<td>Yes</td>
<td>0.16</td>
<td>[163]</td>
</tr>
<tr>
<td>Method</td>
<td>Thickness</td>
<td>Film Type</td>
<td>Adhesion</td>
<td>Cell Growth</td>
<td>Ellipsometry</td>
<td>AFM/SEM-3D-MEX</td>
</tr>
<tr>
<td>------------------------</td>
<td>-----------</td>
<td>----------------------</td>
<td>----------</td>
<td>-------------</td>
<td>--------------</td>
<td>----------------</td>
</tr>
<tr>
<td>ATRP (PEGMA-b-pNIPAm)</td>
<td>19+</td>
<td>n/a</td>
<td>n/a</td>
<td>Yes</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>ATRP pNIPAm</td>
<td>26+</td>
<td>n/a</td>
<td>n/a</td>
<td>Yes</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>UV crosslinked Poly (NIPAm-co-AcBzPh)</td>
<td>13*</td>
<td>n/a</td>
<td>n/a</td>
<td>Yes</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>UV crosslinked Poly (NIPAm-co-AcBzPh)</td>
<td>188*</td>
<td>n/a</td>
<td>n/a</td>
<td>No</td>
<td>n/a</td>
<td></td>
</tr>
</tbody>
</table>

*=AFM, “= SEM-3D-MEX, += Ellipsometry, **= cell growth less than control

As mentioned earlier pNIPAm coatings prepared via EBP are only conducive to cell adhesion and proliferation if their film thickness is less than 30 nm. It was proposed that the thinner polymer film allows for the interaction of the underlayer substrate with the seeded cells. Okano et al. [148] showed that the TCP underlayer substrate affects the wettability of polymer films that are less than 30 nm in thickness. As film thickness increases the underlayer substrate has less of an influence on film properties [148, 155, 156, 198]. While the exact thickness range for optimal growth on either SI-ATRP or RAFT surfaces has yet to be identified, brush thickness plays a vital role in whether a surface is capable of cell adhesion and subsequent temperature mediated cell detachment [17, 168, 169, 199]. Film thickness does not appear to influence the biocompatibility of plasma polymerized films [186]. Thickness appears to be a limiting factor for spin coated films which are chemically bound to their underlayer substrate [180]. It is not clear why films which are not chemically bound do not exhibit the same thickness sensitivity. The innermost layer of a hydrogel which is confined or
bound to an underlayer substrate behaves differently than the bulk layer of the hydrogel [200, 201]. This manifests as a change in the glass transition temperature ($T_g$) of the hydrogel. The innermost layer of the hydrogel has a lower polymer density due to the nano-confinement effect. Consider a hydrogel as composed of two distinct layers, layer 1 is the lower polymer density layer i.e. the innermost layer and this layer possesses a lower $T_g$. The 2nd layer is the bulk polymer layer which possesses higher polymer density and a higher $T_g$ [202]. It is thought that ultra-thin nanometer thick hydrogels are comprised of only the layer 1 portion; if this theory is correct when cells that are interacting with ultra-thin films would be interacting with a more hydrated surface compared to thicker polymer films [203]. Nash et al.’s [180] study showed only a negligible difference in surface wettability between the 188 nm thick cell resistant poly (NIPAm-co-AcBzPh) spin coated films and the cell adhesive 13 nm thick films [180]. As suggested by Cole et al. [142] it might be more pertinent to investigate the level of hydration of a polymer film in conjunction with surface wettability measurements [142]. The level of hydration may cause changes in mechanical properties depending on the films thickness and this in turn may influence cell adhesion and detachment. Non grafted or covalently bound polymer films would not experience the same mechanical forces and as such they would experience different thickness limitations.

As is evident from the studies mentioned above a great deal of research has been conducted on the use of thermoresponsive polymer films for cell culture. Grafted polymer films appear to be the most attractive due to the lack of polymer dissolution upon temperature reduction. The main disadvantage of grafted films is the large investment required in equipment and specialist training. The crosslinkable spin coated systems offer an alternative to grafting techniques but this approach requires the expertise and equipment necessary to conduct polymer synthesis. Solvent casting is one of the simplest methods available for producing pNIPAm films; the addition of animal sourced CAPs makes these films undesirable from a tissue engineering standpoint. Physical adsorption as a method of thermoresponsive film preparation offers many advantages. It is a simple coating technique which many cell culture users already utilize. Previous studies have shown that it is possible to produce nanometer thick pNIPAm films
using this method of film preparation. A variety of pNIPAm copolymers could be adsorbed exploring cell adhesion and detachment from crosslinkable and non-crosslinkable physical adsorbed thermoresponsive polymer films.

1.15 MECHANISM OF CELL ADHESION AND DETACHMENT FROM THERMORESPONSIVE POLYMERS

Okano et al. [144] studied the cell adhesion and detachment of rat hepatocytes and human endothelial cells on electron beam polymerized pNIPAm films. They concluded that cell adhesion and detachment from thermoresponsive surfaces is composed of two stages: a passive stage and an active stage. Passive cell adhesion involves the seeded cells approaching the thermoresponsive surface. Once the cells have made contact with the surface they begin the process of altering their membranes and morphology in order to adhere to the surface, this is active adhesion. pNIPAm must be hydrated in order to facilitate cell detachment. This is achieved by lowering the temperature below the polymers LCST. In the case of the rat hepatocytes and the human endothelial cells Okano et al. [144] lowered the temperature to 10 °C and 20 °C respectively. The polymer film was successfully hydrated upon temperature reduction but only 15% of the adhered cells detached within 30 minutes of temperature reduction. Okano et al. [144] raised the temperature of both the rat hepatocytes and the human endothelial cells and they found that 100% of the cell had detached within 10 minutes. These findings lead Okano et al. [144] to the conclusion that cell detachment from thermoresponsive polymer surfaces must require cell metabolic activity. They proposed that the initial temperature reduction inhibited cell metabolic activity and this is why the majority of cells (85%) did not detach until the temperature was increased. Passive cell detachment involves the hydration of the thermoresponsive polymer. Active cell detachment requires cell rearrangement and ATP consumption and thus it cannot proceed below a certain temperature. Okano et al. [144] state that the exact temperature required for active and passive cell detachment is cell dependent. The above mentioned mechanism for cell adhesion and detachment was developed using grafted
pNIPAm films. Consequently the mechanism may be slightly different for non-grafted films as the polymer layer will dissolve upon temperature reduction [144].

1.16 MAIN AIMS/OBJECTIVES

- The main aim of this project is to develop and characterize thermo-responsive polymer films suitable for cell culture. This investigation aims to determine whether physical adsorption can be used to produce thermo-responsive polymer substrates suitable for cell adhesion and temperature controlled cell detachment. The physically adsorbed films will be prepared above and below the chosen polymer’s LCST in order to determine whether or not polymer conformation affects the physical and chemical properties of the films. The resultant films’ roughness, thickness, percentage composition and wettability will be characterized using a variety of techniques.

- The biocompatibility of the physically adsorbed films will be assessed by seeding human pulmonary microvascular endothelial cells (HPMEC) on the prepared films. Cell activity and cell number will be measured in order to determine the cytocompatibility of the physically adsorbed films.

- Temperature controlled cell detachment from the physically adsorbed films will be assessed.

- The detached cells and cell sheets will be examined using electron and fluorescent microscopy in order to determine the viability of cell and cell sheets which have been detached via temperature control.

- The physical, chemical and cell application effects of film immobilization via the use of the photocrosslinkable monomer AcBzPh will be investigated for the physically adsorbed films.

- The best physically adsorbed polymer system in terms of cell adhesion and detachment will be determined.

- The cell culture potential of a new class of thermo-responsive polymer that swell rather than dissolving upon temperature reduction without the need for a covalent bond to the underlayer substrate will be investigated.


69. Zhu, P.W. and D.H. Napper, *Conformational transitions of poly (n-isopropylacrylamide) chains loopily absorbed at the surfaces of poly (n-tert-
96. Anselme, K., M. Bigerelle, B. Noel, E. Dufresne, D. Judas, A. Iost, and P. Hardouin, Qualitative and quantitative study of human osteoblast adhesion on materials


119. Freshney, R.I., Culture of animal cells, a manual of basic technique, John Wiley and Sons, inc.


The materials and methods used in each chapter varied and this section acts as a summary of the techniques used. Each chapter will contain a detailed description of the materials and methods used to investigate the particular polymer system outlined in said chapter.

2.1 MATERIALS

Poly(N-isopropylacrylamide) (Mn 20,000-25,000), Dulbecco’s Modified Eagle Medium (DMEM) cell culture medium, M199 cell culture medium, Hank’s balanced salts solution (HBSS), trypsin-EDTA, penicillin-streptomycin, fetal bovine serum (FBS), fetal calf serum (FCS), trypan blue stain, sodium heparin, 3T3 fibroblasts, triton X-100, phosphate buffered saline, poly L lysine, fibronectin, collagen, dimethyl sulfoxide (DMSO) and sodium cacodylate trihydrate were purchased from Sigma-Aldrich and used as received. Quant-iT™ PicoGreen® dsDNA assay kit and glutamax from Invitrogen, alamarBlue™ assay from Biosciences, endothelial growth factor supplement were purchased from Becton Dickinson. Agar low viscosity resin kit and 2 % osmium tetroxide from Agar Scientific, 25 % Glutaraldehyde solution in water from AMSBIO and Thermanox™ plastic 35 mm disks from Nunc™ were all used as received. Glass bottomed Petri dishes from MatTek™ and 8 well chamber slides from Ibidi™. All other plastic consumables were purchased from Sarstedt. Primary rabbit anti-paxillin antibodies were purchased from Abcam. Secondary goat anti-rabbit antibodies, phalloidin, Hoechst and normal goat serum were purchased from Invitrogen. An Ectotherm chilling and heating dry bath from Torrey Pines Scientific was used for careful temperature control. Human pulmonary microvascular endothelial cells (HPMEC) were kindly provided by Prof. James Kirkpatrick from the Institute of Pathology, Johannes-Gutenberg University.
2.2 THERMORESPONSIVE FILM PREPARATION

Table 2.1: Substrate type used during film preparation and application

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermanox™ disks</td>
<td>Cell culture, AFM, contact angle, SEM and TEM</td>
</tr>
<tr>
<td>TCP Petri dish</td>
<td>Cell culture and XPS</td>
</tr>
<tr>
<td>Aluminum coupons</td>
<td>FT-IR</td>
</tr>
<tr>
<td>High quality quartz glass</td>
<td>Profilometry</td>
</tr>
<tr>
<td>Ibidi™ 8 well slides</td>
<td>Fluorescent Microscopy</td>
</tr>
</tbody>
</table>

2.2.1 Physical Adsorption

Physically adsorbed thermoresponsive films were fabricated by adding 2 ml of a 0.1 mg/ml aqueous solution of polymer to the desired substrate, which in this case was either tissue culture polystyrene (TCP Petri dish) or Thermanox™ (cover slip placed in a TCP Petri dish). After an incubation period of three hours, at either above or below the polymers' LCST, (10 °C and 40 °C for poly (NIPAm-co-NtBAm) and poly (NIPAm-co-NtBAm-co-AcBzPh), and 20 °C and 40 °C for pNIPAm and poly (NIPAm-co-AcBzPh), the remaining polymer solution was removed. A chilling and heating dry bath was used to ensure that the samples were maintained at an appropriate temperature. The samples were then washed twice with 2 mls of Millipore water and dried for two hours on the chilling and heating dry bath in ambient air followed by complete drying in a vacuum oven at 40 °C for 24 hrs. In the case of the TCP dishes the lids were removed during both deposition and drying. Adsorption may be slightly different on TCP and Thermanox™. In this investigation it was found that both cell growth and detachment were identical on TCP and Thermanox™ and consequently they will be used interchangeably.
2.2.2 Spin Coating

The poly (NIPAm-co-ODMA) and poly (NIPAm-co-NtBAm-co-ODMA) films were fabricated by initially depositing a 150 μl aliquot of an ethanolic polymer solution onto a slowly spinning substrate, (150 RPM) on a Laurell Technologies WS-400B-6NPP/LITE spin coater. Initially the substrate rotates slowly in order to allow the solution to spread evenly across the surface. Once the short period of slow rotation is complete the sample begins to rotate rapidly and this results in the evaporation of the volatile ethanol solvent, leaving behind a thin polymer film. The following parameters were used; concentration 2 % w/v pNIPAm in ethanol, final spin speed 6000 RPM, final spin time 30 s. Films were sterilized by exposure to UV light for two hours prior to cell culture [1].

2.2.2.1 FILM DRYING

Polymer films deposited from volatile solvents such ethanol need to be dried slowly in a solvent rich atmosphere in order to prevent surface irregularities. A solvent rich atmosphere such as an ethanol soaked dessicator slows down the rate of solvent evaporation leading to uniform and transparent films. If the films are dried in air the rate of solvent evaporation is too high and this can cause concentration gradients to develop within the film, this can lead to a mass transfer effect known as the Marangoni effect [2]. The Marangoni effect is the result of mass transfer in a liquid due to surface tension differences which will arise if the sample isn’t properly dried. All films prepared via spin coating were placed in an ethanol soaked dessicator overnight. The films were then transferred to a vacuum oven set to 40 °C and 600 mbar for a minimum of 4 hours in order to ensure that any residual solvent was removed. This drying method also prevents the samples from coming into contact with air borne water vapor, which would result in a phase separation of the films. pNIPAm is soluble in either water or ethanol but it is insoluble in a mixture of both. The reason for this insolubility is thought to be due to the formation of water and alcohol complexes rather than polymer solvent complexes forming and this leads to the precipitation of pNIPAm below the LCST, which is undesirable. The absorption of water vapor by the spin coated films would result in non-reproducible films as
the amount of air borne water vapor varies from day to day and thus the amount of water absorbed by the film would also vary. As the physically adsorbed films were prepared from aqueous solution there was no need to expose them to the ethanol soaked environment and as a result they were placed directly in the vacuum oven [1, 3].

2.2.3 Film Sterilization

All films were sterilized prior to cell culture by exposure to UV irradiation in a laminar flow hood for 2 hours. Films prepared from UV crosslinkable poly (NIPAm-co-AcBzPh) and poly (NIPAm-co-NtBAm-co-AcBzPh) were not sterilized in this manner instead the films were exposed to a more powerful lamp during the crosslinking procedure and this also sterilized the films [1, 4].

2.3 POLYMER SYNTHESIS

2.3.1 Poly (NIPAm-co-NtBAm), poly (NIPAm-co-AcBzPh) and poly (NIPAm-co-NtBAm-co-AcBzPh)

Poly (NIPAm-co-NtBAm) (0.65 molar ratio NIPAm and 0.35 molar ratio of NtBAm) was prepared by free radical polymerization using 2, 2′-Azobis (2-methylpropionitrile) (AIBN) (0.5 mol %) as the initiator in benzene (10 %, w/w) under Ar gas. After polymerization at 60 °C for 24 hours, the mixture was precipitated in n-hexane. Precipitation was repeated three times using acetone as a solvent and n-hexane as a non-solvent, and the product was dried at 45 °C in a vacuum oven [5]. Poly (NIPAm-co-AcBzPh) (0.99 molar ratio NIPAm and 0.01 molar ratio AcBzPh) was synthesized using the same procedure as poly (NIPAm-co-NtBAm) [6]. The synthesis of poly (NIPAm-co-NtBAm-co-AcBzPh) (0.65 molar ratio of NIPAm, 0.338 molar ratio of NtBAm and 0.012 molar ratio of AcBzPh) was identical to the synthesis of poly (NIPAm-co-AcBzPh) and poly (NIPAm-co-NtBAm) where a certain amount of NIPAm was replaced by NtBAm to obtain the desired composition.
2.3.2 Poly (NIPAm-co-ODMA) and poly (NIPAm-co-NtBAm-co-ODMA)

The synthesis of the copolymer of octadecyl methacrylate (ODMA) and N-isopropylacrylamide (NIPAm) was carried out according to the procedure outlined below. The ODMA from Merck was used as received. NIPAm from Aldrich was recrystallized three times from n-hexane. AIBN was used as the initiator at a concentration of 1 mol % of the monomers. 1, 4-dioxane was used as the solvent for the polymerization solution. The concentration of monomers was 20% (w/v). Oxygen was removed by passing argon for 40 minutes. The reaction was started by placing the reaction vessel fitted with a condenser in an oil bath at 63 °C. The polymerization was carried out for 22 hours under argon atmosphere. The resulting polymer was purified by a precipitation from acetone to n-hexane and the dried in vacuum oven at 60 °C.

The synthesis of poly (NIPAm-co-NtBAm-co-ODMA) (0.65 molar ratio of NIPAm, 0.3425 molar ratio of NtBAm and 0.0075 molar ratio of ODMA) was carried out according to the procedure identical to the synthesis of poly (NIPAm-co-ODMA) where a certain amount of NIPAm was replaced by NtBAm to obtain the desired composition.
Figure 2.1: Chemical structure of a) N-isopropylacrylamide, b) N-tert-butylacrylamide, c) acrylamidebenzophenone and d) octadecyl methacrylate

*The synthesis of poly (NIPAm-co-NtBAm), poly (NIPAm-co-AcBzPh), poly (NIPAm-co-NtBAm-co-AcBzPh), poly (NIPAm-co-ODMA) and poly (NIPAm-co-NtBAm-co-ODMA) was performed by Dr. Alexander Gorelov, School of Chemical Biology and Chemistry, UCD, Dublin 4, Ireland*
2.4 POLYMER CHARACTERIZATION

2.4.1 Gel Permeation Chromatography (GPC)

The number average molecular weight ($M_n$) and polydispersity index (PDI) of the copolymers was determined by gel permeation chromatography (GPC). GPC analyses were carried out with PL-gel mixed-D (300*7.8 mm, 5μm nominal particle size) Polymer Labs columns. The mobile phase used was dimethylformamide (DMF) with 30 mM KBr. Separations were performed at 35 °C at a flow rate of 1 mL/min using a RI detector. Molecular weights of polymers were referenced to polystyrene standards [5].

This work was kindly carried out by Dr. Maria Nash, Institute of Polymer Science and Technology CSIC, Juan de la Cierva 3, Madrid, Spain

2.4.2 Lower Critical Solution Temperature (LCST)

The LCST of the polymer solutions was determined by turbidity measurements in aqueous solutions (1 % w/v) ($\lambda = 500$ nm) using a Cary 3 Bio UV-Visible spectrophotometer equipped with a Cary temperature controller. The heating rate was 0.5 °C/min [1]. The LCST of the physically adsorbed films is approximately 2 °C lower than the LCST of the polymer solution. This difference in LCST may be due to the method used to measure the LCST. The LCST of the polymer solution is measured by cloud point which measures the end point of the LCST transition. The LCST of thermoresponsive polymer films is usually measured by calorimetry which records the start point of the LCST transition, the breaking of the water polymer bonds.
AFM is a type of scanning probe microscopy (SPM) used to measure surface features at the atomic level. AFM utilizes a sharp force sensitive tip which is attached to a cantilever. Figure 2.2 shows a schematic of an AFM. Depending on the distance between the sample and the tip, the tip experiences either attraction or repulsion. These attractive and repulsive forces cause the deflection of the cantilever. The deflections can be measured and these measurements are then used to construct a topographical image of the surface of interest [7].

The two main modes of operation in atomic force microscopy are contact mode and tapping mode. In contact mode the force sensitive tip is in direct contact with the sample and it moves slowly across the surface gathering topographical information. Contact mode is suitable for hard substrates such as metals and crystals. In tapping mode the tip is close to the surface and the tip intermittently taps the surface as repulsive forces are experienced. Tapping mode is more suitable for soft substrates such as polymer films and as such it is the mode used in this investigation. AFM was used to determine both roughness and thickness in this research.
Figure 2.2: Schematic of an AFM [7]. This figure has been removed due to copyright restrictions and can be viewed in the reference given here.
2.5.2 Roughness

AFM was used to assess the roughness of the thermoresponsive polymer films using 10 µm × 10 µm scans with a scan rate of 1 Hz. Images were taken with a Veeco Dimension 3100 AFM (Digital Instruments, Santa Barbara, CA, USA) using a 1-10 Ohm-cm phosphorus (n) doped Si tip and a matrix of 512 × 512 points along the x-y plane were analyzed in a single scan. Figure 2.3 above is an image of the AFM used in this investigation. Film roughness was recorded as
the route mean square (RMS) roughness, where RMS denotes the standard deviation of the Z-value along the reference line [1, 6].

\[ \text{RMS} = \sqrt{\frac{\sum_{i=1}^{N} (Z_i - Z_{\text{ave}})^2}{N}} \]  

(24)

Where; Z ave is the average Z value within the given area, Z i is the current Z value and N is the number of points within the given area.

2.5.3 Thickness-AFM Scratch Method

Physically adsorbed films were prepared according to the procedure outlined above. Thermanox™ disks were used as the under layer substrate. The thickness of the physically adsorbed films was measured using an AFM operating in scratch mode. The following parameters were used; ramp size 1.229 µm, scan rate 1 Hz, trigger threshold 0.2 V, scan angle 90°, scratch length 1 µm and scan size 3 µm. Samples were scratched in three random areas and fifteen thickness measurements were taken from each scratch [8, 9].

2.5.4 Profilometry

There are three different types of interferometer white light scatter plane, diffraction grafting and vertical scanning or coherence probe which is the type used in this investigation. Vertical scanning interferometers can reach very high resolution, down to a quarter of a wavelength though they possess a limited dynamic range. Profilometers measure the inference produced after white light has passed through a beam splitter and been reflected through a microscope objective and then on to the sample of interest and a reference sample. The interference fringes produced when these two signals interact provide information on the sample thickness and roughness. A white light source is often employed in profilometry as the resultant fringes will be easy to find.

The Zygo Newview 100 surface profiler provides film thickness measurements to an accuracy of 0.1 nm. Figure 2.4 shows a schematic of the profilometer used. The Zygo Newview 100 surface profiler’s field of view can vary from 6 mm × 4.5 mm down to 0.18 mm × 0.14 mm. It has a vertical range of 100 µm and it
can measure roughness and step-heights in the range 0.1 nm up to 10's of microns. Scans of three randomly selected areas along each scratch were recorded on three different samples in order to insure statistical accuracy. The objective used for the measurements was a 20X Mirau, with zoom set at 0.5X. The profile outlines the step height and this is obtained from the interference patterns produced as the objective scans through the vertical range. The variation of the interference patterns is recorded by a CCD camera, the data is analyzed by fast Fourier transform and the height distance can is measured from the generated surface profile. Poly (NIPAm-co-ODMA) and poly (NIPAm-co-NtBAm-co-ODMA) films were prepared on high quality quartz glass via spin coating and then scratched using a micropipette tip.

Figure 2.4: Optical schematic of interference microscope used for measurement of a sample surface [10]. This figure has been removed due to copyright restrictions and can be viewed in the reference given here.
2.5.5 Contact Angle

The wettability of a surface is an important parameter in terms of cell adhesion and growth. The wettability of the thermoresponsive films produced in this investigation were measured using advancing contact angle. In order to determine the wettability of a substance, the balance of the adhesive and cohesive forces acting on a liquid deposited on the surface must be measured. Adhesive forces will try and spread the liquid across the surface, while cohesive forces will cause the drop to bead. Contact angle is a measure of the wettability of a solid by an applied liquid and it is defined as the angle formed by the liquid at the intersection of the three phase boundary [11].

The Young equation for surface wetting below describes the relationship between contact angle and the surface energies of a substance at the point where the solid, liquid and gas phase meet.

\[ \gamma_{sv} - \gamma_{sl} - \gamma_{lv}\cos\theta = 0 \]  

(25)

Where \( \gamma_{sv} \) = solid/vapor interface surface tension, \( \gamma_{sl} \) = solid/liquid interface surface tension, \( \gamma_{lv} \) = liquid/vapor interface surface tension and \( \cos\theta \) is the contact angle. Surface tension can also be defined as the surface energy per unit area.

There are a number of factors which can influence the contact angle of a surface and these include [11, 12]:

- Contamination of the liquid
- Surface roughness
- Contamination of the surface
- Mechanical deformation of the surface by liquid
• Adsorption of the liquid by the surface leading to altered surface properties

There are a variety of different techniques which may be used to determine the contact angle of a substance. The simplest of these is static contact angle; this method simply involves placing a liquid drop on the sample surface and determining the contact angle. The volume of the drop does not change during the course of this experiment; though the shape of the drop may change due to interactions between the liquid and the sample surface, as a result there is some debate as to whether or not static contact angles provide a true reflection of surface dynamics [13].

There are two types of dynamic contact angle, advancing and receding. In advancing contact angle liquid is constantly added to the drop at a controlled and constant rate allowing the drop to advance across the surface of interest. In receding contact angle measurements liquid is removed from the drop at a controlled and constant rate. Dynamic contact angle measurements establish a new boundary surface each time the drop advances or recedes. Figure 2.5 shows the formation of a new boundary line as the drop advances or recedes. It is important that the rate of either liquid insertion or retraction is not too quick as the new boundary requires time in order to establish equilibrium. It is thought that dynamic contact angle measurements are more reproducible as a new contact angle is measured each time the drop advances or recedes.
Figure 2.5: A) advancing contact angle measurements, b) receding contact angle measurements. A new boundary line is formed as the drop advances or recedes and a new contact angle is formed each time [13]. This figure has been removed due to copyright restrictions and can be viewed in the reference given here.

Axisymmetric drop shape analysis (ADSA) uses the Laplician equation of capillarity to fit a shape to the sessile drop. While ADSA provides a more accurate fit, it is experimentally more difficult to perform as this method of contact angle measurement is particularly sensitive to surface imperfections. The drop must be injected on to the sample which is housed in a quartz chamber; this is done in order to minimize disturbances to the drop [14]. The experimental setup for ADSA can be seen in figure 2.6.
Figure 2.6: Experimental setup for axisymmetric drop shape analysis [15]. This figure has been removed due to copyright restrictions and can be viewed in the reference given here.

The Wilhelmy balance technique measures the force on a sample as it either advances or receded through a liquid and these measurements are then used to determine the contact angle of the sample [16].

Advancing contact angle measurements were carried out using a home built goniometer at 40 °C. The water delivery system was thoroughly cleaned before
each sample measurement, as contact angle measurements are very sensitive to environmental contaminants. The Teflon capillary tubing and stainless steel needle were cleaned by sonicating them in surfactant containing water for ten minutes; they were then rinsed thoroughly with tap water, followed by a final rinse with Millipore water. The polymer films were placed in a temperature-controlled environmental chamber mounted on a tilt stage. The temperature was monitored using a thermocouple attached to the stage. A syringe pump, capillary tube and syringe needle were used to deliver the distilled water to the sample. A drop was deposited onto the substrate surface and a thin stainless steel needle was then inserted in the center of the drop perpendicularly from above. The volume of the drop was increased by the controlled application of liquid into the drop using a syringe pump system. Drop images were acquired every 3 s using a CCD camera. DROPimage™ software marketed by Rame Hart and developed by F. K. Hansen was used to determine the contact angles by mathematically fitting a curve to the side of the drop and then plotting the tangent to the curve at the triple point [5]. The experimental setup can be seen in figure 2.7.
Figure 2.7: Homemade goniometer used for advancing contact angle measurements, UCD school of chemistry and chemical biology.

2.5.6 Fourier Transform Infrared (FT-IR) Spectroscopy

FT-IR involves a beam of infrared light from a laser which is directed at the sample of interest. The reflected light which is directed towards the detector contains information about the sample composition. Figure 2.8 below is a schematic of the components of a typical FT-IR spectrometer. FT-IR can be used to determine the composition of a substance as different bond types possess characteristic vibrational and stretching frequencies which can then be used to identify a particular bond for example C=O vibrates at approximately 1650 cm\(^{-1}\) and the N-H bond in a primary amine vibrates at approximately 3500 cm\(^{-1}\) \[17\]. When infrared light hits the sample certain frequencies are absorbed due to the composition of the sample. When the light reaches the detector it is possible to identify which frequencies have been absorbed and thus the
composition of the molecule can be identified by comparing the spectra produced to other spectra in a peak library [18]. FT-IR is a non-destructive technique. Traditional infrared spectroscopy is operated in dispersive mode and as a result the detector analysis every frequency individually. This can be quiet time consuming but fortunately FT-IR greatly improved analysis time by making use of an interferometer, which is capable of measuring all the frequencies needed at once. The signal from the interferometer is split in two by a beam splitter and one beam shines onto a mirror and then onto a camera, while the other shines onto the sample of interest then onto a mirror, through the beam splitter and finally it hits the same camera as the other beam of light. An interferogram is produced when the two signals meet and interfere with each other, either constructively or destructively. The interferogram must be read by a computer using a mathematical transformation known as the Fourier Transform. The computer converts the sample data into an infrared spectra i.e. a plot of wavenumber vs absorbance [18].
FT-IR was carried out on a Shimadzu FT-IR 830 Fourier transform infrared spectrometer with a golden gate diamond ATR accessory. Spin coated films were prepared on aluminum disks in order to prevent the interference associated with non-metallic substrates. A background scan was taken before every sample scan and this background was then subtracted.

Figure 2.8: Components of an FT-IR [16]. This figure has been removed due to copyright restrictions and can be viewed in the reference given here.
XPS is a quantitative and qualitative technique used to determine the elemental composition of a sample. XPS can also provide information on the oxidation state of the elements present in the sample. The sampling depth of XPS is 10 nm and thus it can only provide information on about the top few nanometers of a sample's surface. In XPS x-rays produced by thermionic emission from an aluminum or magnesium filament interact with the chosen sample. This interaction ionizes electrons in the core levels and as a result an electron may be ejected. The kinetic energy (KE) of this electron is described in equation 26; where Ebinding is the binding energy of the electron and Ephoton is the energy for the monochromatic x-rays.

\[
KE = \text{Ephoton} - \text{Ebinding} \tag{26}
\]

It is possible to determine the binding energy of the electron which has been ejected from the surface as the kinetic energy of the electron is detectable and the energy of the photon is known. Each electron in each atomic orbital possesses a characteristic binding energy and thus it is possible to determine the elemental composition of a material based on the binding energies. The percentage composition of the sample can be determined using the photoelectron intensities of each element. A change in oxidation state causes a shift in the binding energy, this results in an electron being emitted with a slightly lower kinetic energy. XPS cannot be used to detect hydrogen or helium but it will work for the remaining elements in the periodic table. XPS analysis is always carried out under high vacuum (approximately \(10^{-8}\) Torr).

Below are the specifications which were used for XPS analysis in this investigation;

**Sample Temperature:** 20-30 °C

**X-ray Gun:** 150 W (10 mA, 15 kV) mono Al Kα 1486.58 eV
**Pass Energy:** 160 eV for the survey spectrum and 20 eV for narrow regions

**Step:** 1 eV (survey), 0.05 eV (regions)

**Dwell:** 50 ms (survey), 100 ms (regions)

**Sweeps:** Surveys (3) C1s (4), N 1s (15), O 1s (15), F1s (15)

**Calibration:** Adventitious carbon was used for charge reference: the C 1s line of adventitious hydrocarbon was assumed to have a binding energy of 284.8 eV.

**Other:** Spectra were collected in the normal to the surface direction. XPS detection limit is estimated to be at 0.1-0.5 %

*This work was kindly carried out by Dr. Sergey Beloshapkin of the Materials and Surface Science Institute (MSSI), University of Limerick, Ireland.*

---

### 2.6 CELL CULTURE TECHNIQUES

---

#### 2.6.1 Defrosting Cryo-Preserved Cells

For long term cell storage cells were stored in cryo-preservation vials and then stored in liquid nitrogen. The cells were reconditioned in the following manner; the cryo-vial was removed from liquid nitrogen and placed in a water bath at 37 °C. Once the cells were completely defrosted the contents of the cryo-vial were transferred to a T75 flask. Pre-warmed (37 °C) media was then added to the flask drop wise. The flask was then placed in an incubator at 37 °C and 5 % CO₂ for 24 hours. After 24 hours the media was replaced with fresh pre-warmed (37 °C) media in order to remove the DMSO.

---

#### 2.6.2 Cryo-Preservation of Cells

It is necessary to cryo-preserve cells in order to maintain a supply of cells for future use. Once the cells had a reached the desired level of growth they were trypsinized and centrifuged in order to generate a pellet. The pellet was then re-
suspended in 1 ml of freezing media. The freezing media for 3T3 consists of 10 % DMSO in fetal bovine serum. The freezing media for HPMEC contains 20 % DMSO in fetal calf serum. The cell suspension was then transferred to a cryo-vial and slowly frozen. The vial was wrapped in tissue paper and placed in a -80 °C freezer overnight. The vial was then moved to liquid nitrogen for long term storage.

2.6.3 Subculture of 3T3 Fibroblast Cells

3T3 cells were cultured in DMEM which had been supplemented with 10 % fetal bovine serum and 1 % penicillin and streptomycin. To subculture a T75 flask, the media was aspirated from the flask and the flask was then rinsed twice with 7 mls pre-warmed HBSS (37 °C). A 1 % trypsin solution (3 mls) was then added and the flask was placed in the incubator at 37°C and 5% CO₂. The flask was observed periodically in order see if the cell layer had detached. Pre-warmed media (37 °C) (4 mls) was then added to the flask in order to neutralize the trypsin. The cell suspension was then removed and added to a 15 ml tube. The cell suspension was then centrifuged at 1500 rpm. The resultant cell pellet was re-suspended in fresh media. A haemocytometer was used to calculate the number of cells in the suspension and the appropriate amount of cells were then re-seeded. Subculturing of the 3T3 cells was carried out every three days or once the cells had attained 80 % confluence. Incubation conditions were an atmosphere of 95 % air and 5 % CO₂ at 37 °C.

2.6.4 Preparation of Human Pulmonary Microvascular Endothelial Cell Media

HPMEC were cultured in M199 media which had been supplemented with 20 % fetal calf serum, Glutamax (2 mM), sodium heparin (12 µg/ml), penicillin/streptomycin (100 µg/ml) and endothelial growth factor supplement (50 µg/ml). In order to prepare the media 1 ml of M199 media was added to the sodium heparin, in order to dissolve it. The endothelial growth factor supplement was dissolved in 5 mls of M199 media and 1ml was added to the media to be used for subculturing. The remaining 4 mls were frozen as 1 ml aliquots for
future use. The fetal calf serum, penicillin/streptomycin and glutamax were then added to the 500 ml bottle of M199 media. Once the sodium heparin and glutamax had dissolved they were also added to the mixture. The media was then filtered.

2.6.5 Subculture of HPMEC

The HPMEC used in this investigation were a kind gift from Prof. James Kirkpatrick. Once the HPMEC had reached 80 % confluence the old media was removed and the cells were rinsed twice with pre-warmed HBSS (37°C). A 1% trypsin solution was then added to the flask. The flask was placed in an incubator at 37 °C and a bright field microscope was used to observe the cells in order to determine when cell detachment was complete. Pre-warmed cell culture media was then added to the flask to neutralize the trypsin and the cell suspension was centrifuged at 1500 rpm for five minutes. The cell pellet was re-suspended in fresh media and a haemocytometer was used to count the number of cells in the suspension. An appropriate number of cells were then seeded. Cells were incubated in an atmosphere of 95 % air and 5 % CO₂ at 37 °C. The cell culture media was changed after 48 hours and cells were subcultured after 96 hrs or once 80 % confluence was reached.

2.6.6 Seeding Cells on Thermoresponsive Polymer Films

Thermoresponsive films were placed on a dry chilling heating bath set to 37 °C for ten minutes prior to cell seeding in order to ensure complete temperature equilibration. Cells were subcultured as outlined above in sections (2.6.3 and 2.6.5) and counted using a haemocytometer. The cell suspension was placed in a water bath a 37 °C. The top of the vial containing the cell suspension was parafilmed™ in order to prevent contamination. Two mls of pre-warmed media (37 °C) was then added to the thermoresponsive films. The desired number of cells was then seeded on each thermoresponsive polymer film in triplicate. Incubation conditions were an atmosphere of 95 % air and 5 % CO₂ at 37 °C.
2.7 QUANTITATIVE ASSESSMENT OF CELL GROWTH

2.7.1 Quant-iT™ PicoGreen® Assay

The Quant-iT™ PicoGreen® Assay kit was used to determine the total amount of DNA in cells. PicoGreen® is a fluorescent dye which selectively binds to solubilized double strand DNA. In order to release the DNA the cells must be repeatedly freeze-thawed. The procedure described here is suitable for 6 well plates or 35 mm Petri dishes.

Procedure

- Cells were seeded on the polymer samples and controls as described earlier and incubated until the desired level of growth was attained.
- A chilling heating dry bath was utilized here in order to ensure that the temperature of the cell samples didn’t fall below 37 °C before the freeze-thaw step as this would initiate unwanted cell detachment.
- The samples were placed on the chilling heating dry bath set to 37 °C.
- Old media was removed and the samples were rinsed three times with pre-warmed HBSS (2 mls).
- 1.5 mls of double distilled H₂O was then added to each sample.
- The cells were then repeatedly freeze-thawed according to the following procedure; the cells were placed in a -80 °C freezer for 30 minutes. After 30 minutes the cells were removed and allowed to defrost on the bench for 30 minutes. This cycle of freeze-thawing was repeated three times in order to ensure complete cell lysis.
- During the 2nd freeze-thaw step the following solutions were prepared.

1. 1*TE buffer; A 1/20 dilution of the 20*TE buffer was prepared for example 8.075 ml of double distilled water and 425 µl of 20*TE buffer.
2. 2 µg/ml DNA stock; A 1/50 dilution of the 100 µg/ml DNA stock was prepared, 588 µl of tube 1 and 12 µl of the DNA stock
3. 50 ng/ml DNA stock; A 1/40 dilution of the 2 µg/ml DNA stock was prepared, 585 µl of tube 1 and 15 µl of tube 3
4. PicoGreen®; A 1/200 dilution of the PicoGreen® dye was prepared, 5.174 mls of tube 1 and 26 µl of the PicoGreen® dye. The PicoGreen was not added until just before the solution was required as it is very photosensitive. The tube the PicoGreen® dye was prepared in was also covered in aluminum foil.

- The quantities of solution above are sufficient for 26 samples and the calibration curve.
- The calibration curve was prepared in a 96 well plate during the final freeze-thaw step. **Table 2.2** contains a guide for preparing the calibration curve.
- 100 µl of cell lysate from each sample was transferred to a 96 well plate after the final freeze-thaw step.
- 100 µl of the diluted PicoGreen® was then added to each well containing either cell lysate or the standard curve.
- Fluorescence was measured using a Wallac Victor Plate Reader at an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

**Table 2.2: PicoGreen® Standard Curve Calculations**

<table>
<thead>
<tr>
<th>DNA concentration (ng/ml)</th>
<th>DNA Stock= 50 ng/ml</th>
<th>DNA Stock 2 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA (µl)</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Buffer(µl)</td>
<td>100</td>
<td>80</td>
</tr>
</tbody>
</table>

---

127
2.7.2 alamarBlue™ assay

alarBlue™ or Resazurin is a weakly fluorescent blue dye that takes the place of oxygen in the electron transport chain and in so doing is reduced to its highly fluorescent pink form, resorufin. The extent of the colorimetric change is indicative of metabolic activity. alamarBlue™ is a non-destructive assay and as such it is possible to simply rinse the cells with HBSS at the end of the protocol to remove any remaining dye and then re-use the cells for further experimentation. The alamarBlue™ dye must be protected from light during the course of the experiment.

- Cells were seeded according to the procedure outlined earlier in section 2.6.6 and allowed to reach the desired level of growth.
- The samples were placed on a chilling heating dry bath set to 37 °C in order to prevent premature cell detachment.
- A 10 % dilution of alamarBlue™ in HBSS was then prepared in a laminar flow hood with the lights off. The tube containing the 10 % alamarBlue™ was wrapped in aluminum foil in order to protect it from light. The amount of dye prepared depended on the number of samples. As a guide 2 ml of the 10 % dilution is suitable for one well of a six well plate or for a single 35 mm Petri dish.
- The media was removed from the samples.
- The samples were then rinsed three times with pre-warmed HBSS (2 mls) in order to remove any residual media.
- The 10 % alamarBlue™ dilution (2 mls) was then added to each sample.
- The samples were then covered with aluminum foil and transferred to the incubator for 1 hour.
- After an hour the samples were removed from the incubator and 100 µl was taken from each sample in triplicate and transferred to a well in a 96 well plate.
- The 10 % alamarBlue™ dilution was then added to the 96 well plate in triplicate, as this was the negative control.
- Fluorescence was measured using a Wallac Victor Plate Reader at an excitation wavelength of 530 nm and an emission wavelength of 590 nm.
2.7.3 Trypan Blue Viability Studies

Trypan blue dye was used to assess the viability of detached cells and during routine subculturing in order to assess the viability of the cell line. Trypan blue is a dye which selectively stains dead cells. Trypan blue is unable to react with healthy cells due to its negative charge and consequently healthy cells will appear as rounded and refractile. Conversely it is able to react with cells with damaged membranes; dead cells will appear large in comparison to their healthy counterparts, dark blue and non-refractile. The protocol for staining is as follows; 100 µl of cell suspension was placed in an eppendorf tube. Trypan blue dye (100 µl) was then added to the eppendorf. The mixture was allowed to equilibrate at room temperature for five minutes; after which a haemocytometer was used to count the number of healthy and dead cells. The percentage viability was then calculated.

2.8 TEMPERATURE INDUCED CELL DETACHMENT AND IMAGING TECHNIQUES

2.8.1 Temperature Controlled Cell Detachment

Once the desired level of cell growth was obtained the samples and controls were removed from the incubator and placed on a heating chilling dry bath set to 37 °C. The media was then removed from the samples and controls. The samples and controls were then rinsed three times with pre-warmed HBSS. This was done to insure that all traces of media had been removed as this impedes the detachment process. After the final HBSS rinse 2 mls of cold (4 °C) HBSS was added in order to initiate cell detachment. The samples and controls were then transferred to a chilling heating dry bath set to 4 °C. Periodic bright field observation was utilized in order to determine the point of complete cell detachment.
2.8.2 Scanning Electron Microscopy

Scanning electron microscopy uses a beam of electrons instead of light like traditional microscopes to analysis the microstructure of solid objects. **Figure 2.9** is a schematic of a basic SEM. The main components of an SEM are an electron source, condenser lens, scan coils, objective lens, sample stage and detector. A tungsten filament is used as the electron source as it is capable of withstanding high temperatures without degrading. In order for thermionic emission to occur a voltage of between 3-4 amps is applied to the tungsten filament and this causes the filament to heat to approximately 2650 °C. The resultant electrons from thermionic emission pass through anodic plates before they are focused through the Wehnelt cap. The electron beam that emerges from the anionic plates must be focused and this is done by passing the beam through a series of electromagnetic condenser lens and apertures, this creates a parallel stream of electrons. The scanning coils enable the beam of electrons to be swept across the surface of the sample of interest. Finally the beam is focused on the sample using the objective lens. The sample is positioned on a goniometer stage which allows the user to move the freely in the x, y and z directions.
Figure 2.9: Cartoon showing the inside of a SEM [19]. This figure has been removed due to copyright restrictions and can be viewed in the reference given here.

The interaction of the electron beam with the sample results in two different events and these are called elastic and inelastic events. When an electron that is travelling at high speeds hits a sample, it is possible that the electron may hit the sample too close to the nucleus and as a result the electron is backscattered. Backscattered electrons possess the same amount of energy as the incident electron; their trajectory has simply been altered. The amount of
backscattered electrons generated depends on atomic number, the higher the atomic number the larger the amount of backscattering. If an SEM possess a backscattered electron detector it is possible to generate an image of the sample using backscattered electrons as the amount of backscattered electrons depends on the elemental composition of the sample. Inelastic events generate secondary electrons. Typically secondary electrons possess energies of less than 50 eV. Secondary electrons are created by the collision of an inelastic electron with an electron in the sample. When the two electrons collide there is an energy transfer from the inelastic electron to the sample electron and the loosely bound electrons typically located in the valence shell are knocked out of their orbit. The energy of a secondary electron is indicative of its former position in an atom and these electrons can be detected by a positively charged detector and then used to construct an image of the sample surface. The secondary electrons interact with a phosphorus containing scintillator in the detector, this interaction results in flashes of light whose brightness is proportional to the energy of the secondary electrons. The flashes pass through a photomultiplier tube where they are amplified into an electronic signal and this signal is then displayed using a cathode ray tube.

Samples must be conductive otherwise a charge will build up on the sample from the incident electrons and this will cause the electron beam to deflect. The deflection of the electron beam will cause image distortion. All non-conducting samples are coated with an ultra-thin layer (20 nm) layer of gold. A high vacuum must be maintained in order to prevent beam scattering on route to the sample or impediment of the secondary electrons on their path to the detector. All samples must be thoroughly dried as a result, otherwise any remaining water or solvent may interact with the electron beam.
Figure 2.10: Schematic illustrating the effect of electron bombardment on a sample. The backscattered and secondary electrons are of interest in this investigation [20]. This figure has been removed due to copyright restrictions and can be viewed in the reference given here.
SEM was used to assess the cell sheet integrity of the HPMEC grown and detached from the physically adsorbed films and the Thermanox™ controls i.e. cell detachment was initiated prior to sample dehydration for SEM preparation, this ensured that cell sheet detachment was captured mid-stream, therefore it was possible to observe cells still attached to the polymer surface and the integrity of the sheet section that had detached. The fixation protocol is as follows;

- Cells grown on both the thermoresponsive films and the controls were rinsed twice with prewarmed HBSS.
- Primary fixation with 2% glutaraldehyde in 0.1 M sodium cacodylate was then carried out for 1 hour at room temperature.
- Samples were then twice rinsed with 0.1 M cacodylate buffer (5 minutes per rinse).
- Secondary fixation with 1% osmium tetroxide in 0.1 M cacodylate buffer was then allowed to proceed for 1 hr at room temperature.
- After an hour the samples were rinsed twice with cacodylate buffer (5 minutes per rinse).
- Samples were then dehydrated through a graded alcohol series, as outlined below.

1. 50% ethanol for 10 minutes
2. 70% ethanol for 10 minutes
3. 95% ethanol for 10 minutes
4. 100% ethanol for 10 minutes
5. 100% ethanol for 10 minutes

- After dehydration, the samples were placed in a critical point dryer and then sputter coated with gold.
- The coated samples were examined on a Hitachi S2600N variable pressure scanning electron microscope.
A transmission electron microscope consists of the following parts; an electron gun, condenser lens system, deflector coils, objective lens, diffraction lens, projector lens, camera, specimen holder and a viewing system. The components of a typical TEM can be seen in figure 2.11. As the name suggests a beam of electrons is passed through a thin sample, which has been impregnated with heavy metals. The interaction of the electron beam with the heavy metal staining gives rise to secondary electrons which are responsible for the contrast [21, 22].
There are three different sources for the generation of electrons in a TEM; the simplest of these is a tungsten filament which liberates electrons as it is heated. A single pointed crystal of lanthanum hexaboride (LaB₆) may also be used. A LaB₆ electron source gives a brighter image and it lasts longer than a tungsten filament (400-600 hours vs. 100-200 hours) but it is about 20 times more expensive. The final electron source is a field emission gun (FEG). FEG’s also generate brighter images and they also give better resolution, as a FEG is a

Figure 2.11: Components of a TEM [22]. This figure has been removed due to copyright restrictions and can be viewed in the reference given here.
monochromatic electron source with a finer probe than a tungsten filament or a LaB$_6$ crystal [23].

Once the electron beam has been generated the Wehnelt cap focuses the beam to a crossover. There are two condenser lenses in a TEM and these are denoted C1 and C2 here. C1 is a stronger lens and it is generally set to the same specifications for all magnifications as it is responsible for de-magnifying the crossover to give a smaller point source. C2 projects the beam spot onto the sample and it possesses a series of apertures, thus the C2 lens is adjusted depending on the magnification required [23]. The deflector coils are responsible for beam alignment and the objective lens focus and magnify the sample. Projector lenses then magnify the image produced by the objective lens. The focal length of the projector lenses is adjusted by varying the current in the windings of the copper wire, if the current is increased the focal length of the lens will decrease and conversely if the current is decreased the focal length increases. Samples are inserted into the TEM via either a side entry or top entry sample holder. A high vacuum must be used in order to prevent electron scattering [24].

TEM was used to assess the integrity and structure of junctions and cell organelles in HPMEC grown on both the physically adsorbed pNIPAm films and the Thermanox™ controls. The fixation protocol is outlined below;

- HPMEC grown on both the physically adsorbed thermoresponsive films and the TCP controls were rinsed twice with warm (37 °C) HBSS.
- The monolayers were then fixed with 2 % glutaraldehyde in 0.1 M cacodylate buffer for one hour at room temperature.
- Samples were then rinsed twice with cacodylate buffer (5 minutes per rinse) and post-fixed with 1 % osmium tetroxide in cacodylate for one hour at room temperature.
- Another cacodylate rinse was performed after which the samples were dehydrated through a graded alcohol series as outlined in the SEM section.
The samples were then embedded in low viscosity resin (R1078A Agar Scientific)

1. 75 % ethanol:25 % resin for 30 minutes
2. 50 % ethanol:25 % resin for 30 minutes
3. 25 % ethanol:25 % resin for 30 minutes
4. 100 % resin for 16 hours

Transfer pipettes were used to add and remove the ethanol: resin mixtures as needed.

- After 16 hours the 100 % resin was removed and the samples were placed in the bottom of tear away molds. Fresh 100 % resin was then added and the samples were placed in an oven at 60 °C for 72 hours in order to allow polymerization to take place.
- Once polymerization was complete the samples were removed from the oven and allowed to cool slightly.
- Ultra-thin sections were cut on an ultra-microtome (Leica Reichert Jung Ultracut) using a diamond knife.
- The sections were then stained with uranyl acetate and lead citrate and examined on a Hitachi H7000 transmission electron microscope.

---

2.8.4 Fluorescent Microscopy

Fluorescent microscopes allow the user to examine tissue and cells in minute detail. The main function of a fluorescent microscope is to transmit excitation energy to the fluorescent probe in the sample and then separate the weaker emission fluorescence from the excitation [25].

Modern fluorescent microscopes use epi-illumination, where by the light used for excitation comes from either a laser, Hg lamp, Xe lamp or a combination Hg/Xe lamp and the light is reflected by a dichromatic beam-splitting mirror onto the back aperture which acts as a condenser and then on to the sample. The light from the excitation light source may pass through a short pass filter which only
permits light below a certain wavelength to pass through. The emitted fluorescence is collected by the objective lens and then passes through the dichromatic beam-splitting mirror; as the wavelength of the emitted fluorescence is longer than that of the of the excitation due to Stokes shift. The dichromatic mirror acts as a long pass filter reflecting any excitation fluorescence due to the difference in wavelength thus allowing the weaker emission fluorescence through. Finally the fluorescent signal passes through another long pass filter which should remove any remaining excitation fluorescence and then on to the detector and eye piece [26].

Fluorescence microscopy was used to assess cell sheet integrity and cellular adhesion after temperature controlled detachment. The protocol used is as follows;

- Cells were seeded and allowed to reach the desired level of growth.
- The samples and controls were then transferred from the incubator to a chilling heating dry bath set to 37 °C.
- Media was removed from the cells and they were then rinsed twice with pre-warmed HBSS.
- The temperature controlled cell detachment protocol was followed for cells seeded on thermoresponsive polymer films.
- Once complete cell detachment had taken place the cells or cell sheet were transferred to another polymer coated surface with the aid of a 1000 µl pipette tip.
- The cells were given 24 hours to re-adhere before progressing with the experiment.
- The control samples were detached using trypsin and they were re-seeded in uncoated Petri dishes.
- After 24 hours the media was removed and the cells were then rinsed twice with prewarmed (37 °C) PBS.
- The samples were fixed with 4 % paraformaldehyde in PBS for 15 minutes.
- After fixation the samples were permeabilized with 0.1 % Triton X-100 in PBS (3 mins) and blocked with 3 % Normal Goat Serum (NGS) in PBS (20 mins).
- The primary rabbit monoclonal anti-paxillin antibody (focal adhesions) (Abcam) was then added for an hour at room temperature (1:250 in block).
- The Goat Anti-Rabbit secondary antibody (Alexa 594 nm at 1:200 in PBS) (Invitrogen) was incubated with the samples for 1 hour at room temperature.
- Samples were counterstained with Phalloidin, for F-Actin, (Alexa 488 nm 1:200) and Hoechst, for Nuclei, (1:1000) (both Invitrogen).
- Cells were stored in PBS and imaging was carried out less than 24 hrs.’ after staining.
- All samples were imaged using an Olympus IX81 microscope and Volocity™ software. Low magnification overviews were captured at 40x magnification, with detailed views captured using a 100x oil lens.
- Appropriate primary and secondary antibody, and polymer controls were also included in this work in order to confirm that there was no non-specific binding on the part of either the primary or secondary antibody.

2.9 STATISTICAL ANALYSIS

Statistical analysis was carried out using Minitab version 17. One way ANOVA and post hoc Tukey’s tests were employed to investigate statistical differences between groups under study. P values were deemed significant if found to be ≤0.05.
2.10 REFERENCES


3 NANOMETRE SCALE PHYSICALLY ADSORBED THERMORESPONSIVE FILMS FOR CELL CULTURE

3.1 INTRODUCTION

In this investigation physical adsorption was used to produce nanometer thick thermoresponsive polymer films for cell culture. To the best of the authors’ knowledge this is the first in-depth investigation into physically adsorbed thermoresponsive films with a view to the adhesion and detachment of human cell sheets. There have been some investigations into the adsorption of poly-(N-isopropylacrylamide (pNIPAm)) on both hydrophobic and hydrophilic substrates, the results of which confirm that it is possible to produce ultra-thin coatings on both substrate types [1-5]. Due to its amphiphilic nature, pNIPAm will adsorb strongly on various polymeric substrates, for example polystyrene, which is the common material used as tissue culture plastic-ware. The extent of the adsorption can be controlled by the solution concentration and the deposition temperature [3, 4, 6, 7]. This study seeks to answer the question of whether or not this simple method of film preparation is capable of yielding thermoresponsive surfaces suitable for both cell adhesion and detachment.

As a method of film preparation physical adsorption possess several advantages, it is a simple method of film preparation which requires no specialized equipment or training, it is possible to use this technique to coat non-planar surfaces and complicated geometric profiles and it is a technique which many cell culture practitioners already use, for example, to routinely coat surfaces with fibronectin, collagen or other cell adhesion promoters. There are of course some limitations; one of these limitations is the lack of control of the amount of polymer adsorbed. To address this problem polymer adsorption was carried out at two different temperature points; one below and one above the polymers lower critical solution temperature (LCST) in order to investigate the
effect of temperature and polymer conformation on the amount of polymer adsorbed. Studies show that the presence of pNIPAm in cell culture media, in much higher concentrations than used within this study, is not detrimental to cell viability and as a result the ease of preparation and accessibility of this technique out-weigh the short-coming of possible polymer desorption [8, 9].

Due to the aforementioned advantages, physical adsorption could be a valuable addition to the arsenal of thermoresponsive film preparation methods, developed with a view to thermal-controlled cell harvesting, which include; spin coating, solvent casting, dip coating, spray coating, electron beam polymerization, (EBP) plasma polymerization, surface initiated atom transfer radical polymerization (SI-ATRP) and reversible addition fragmentation chain transfer (RAFT) [10, 11].

Pioneered by Yamada et al. [12] and Takezawa et al. [9] pNIPAm based coatings have been used for cell culture recovery since the early 1990s [9, 12]. Above their lower critical solution temperature pNIPAm films exist in a condensed, hydrophobic state, which can be conducive to cell adhesion and subsequent proliferation. The depression of the ambient temperature causes the pNIPAm coatings to become hydrophilic and this sudden increase in hydration induces spontaneous cell detachment. Unlike traditional cell culture detachment methods, detachment from thermoresponsive substrates allows for the detachment of cells without the disruption of cell-to-cell junctions. Therefore this approach can facilitate the harvesting of cell sheets, which can be applied for tissue engineering purposes. Depending on the type of cells seeded, the resultant cell sheets can be used for a variety of biomedical applications for example in the repair of cardiac tissue, the creation of corneal grafts or 2D or 3D biomedical constructs [13-16].

Two different pNIPAm based polymer systems were used in this study, both with their own merits; the pNIPAm (LCST 32°C) polymer used in the study was the commercially sourced homopolymer (Sigma Aldrich). The principal advantages of employing the commercial polymer are availability and polymerization circumvention. Poly (NIPAm-co-N-tertbutylacrylamide (NtBAm)) (LCST 16°C) is a more hydrophobic copolymer of pNIPAm. Studies show that cells preferentially
grow on slightly more hydrophobic surfaces than pNIPAm alone, and thus this advantage is comparatively explored for the physically adsorbed surfaces [17-19]. Polymer adsorption was carried out in aqueous solution without the presence of any organic solvent, which removes the risks and costs associated with the disposal of organic solvents. Furthermore, using aqueous solvent ensures that no residual toxic solvents, which could be detrimental to growing cells, remained trapped within the deposited polymer layer. Finally, polymer films were formed both above and below the LCST in order to determine whether or not the temperature induced changes in the polymers’ conformation affected the films’ physical and chemical properties and their ability to successfully host cells.

Thus, in total, four types of films were prepared (two different polymers physically adsorbed both above and below the polymers’ LCSTs) and their physical and chemical properties were characterized before finally being assessed in terms of their suitability as cell culture substrates. The films were characterized using atomic force microscopy (AFM) which was employed to determine surface roughness and thickness, advancing contact angle (surface wettability). X-ray photoelectron spectroscopy (XPS) was used to determine percentage composition and gel permeation chromatography was used to determine the molecular weight of the polymers. Human pulmonary microvascular endothelial cells (HPMEC) were seeded on the physically adsorbed films and cell viability was assessed quantitatively through cell viability assays, and qualitatively using light microscopy. Transmission electron microscopy (TEM) was used to assess the cell-cell junction integrity and cell organelles post detachment. Scanning electron microscopy (SEM) was used to analyze the cell sheets post cell detachment. Fluorescence microscopy was used to assess cell and cell sheet re-adhesion after detachment from thermoresponsive polymer films.
3.2 MATERIALS AND METHODS

3.2.1 Materials

Poly (N-isopropylacrylamide) (M_n 20,000-25,000), M199 cell culture medium, Hank’s balanced salts solution (HBSS), trypsin-EDTA, penicillin-streptomycin, fetal calf serum (FCS), trypan blue stain, sodium heparin, triton X-100, phosphate buffered saline (PBS), dimethyl sulfoxide (DMSO) and sodium cacodylate trihydrate were purchased from Sigma-Aldrich and used as received. Quant-iT™ PicoGreen® dsDNA assay kit and glutamax from Invitrogen, alamarBlue™ assay from Biosciences, endothelial growth factor supplement were purchased from Becton Dickinson. Agar low viscosity resin kit and 2 % osmium tetroxide from Agar Scientific, 25 % Glutaraldehyde solution in water from AMSBIO and Thermanox™ plastic 35 mm discs from Nunc™ were all used as received. Glass bottomed Petri dishes from MatTek™ and 8 well chamber slides from Ibidi™. All other plastic consumables were purchased from Sarstedt. Primary rabbit anti-paxillin antibodies were purchased from Abcam. Secondary goat anti-rabbit antibodies, phalloidin, Hoechst and normal goat serum were purchased from Invitrogen. An Ectotherm chilling and heating dry bath from Torrey Pines Scientific was used for careful temperature control. Human pulmonary microvascular endothelial cells (HPMEC) were kindly provided by Prof. James Kirkpatrick from the Institute of Pathology, Johannes-Gutenberg University.

3.2.2 Polymer Synthesis and Characterization

The synthesis of poly (NIPAm-co-NtBAm) (0.65 molar ratio of NIPAm – 0.35 molar ratio of NtBAm) has been previously reported and thus it will not be discussed in detail here.[20] The number average molecular weight (M_n) (350,000 g/mol) and polydispersity index (PDI) (2.7) of the copolymer was determined by gel permeation chromatography (GPC). GPC analyses were carried out with PL-gel mixed-D (300*7.8 mm, 5 μm nominal particle size) Polymer Labs columns. The mobile phase used was dimethylformamide (DMF) with 30 mM KBr. Separations were performed at 35 °C at a flow rate of 1
mL/min using a RI detector. The molecular weights of the polymers were referenced to polystyrene standards [20].

3.2.3 Film Preparation

Physically adsorbed thermoresponsive films were fabricated by adding 2 ml of a 0.1 mg/ml aqueous solution of pNIPAm to the desired substrate, which in this case was either tissue culture polystyrene (TCP Petri dish) or Thermanox™ (cover slip placed in a TCP dish). After an incubation period of three hours, at either above or below the polymers' LCST, (10 °C and 40 °C for poly (NIPAm-co-NtBAm) and 20 °C and 40 °C for pNIPAm), the remaining polymer solution was removed. A chilling and heating dry bath was used to ensure that the samples were maintained at an appropriate temperature. The samples were then washed twice with 2 mls of Millipore water and dried for two hours on the chilling and heating dry bath in ambient air followed by complete drying in a vacuum oven at 40 °C for 24 hours. In the case of the TCP dishes the lids were removed during both deposition and drying. Films were sterilized by exposure to UV light for two hours prior to cell culture. TCP Petri dishes were used as the underlayer substrate for cell culture work, x-ray photo electron spectroscopy (XPS) and the alamarBlue™ and PicoGreen® assays. Thermanox™ coverslips were used as the underlayer substrate for advancing contact angle, atomic force microscopy (AFM), transmission electron microscopy (TEM) and scanning electron microscopy (SEM). Ibidi™ chamber slides or MatTek™ Petri dishes were used for fluorescence microscopy.
3.2.4 Physical Characterization

3.2.4.1 ATOMIC FORM MICROSCOPY (AFM)
AFM was used to assess the roughness of the adsorbed films using 10 µm × 10 µm scans with a scan rate of 1 Hz. Images were taken with a Dimension 3100 AFM (Digital Instruments, Santa Barbara, CA, USA) Veeco 1-10 Ohm-cm phosphorus (n) doped Si tip and a matrix of 512 × 512 points along the x-y plane were analyzed in a single scan. Film roughness was recorded as the root mean square (RMS) roughness, where RMS denotes the standard deviation of the Z-value along the reference line [21, 22].

3.2.4.2 THICKNESS
Films were prepared according to the procedure outlined above. Theranox™ discs were used as the under layer substrate. The thickness of the physically adsorbed films was measured using an AFM operating in scratch mode. The following parameters were used; ramp size 1.229 µm, scan rate 1 Hz, trigger threshold 0.2 V, scan angle 90°, scratch length 1 µm and scan size 3 µm. Samples were scratched in three random areas and fifteen thickness measurements were taken from each scratch [23, 24].

3.2.4.3 CONTACT ANGLE
Advancing contact angle measurements were carried out using a home built goniometer at 40 °C, i.e. > the polymers’ LCST. Contact angle measurements were carried out at 40 °C in order to provide direct comparison to previously measured contact angles for thermoresponsive films produced via alternative fabrication methods by this research group. A detailed description of the procedure has been published elsewhere [11, 20, 21]. Briefly, the physically adsorbed films were placed in a temperature-controlled environmental chamber mounted on a tilt stage. The temperature was monitored using a thermocouple attached to the stage. A drop was deposited onto the substrate surface and a thin stainless steel needle was then inserted in the center of the drop.
perpendicularly from above. The volume of the drop was increased by the controlled application of liquid into the drop using a syringe pump system. Drop images were acquired every 3 s. DROPimage software marketed by Rame Hart and developed by F. K. Hansen was used to determine the contact angles.

3.2.4.4 X-RAY PHOTOELECTRON SPECTROSCOPY (XPS)

X-ray photoelectron spectroscopy (XPS) was performed with a Kratos ULTRA DLD spectrometer using monochromatic Al Kα radiation ($h\nu = 1486.58$ eV) and a fixed analyzer pass energy of 20 eV; the binding energy scale was referenced against the C 1s line (284.8 eV).

3.2.4.5 LCST DETERMINATION

The LCST of polymer solutions was determined by turbidity measurements in aqueous solutions (1% w/v) at $\lambda = 500$ nm with Cary 3 Bio UV-Visible spectrophotometer equipped with a Cary temperature controller. The heating rate was 0.5 °C/min [21].

3.2.5 Cell Culture

HPMEC were cultured in M199 medium supplemented with 20 % fetal calf serum (FCS), 1 % penicillin and streptomycin antibiotics, glutamax (2 mM), endothelial growth factor supplement (50 µg/ml) and sodium heparin (25 µg/ml). For experimentation HPMEC were seeded in triplicate on physically adsorbed thermoresponsive films and on TCP or Thermanox™ controls at a density of 20,000 cell/cm² and incubated for 96 hours. The cell culture medium was changed every two days. Incubation conditions were an atmosphere of 5 % CO₂ and 95 % air at 37 °C. A chilling and heating dry bath was used to maintain a working temperature above the polymers’ LCST, except when they were housed under incubation conditions. Care was taken when changing the medium and handling the samples to ensure that the temperature did not fall below the LCST which would initiate unwanted and premature cell detachment.
3.2.6 Cell Detachment and Imaging

Cell growth was observed and images were captured using an Olympus BX51 phase contrast microscope equipped with an Image Pro-Plus analysis system. Detachment was initiated by removing the old warm medium followed by rinsing with pre-warmed HBSS (37 °C), before finally adding cold HBSS (4 °C). The samples were then placed on a chilling and heating dry bath set to 4 °C. Cell detachment was monitored through bright field microscopic observation and several images were captured as the process progressed. Cell detachment was defined as the time point at which all cells have been detached from the surface in question.

3.2.7 Cell Activity Assays

The alamarBlue™ assay was used to assess cell metabolic activity in both the HPMEC grown on the thermoresponsive polymer films and the controls. Total DNA content of the HPMEC grown on both the thermoresponsive polymer films and the controls was determined using the Quant-iT™ PicoGreen® dsDNA assay kit. Both assays were performed according to the manufacturer’s instructions and all experiments were performed in triplicate.

3.2.8 Transmission Electron Microscopy (TEM)

TEM was used to assess the integrity and structure of junctions and cell organelles in HPMEC grown on both the physically adsorbed pNIPAm films and the Thermanox™ controls. HPMEC grown on both the physically adsorbed thermoresponsive films and the Thermanox™ controls were rinsed twice with warm (37 °C) HBSS. The monolayers were then fixed with 2 % glutaraldehyde in 0.1 M cacodylate buffer for one hour at room temperature. Samples were then rinsed with cacodylate buffer and post-fixed with 1 % osmium tetroxide in cacodylate for one hour at room temperature. Another cacodylate rinse was performed after which the samples were dehydrated through a graded alcohol
series and then embedded in low viscosity resin (R1078A Agar Scientific). Ultra-thin sections were cut on an ultra-microtome (Leica Reichert Jung Ultracut) using a diamond knife. The sections were then stained with uranyl acetate and lead citrate and examined on a Hitachi H7000 transmission electron microscope.

3.2.9 Scanning Electron Microscopy (SEM)

SEM was used to assess the cell sheet integrity of the HPMEC grown and detached from the physically adsorbed films and the Theranox™ controls i.e. cell detachment was initiated prior to sample dehydration for SEM preparation, this ensured that cell sheet detachment was captured mid-stream, therefore it was possible to observe cells still attached to the polymer surface and the integrity of the sheet section that had detached. HPMEC monolayers were rinsed with HBSS, fixed with 2% Glutaraldehyde, rinsed with cacodylate buffer, post-fixed with 1% osmium tetroxide, rinsed again with cacodylate buffer and dehydrated through a graded alcohol series, as outlined above. After dehydration, the samples were placed in a critical point dryer and then sputter coated with gold. The coated samples were examined on a Hitachi S2600N variable pressure scanning electron microscope.

3.2.10 Fluorescence Microscopy

Fluorescence microscopy was used to assess cell sheet integrity and cellular adhesion after temperature controlled detachment. HPMEC’s were seeded at a density of 20,000 cell/cm² on either polymer coated 8 well chamber slides (Ibidi™) or, on glass bottomed Petri dishes (MatTek™) (controls) and incubated for 48 hours. The HPMEC grown on the polymer coated 8 well chamber slides were detached via temperature control and transferred to new polymer coated 8 well chamber slides. The control samples were detached using trypsin and they were re-seeded in uncoated glass bottom Petri dishes. The HPMEC were given 24 hrs to adhere after which the samples were treated according to the procedure outlined below. Media was removed and the cells were then rinsed quickly with
prewarmed (37 °C) phosphate buffered saline (PBS), and were fixed with 4 % paraformaldehyde in PBS for 15 mins. After fixation the samples were permeabilized with 0.1 % Triton X-100 in PBS (3 mins) and blocked with 3 % Normal Goat Serum (NGS) in PBS (20 mins). The primary rabbit monoclonal anti-paxillin antibody (focal adhesions) (Abcam) was then added for an hour at room temperature (1:250 in block). The Goat Anti-Rabbit secondary antibody (Alexa 594 nm at 1:200 in PBS) (Invitrogen) was incubated with the samples for 1 hour at room temperature. Samples were counterstained with Phalloidin, for F-Actin, (Alexa 488nm 1:200) and Hoechst, for Nuclei, (1:1000) (both Invitrogen). Cells were stored in PBS and imaging was carried out less than 24 hours after staining. All samples were imaged using an Olympus IX81 microscope and Volocity™ software. Low magnification overviews were captured at 40x magnification, with detailed views captured using a 100x oil lens. Appropriate primary and secondary antibody, and polymer controls were also included in this work in order to confirm that there was no non-specific binding on the part of either the primary or secondary antibody.
3.3 RESULTS AND DISCUSSION

3.3.1 Physical Characterization

The roughness of the physically adsorbed samples was assessed via AFM analysis and the comparative root mean square (RMS) roughness of the films is detailed in Table 3.1, where RMS denotes the standard deviation of the Z-values along the reference line.

Table 3.1: Roughness measurements for the physically adsorbed films measured using AFM in tapping mode. Three samples of each surface type were analyzed in three random areas.

<table>
<thead>
<tr>
<th>Surface Type</th>
<th>RMS Roughness (Mean ± SD) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermanox™</td>
<td>17.0 ± 2.3</td>
</tr>
<tr>
<td>pNIPAm &lt;LCST</td>
<td>9.8 ± 2.5</td>
</tr>
<tr>
<td>pNIPAm &gt;LCST</td>
<td>7.7 ± 5.1</td>
</tr>
<tr>
<td>Poly (NIPAm-co-NtBAm) &lt;LCST</td>
<td>8.6 ± 3.5</td>
</tr>
<tr>
<td>Poly (NIPAm-co-NtBAm) &gt;LCST</td>
<td>26.9 ± 4.8</td>
</tr>
</tbody>
</table>

The variations in the roughness of the polymer films prepared by adsorption at different temperatures are probably due to the differences in the polymer structure above and below LCST. In very dilute solutions, above the LCST, thermoresponsive polymers exist in a condensed or globular conformation whereas below the LCST the polymers adopt an open chain conformation; such chains are commonly referred to as to as Flory coils. Below the LCST of the thermoresponsive polymers, adsorption of these Flory coils takes place which results in the formation of a thin polymer film. Adsorption above the polymers’
LCST can occur via two different scenarios depending on the molecular weight of the polymer as depicted in figure 3.1. In one of the scenarios inter-chain collapse takes place before individual chain collapse above the LCST, this is thought to be the case for high molecular weight polymers. A stable suspension of solid polymer particles is formed, ranging in size from 30 to 300 nm, depending on the polymer concentration and the kinetics of aggregate formation [25]. The adsorption of such particles may result in the formation of a surface with a roughness which is correlated to the size of the particles. We believe this is the adsorption scenario for the formation of the poly (NIPAm-co-NtBAm) films due to its higher comparative molecular weight (350,000 g/mol). In the second scenario, which pertains to low molecular weight polymers, individual chain collapse precedes aggregation which may lead to the adsorption of numerous polymer particles due to intra-chain collapse [26]. We hypothesize that this is the adsorption scenario which occurs for the pNIPAm films produced above the LCST. Roughness is determined by the size of the individual polymer globules, which depends only on the molecular weight of the polymer. [26] poly((NIPAm-co-NtBAm)’s molecular weight (350,000 g/mol) is much larger than that of pNIPAm (20,000-25,000 g/mol) and as a result above the LCST it most likely undergoes inter-chain collapse where as pNIPAm adsorption probably proceeds via intra-chain collapse.
Figure 3.1: Schematic illustrating the differences in film roughness for high molecular weight (left hand side of illustration) and low molecular weight (right hand side of illustration) polymers deposited above the polymers LCST. There are two principle modes of polymer collapse. In the case of high molecular weight polymers the various chains collapse prior to the chain to globule collapse, giving rise to a mesoglobule. Intra-chain collapse, which low molecular weights polymers undergo, results in the formation of particles that are much smaller and are dependent only on the molecular weight of the polymer. It is thought that this difference in conformation affects the amount of polymer adsorbed and also the roughness of the deposited surface.
Table 3.2: Thickness of the physically adsorbed films measured using AFM in scratch mode. Three samples of each surface type were scratched in three random areas and fifteen thickness measurements were taken from each scratch.

<table>
<thead>
<tr>
<th>Surface Type</th>
<th>Thickness (Mean ± SD) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pNIPAm &lt; LCST</td>
<td>7.1 ± 3.4</td>
</tr>
<tr>
<td>pNIPAm &gt; LCST</td>
<td>9.1 ± 4.1</td>
</tr>
<tr>
<td>Poly (NIPAm-co-NtBAm) &lt; LCST</td>
<td>11.7 ± 2.9</td>
</tr>
<tr>
<td>Poly (NIPAm-co-NtBAm) &gt; LCST</td>
<td>38.2 ± 2.1</td>
</tr>
</tbody>
</table>

This mechanism is also in agreement with experimental results obtained for the film thickness. Below the LCST pNIPAm films are slightly thinner than poly (NIPAm-co-NtBAm) films, most likely due to the weak dependence of the adsorbed amount of polymer on molecular mass. Above the LCST polymers adsorb in the globular state, where the size of globules depends on the molecular weight of polymer according to the mechanism outlined above. Large poly (NIPAm-co-NtBAm) globules formed above the LCST, forming thicker films with higher roughness than their lower temperature-fabricated counterparts and pNIPAm films adsorbed above LCST.

The LCST data for the polymers was obtained from turbidimetric changes for the aqueous polymer solutions with temperature using a UV-vis spectrophotometer, (λ = 500 nm). The relative LCSTs’ of the thermoresponsive polymers depend on the relative contribution of hydrophobic and hydrophilic monomers present in the polymer. As expected, the introduction of a hydrophobic comonomer reduces
the LCST of the polymer system giving rise to a LCST of 16 °C for poly (NIPAm-co-NtBAm) compared to a LCST of 32 °C for the homopolymer [27]. The comparatively lower LCST of poly (NIPAm-co-NtBAm) facilitates routine cellular observation without the risk of accidental cell detachment unlike the homopolymer polymer analogue where great care needs to be taken due to the proximity of the LCST to physiological temperature.

Advancing water contact angles were measured for each surface type at 40°C. Two regimes of contact angle behaviour with time were observed as presented in figure 3.2.

The dashed trace in figure 3.2 shows the change in contact angle over time for pNIPAm. This is an example of the normal behaviour observed for polymer advancing contact angle profiles i.e. where the contact angle does not change significantly with the progression of time. The black trace in figure 3.2 is an example of stick and slip type behaviour, which was observed for poly (NIPAm-co-NtBAm) films. The plot shows the change in the contact angle of the poly (NIPAm-co-NtBAm) film produced at 40 °C with advancing time, where the contact angle trace displays a distinct ‘saw tooth’ type pattern. This trend is often observed in advancing contact angle traces of pNIPAm and pNIPAm copolymer films and is referred to as stick and slip type behaviour [20]. The stick angle is the angle formed after the advancing drop has slipped. The angle observed prior to the drop slipping is the slip angle. For the majority of polymer based systems the advancing contact angle does not vary substantially over time as is seen in figure 3.2 (dashed trace) but in the case of so called stick and slip behaviour, again seen in figure 3.2 (black trace); a saw-tooth type contact angle trace is observed over time as the drop progresses across the sample surface. It is thought that stick and slip behaviour may be the result of either surface roughness or liquid absorption into the polymer film [20]. Gilcreest et al. [20] describe the stick and slip effect for similar polymers in detail and proposed that the stick angle should be close to the “true” equilibrium contact angle [20]. Therefore it is reasonable to compare stick angles to contact angles obtained on the systems where stick and slip behaviour is not observed. The lack of stick and slip behaviour observed for pNIPAm homopolymer films here is likely due to the film thickness as a similar trend was observed by Nash et al. [22]. It is
possible for pNIPAm films to absorb water even above the LCST. This absorption of water softens the polymer film and this deformation results in stick and slip behaviour. As film thickness decreases its ability to absorb water also decreases and this may be why pNIPAm films prepared via physical adsorption do not display stick and slip behaviour.

Figure 3.2: Dashed trace - Advancing water contact angle of physically adsorbed pNIPAm < LCST. Black trace - Advancing water contact angle of physically adsorbed poly (NIPAm-co-NtBAm) < LCST
Table 3.3: Advancing water contact angles for physically adsorbed films taken at 40 °C. Three samples of each surface type were measured.

<table>
<thead>
<tr>
<th>Surface Type</th>
<th>Contact Angle (Mean ± SD) (degrees)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermanox™</td>
<td>58° ± 0.2</td>
</tr>
<tr>
<td>pNIPAm &lt;LCST</td>
<td>56° ± 3</td>
</tr>
<tr>
<td>pNIPAm &gt;LCST</td>
<td>68° ± 3</td>
</tr>
<tr>
<td>Poly (NIPAm-co-NtBAm) &lt;LCST</td>
<td>79° ± 1^a</td>
</tr>
<tr>
<td>Poly (NIPAm-co-NtBAm) &gt;LCST</td>
<td>84° ± 1^a</td>
</tr>
</tbody>
</table>

a) stick angle

As expected, the films adsorbed above the LCST display higher contact angles compared to those adsorbed below, as the polymer is in a more hydrophobic state when adsorbed. The larger contact angles values observed for the poly (NIPAm-co-NtBAm) films compared to the pNIPAm films are due to the introduction of a more hydrophobic monomer NtBAm. Another factor which contributes to the higher contact angles values recorded for the poly (NIPAm-co-NtBAm) physically adsorbed films produced above the LCST is surface roughness, as the advancing contact angle of the films produced above the polymer’s LCST are rougher than those produced below the LCST. Both Cassie et al. [28] and Wenzel [29] reported that rougher surfaces give rise to larger contact angles than corresponding smoother surfaces and this appears to true for the poly (NIPAm-co-NtBAm) films [28, 29].

The stick angles observed for the poly (NIPAm-co-NtBAm) films produced above and below the LCST are in good agreement with previously reported stick angles by Fan et al. [30] for poly (NIPAm-co-NtBAm) films, i.e. 80.2° ± 0.9 for
spin coated films of poly (NIPAm-co-NtBAm). The mean contact angle for pNIPAm prepared above the LCST is in-keeping with the mean contact angle reported by Nash et al. [22] for 13 nm UV modified pNIPAm films prepared via spin coating [22].

The contact angle of the pNIPAm film produced below the LCST is similar to that of Thermanox™; this may be because the under layer may be appreciable to the contact angle technique due to the ultra-thin nature of the polymer coating. Considering thickness data measured for the pNIPAm films, it is entirely plausible that the contact angle recorded in this thin film regime would reflect a strong influence of the underlayer substrate character.
Table 3.4: Relative atomic composition of physically adsorbed films compared with the stochiometrically calculated composition of the NIPAm monomer and the poly (NIPAm-co-NtBAm) monomer

<table>
<thead>
<tr>
<th>Surface Type</th>
<th>% Carbon</th>
<th>% Nitrogen</th>
<th>% Oxygen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncoated TCP</td>
<td>85.4</td>
<td>2.9</td>
<td>11.6</td>
</tr>
<tr>
<td>NIPAm monomer a</td>
<td>75.0</td>
<td>12.5</td>
<td>12.5</td>
</tr>
<tr>
<td>NIPAm-co-NtBAm monomer a</td>
<td>76.0</td>
<td>12.0</td>
<td>12.0</td>
</tr>
<tr>
<td>pNIPAm &lt;LCST</td>
<td>84.3</td>
<td>5.8</td>
<td>9.9</td>
</tr>
<tr>
<td>pNIPAm &gt;LCST</td>
<td>79.8</td>
<td>9.4</td>
<td>10.7</td>
</tr>
<tr>
<td>Poly (NIPAm-co-NtBAm) &lt;LCST</td>
<td>80.6</td>
<td>9.4</td>
<td>9.9</td>
</tr>
<tr>
<td>Poly (NIPAm-co-NtBAm) &gt;LCST</td>
<td>78.3</td>
<td>11.3</td>
<td>10.3</td>
</tr>
</tbody>
</table>

*a* Obtained from molecular formula

The composition of the films produced above the LCST for pNIPAm and poly (NIPAm-co-NtBAm) are in good agreement with the theoretical stoichiometric calculations for the NIPAm and NIPAm-co-NtBAm monomer respectively, confirming successful adsorption of the thermo-responsive polymer. The
deviation in the atomic composition of the pNIPAm film produced below the LCST may be due to the sampling depth of the XPS analysis; as XPS analyses the outermost 10 nm and as a result the data for the pNIPAm film may reflect a combination of the compositions of the deposited film and the TCP underlayer. Successful polymer adsorption is corroborated by the presence of an N1 s peak at 399 eV which is indicative of an amide and a C1 s peak at 287.5 eV which is the fingerprint peak for carbonyl functional groups, these peaks were observed for all of the physically adsorbed films but they were absent for the TCP controls.
3.3.2 Biocompatibility assessment

This study sought to investigate the cell culture regenerative potential of physically adsorbed thermoresponsive films. HPMEC were seeded on the prepared thermoresponsive polymer films, TCP and Thermanox™ controls and the relative cell growth was assessed qualitatively via bright-field microscopy and quantitatively via metabolic activity and DNA assays, after 96 hours of incubation. Cell sheet, junctional and organelle integrity were assessed using SEM and TEM respectively. HPMEC were chosen to investigate the cell culture potential of physically adsorbed films as these cells have been shown to proliferate well and produce cell sheets under cell culture conditions after a suitable length of incubation time. The production of an intact cell sheet is of interest as it may be possible to stack the cell sheets creating tissue suitable for transplantation studies.
Figure 3.3: Percentage metabolic activity of HPMEC grown on thermoresponsive polymer films compared to HPMEC grown on the TCP controls after 4 days of incubation. Percentage metabolic activity was measured using the alamarBlue™ assay kit. The error bars refer to standard deviation where 3 separate samples of each type of substrate were used and assays were performed in triplicate. The astrix denote statistical significance.
Figure 3.4: Percentage DNA of HPMEC grown on various thermoresponsive polymer films compared to HPMEC grown on the TCP control after 4 days of incubation. The percentage DNA content of HPMEC was determined using a Quant-iT™ PicoGreen® kit. The error bars refer to the standard deviation where 3 separate samples of each type of substrate were used and assays were performed in triplicate. The astrix denote statistical significance.
The assay results outlined graphically in **figures 3.3 and 3.4** indicate that HPMEC cells seeded on physically adsorbed poly (NIPAm-co-NtBAm) films formed below the polymer’s LCST proliferated and displayed a similar level of metabolic activity to cells seeded on the TCP controls, which was not the case for the poly (NIPAm-co-NtBAm) films deposited above the polymer’s LCST. Previous work by Moran *et al.* [31] and Fan *et al.* [17] indicates that cell growth is better on more hydrophobic thermoresponsive derivatives such as the poly (NIPAm-co-NtBAm) copolymer used here than on pure pNIPAm and these results for the poly (NIPAm-co-NtBAm) films formed below the LCST further support these findings [17, 31].

Statistical analysis has shown that the difference in cell metabolic activity for the HPMEC grown on pNIPAm films adsorbed above and below the LCST, and on the poly (NIPAm-co-NtBAm) film adsorbed above the LCST compared to HPMEC grown on the TCP control, are statistically significant (p value ≤0.05). Cell metabolic activity is not significantly different when comparing HPMEC grown on the poly (NIPAm-co-NtBAm) films absorbed below the LCST to HPMEC grown on the control. This may imply that HPMEC are just as metabolically active on the poly (NIPAm-co-NtBAm) film adsorbed below the LCST, as on the control. The differences in dsDNA content observed for HPMEC grown on the pNIPAm films adsorbed above and below the LCST and on the poly (NIPAm-co-NtBAm) films adsorbed above the LCST, compared to the HPMEC grown on the TCP control, are statistically significant (p value ≤0.05). No statistically significant difference was observed when the HPMEC grown on the poly (NIPAm-co-NtBAm) below the LCST were compared to the HPMEC grown on the control.

The reasons why cell growth is greater on surfaces prepared below the LCST than on those prepared above may be due to a number of factors, such as roughness, hydrophobicity/hydrophilicity balance and thickness, all of which are influenced by the polymers’ adsorption temperature. Cell growth and proliferation is a delicate process, which will only proceed optimally if conditions are suitable for a specific cell type. For example, studies have shown that cells
are resistant to growth on surfaces that are too hydrophobic [32-34]. The same is true for surfaces that are too hydrophilic, as these surfaces hinder protein adsorption, which is necessary to facilitate cell adhesion [35, 36]. If a balance between these two extremes is achieved cell adhesion and proliferation becomes possible, if other conditions are also satisfied [35]. Gessner et al. [37] have reported that protein adsorption on hydrophobic surfaces is elevated compared to hydrophilic analogues, and this increase in protein adsorption promotes cell adhesion [37]. More pertinent to this study, Allen et al. [38] investigated protein adsorption on poly(NIPAm-co-NtBAm) polymer films of varying composition and while they found that increased hydrophobicity decreased the adsorption of the majority of serum proteins, adsorption of fibronectin which is an important cell adhesion promoter was increased on the poly(NIPAm-co-NtBAm) films [38]. The aforementioned studies offer possible explanations as to why the more hydrophobic poly(NIPAm-co-NtBAm) films produced below the LCST perform better in terms of HPMEC cell adhesion and proliferation.

There is a good body of evidence which supports our finding that endothelial cells grow better on smoother surfaces. Hormia et al. and Baharloo et al. report that epithelial adhesion and cell growth is better on smoother titanium surfaces. In the above mentioned investigations vinculin staining of the focal adhesions showed that epithelial cells grown on smooth surfaces possessed both a greater number and larger adhesion points [39, 40]. Xu et al. [41] investigated the effect, the surface roughness of solvent cast and electro-spun poly (L-lactic acid) (PLLA) films had on human vascular endothelial cells proliferation. Human vascular endothelial cell adhesion, function and proliferation were enhanced on the smoother solvent cast films compared to the rougher electro-spun films. While the majority of endothelial cell types grow better on smoother surfaces there are some exceptions including; human umbilical vein endothelial cells which were reported by Chung et al. [42] to have grown better on polyurethane-polyethylene glycol surfaces with increased roughness in the nanometer scale [42]. Cell type dictates a cells response to surface roughness for example, osteoblasts grown on rougher titanium surfaces proliferate better and display a more mature phenotype than those cultured on smoother surfaces. Bächle et al.
[43] studied the effect of surface roughness on osteoblasts and they concluded that proliferation was greater on rougher titanium surfaces than on smoother ones [43]. Xu et al. [41] found that vascular smooth muscle cells proliferated better on rougher electro-spun PLLA films than on the smooth spin coated films [41]. While smoother surfaces are optimal for endothelial cells such as the HPMEC used in this investigation, it is important to note that this may not be the case for all cell types.

There appears to be a polymer film thickness threshold for optimal HPMEC growth with HPMEC preforming better on the thinner poly (NIPAm-co-NtBAm) films produced below the LCST than on the poly (NIPAm-co-NtBAm) films produced above the LCST. The poly (NIPAm-co-NtBAm) film thickness is greater than the cell adhesion inhibiting thickness threshold of 30 nm reported in the literature [11, 44]. There are exceptions to this thickness threshold most notably plasma polymerized thermoresponsive films and previous work in our lab by Nash et al. [45] and Fan et al. [17] on spin coated and solvent cast films [17, 21, 45]. The influence of the thickness threshold appears to be dependent on the method of film preparation and as such it is likely that thickness is not the sole driving force behind successful cell adhesion and proliferation.

In the present study, it is more likely a combination of favorable film factors including thickness, roughness and hydrophobicity that provide an environment which promotes optimum biomaterial-surface interfacial interactions and thus facilitates cell proliferation similar to positive controls, for the poly (NIPAm-co-NtBAm) films prepared below the polymers LCST.

The cell detachment times for HPMEC grown on the physically adsorbed thermoresponsive films can be seen in table 3.5. Cell detachment was initiated by the removal of warm cell culture medium, followed by rinsing with pre-warmed HBSS (37 °C) before finally introducing cold HBSS (4 °C). The initial rinsing with HBSS serves to remove any residual media serum, which would hinder the cell detachment process. The dishes were then placed on the chilling and heating dry bath set to 4 °C. Cell detachment times ranged from 20 minutes, for the pNIPAm films produced above and below the LCST, to 30 minutes from the poly (NIPAm-co-NtBAm) films produced above the LCST and
60 minutes for the poly (NIPAm-co-NtBAm) prepared below the LCST. HPMEC detachment was slower from the poly (NIPAm-co-NtBAm) films due to the wider temperature range of transition for poly (NIPAm-co-NtBAm) compared with pNIPAm, and the presence of a significant quantity of a very hydrophobic component. The faster detachment observed for cells detaching from the poly (NIPAm-co-NtBAm) films produced above the LCST is not completely clear at the moment and most likely is due to the fact that the poly (NIPAm-co-NtBAm) films produced above the LCST are rougher than the poly (NIPAm-co-NtBAm) films produced below the LCST. As such, the rate of polymer dissolution would be faster for the comparatively rougher films produced above the LCST than the smoother films produced below the LCST as the rougher polymer surface facilitates faster infiltration of HBSS when the ambient temperature is dropped below the LCST.

Furthermore, it is likely that HPMEC detachment was comparatively slower from the poly (NIPAm-co-NtBAm) films than from pNIPAm films as protein adhesion is stronger on the more hydrophobic surfaces and as a result a larger $f_{cell}$ would be required to break the protein integrin bonds than in the case of the pNIPAm films. $f_{cell}$, as defined by Haplerin et al. [46], is a disjoining force caused by the confinement of the ventral membrane of an adhering cell by the integrin-ECM bonds [46]. The integrin-ECM bonds are weakened by the disjoining force of $f_{cell}$ resulting in desorption of the ECM proteins and subsequently the detachment of the cell sheet.

Bright field microscopy was used to image the cell sheets before, (Figure 3.5a) and during the detachment of the cell sheets from the polymer films, (Figure 3.5b). Furthermore, an SEM imaging of a cell sheet detaching from poly (NIPAm-co-NtBAm) (prepared below the LCST) clearly shows that cell sheet integrity is maintained during the detachment process, (Figure 3.6). The sheet was detached by lowering the ambient temperature to 4°C and the sample was rapidly submitted for sample dehydration mid-cell sheet detachment and preparation for SEM in order to capture a high quality image of cell sheet detachment. Additionally, the SEM micrograph of the cell sheet clearly shows the basolateral ECM that detaches with the cell sheet. The maintenance of this extracellular matrix ensures that cells that are detached in this manner naturally
facilitate better intercellular biophysical communication compared with cells detached through conventional avenues where the subcellular matrix is digested [45]. HPMEC which were detached from the physically adsorbed films and re-seeded showed near full viability, >95 % when tested with trypan blue dye.

Neither the pNIPAm nor the copolymer films are covalently bound to the underlayer substrate and as such, polymer dissolution as well as polymer swelling will take place upon temperature reduction and these combine to facilitate cell detachment. Grafted films, such as those prepared by grafting techniques, are covalently bound to their underlayer substrate and as such the mechanism of cell detachment proceeds via polymer swelling only. In this study, the amount of free polymer in solution is minimized due to the low amount of polymer adsorbed as evidenced by the thickness of the physically adsorbed films. Moreover, Malonne et al. [8] investigated the toxicity of pNIPAm in mice for 28 days at concentrations of 2,000 mg/kg and found no detectable toxicity [8]. Cytotoxicity studies carried out by Takezawa et al. [9] additionally suggest that pNIPAm is non cytotoxic under cell culture conditions [9].
Table 3.5: Detachment times for HPMEC grown on various thermoresponsive polymer films. Cell detachment was initiated by lowering the temperature of the sample below the particular polymer’s LCST. Three samples of each surface type were measured.

<table>
<thead>
<tr>
<th>Surface Type</th>
<th>Detachment Time (Mean ± SD) (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pNIPAm &lt;LCST</td>
<td>20 ± 2</td>
</tr>
<tr>
<td>pNIPAm &gt;LCST</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>Poly (NIPAm-co-NtBAm) &lt;LCST</td>
<td>60 ± 9</td>
</tr>
<tr>
<td>Poly (NIPAm-co-NtBAm) &gt;LCST</td>
<td>30 ± 4</td>
</tr>
</tbody>
</table>
Figure 3.5: Monolayer of Human Pulmonary Microvascular Cells (HPMEC) growth on a) poly (NIPAm-co-NtBAm) < LCST b) Cell detachment from, poly (NIPAm-co-NtBAm) < LCST. Seeding density of 20,000 cell/cm² and 4 days incubation. Scale bar 200 µm
Figure 3.6: SEM image of a HPMEC sheet detaching from, poly (NIPAm-co-NtBAm) < LCST film. Cell detachment was initiated using temperature control prior to sample preparation for SEM viewing.
Transmission electron microscopy was used to examine the cell and organelle composition of HPMEC grown on both Thermanox™ controls and HPMEC cultured on the thermoresponsive polymers, in order to comparatively observe the absence or presence of healthy cell features in cell sheets grown on both substrate types. The images, compiled in figures 3.7 to 3.9, show that cells grown on the Thermanox™ controls and on the thermoresponsive polymers alike displayed all the signs of healthy and metabolically active cells. In more detail; Cell shape – HPMEC’s can be categorized as a simple squamous epithelium. In culture, the cells adopt a polygonal shape which was observed on all of the polymer samples and the controls used in this investigation. Organelle Content - Organelles including mitochondria, lysosomes, rough endoplasmic reticulum (RER) and ribosomal structures were distributed in the cytoplasm and clearly visible in both cells grown on the Thermanox™ controls and on the thermoresponsive polymers. It can also be inferred from the large quantity of mitochondria in the cells cultured both on TCP and the polymer that the HPMEC appear to be metabolically active. Ribosomal density within the cytoplasm appears equal in both cultures, with elaborate RER’s and Golgi visible. Further to this, plasmalemmal vesicles, now more commonly known as caveolae, appear to be present in both cultures. Caveolae are often deemed a predominant feature of the microvasculature of endothelial cells. They may be described as “infoldings” or micro-invaginations of the cell membrane [47]. Initially, it was thought that these vesicular structures were just involved in transcytosis, or movement of cargo from one cellular surface to another, but it is now believed that they carry out many more complex functions including signal transduction [48, 49]. A variety of junctional complexes were evident between cells. Tight junctions were present in the apical domain. Desmosomes were observed between cells in both cultures. The presence of these junctions indicates that the cells have formed a cohesive monolayer. In summary, the features observed and detailed above indicate the presence of healthy HPMEC and the healthy features which were observed for cells grown on the Thermanox™ controls were mirrored in the cells that were grown on the thermoresponsive polymers in terms of organelle presence, abundance, intercellular junctions and cell morphology.
Figure 3.7: TEM image of a) overview of HPMEC grown on Thermanox™, scale bar 2 µm  b) overview of HPMEC grown on poly (NIPAm-co-NtBAm) < LCST, scale bar 2 µm  c) mitochondria in HPMEC grown on Thermanox™, scale bar 500 nm  and d) mitochondria in HPMEC grown on poly (NIPAm-co-NtBAm) < LCST, scale bar 500 nm. An incubation period of 4 days was used.
Figure 3.8: The black arrows in the TEM images highlight the following organelles observed in the HPMEC a) caveolae in HPMEC grown on Thermanox™, b) caveolae in HPMEC grown on poly (NIPAm-co-NtBAm) < LCST, c) Golgi apparatus in HPMEC grown on Thermanox™ and d) Golgi apparatus in HPMEC grown on poly (NIPAm-co-NtBAm) < LCST. The mentioned organelles are indicative of cell health. The scale bar is 500 nm in all of the above images. An incubation period of 4 days was used.
Figure 3.9: The black arrows in the TEM images highlight the followings organelles a) lysosome in a HPMEC grown on Thermanox™, b) lysosome in a HPMEC grown on poly (NIPAm-co-NtBAm) < LCST, c) cell junction in HPMEC grown on Thermanox™ and d) cell junction in HPMEC grown on poly (NIPAm-co-NtBAm) < LCST. The presences of the mentioned organelles are indicative of cell health and cell sheet integrity. The scale bar is 500 nm in all of the above images. An incubation period of 4 days was used.
Figure 3.10: HPMEC grown on a) poly (NIPAm-co-NtBAm) < LCST X 40 Scale bar 56 µm, b) pNIPAm < LCST X40 Scale bar 56 µm, c) poly (NIPAm-co-NtBAm) < LCST X100 Scale bar 22 µm and d) NIPAm < LCST X 100 Scale bar 22 µm. Red – paxillin staining, green- F-actin staining and blue- nuclear staining. The initial incubation period was 4 days after which the cells were detached and transferred to a new polymer coated substrate and an incubation period of 1 day was used.
Figure 3.11: HPMEC grown on a) uncoated Ibidi™ dish (polymer control) X 40 Scale bar 56 µm, b) uncoated Ibidi™ dish (polymer control) X 100 Scale bar 22 µm, c) poly (NIPAm-co-NtBAm) < LCST (primary antibody control) X40 Scale bar 56 µm and d) poly (NIPAm-co-NtBAm) <LCST (secondary antibody control) X40 scale bar 56 µm. Red – paxillin staining, green- F-actin staining and blue- nuclear staining. The initial incubation period was 4 days after which the cells were detached and transferred to a new polymer coated substrate and an incubation period of 1 day was used.
Fluorescence microscopy was used to investigate the cell adhesion of HPMEC that had been grown on and detached from poly (NIPAm-co-NtBAm), pNIPAm coated surfaces and controls. The polymer coatings were produced below the polymer’s LCST in both cases. Once the HPMEC reached the desired level of growth the cells were detached via temperature control and then transferred to another polymer-coated surface, which had been coated with the same thermoresponsive polymer as the initial dish. This not only allowed us to confirm cell detachment through temperature control but also to observe the behaviour of detached cells post seeding in comparison with cells harvested through conventional means. With this in mind, we specifically stained with a view to observing the focal adhesions of the reseeded cells. Focal adhesions are important points of cell adhesion as they connect the ECM with the cells’ actin cytoskeleton [50]. Focal adhesions function as signaling centers and as such they play an important role in gene expression and cell growth [50-52]. In the case of cells detached from the poly (NIPAm-co-NtBAm) copolymer films, confluent cell sheets were detached and reseeded using a micro-pipette tip to facilitate the transfer. In the case of cells detached from pNIPAm, cell growth was suboptimal and therefore the cells did not detach as a full cell sheet, rather a number of cell clumps, all of which again were transferred using a 1000 µl micro-pipette. Furthermore, for comparative purposes cells were detached from control TCP dishes using conventional trypsinization and similarly reseeded. Rabbit anti-paxillin antibodies were used to successfully mark the focal adhesions, as can be seen in figures 3.10 and 3.11 above (red staining). Unsurprisingly, cell density was much greater for the HPMEC grown, detached and reseeded from and on the poly (NIPAm-co-NtBAm) films produced below the LCST than on the pNIPAm polymer films. This is in agreement with the alamarBlue™ and PicoGreen® assay results for HPMEC grown on pNIPAm and poly (NIPAm-co-NtBAm) films. It is clear from the density and intensity of the focal adhesion staining seen in figure 3.10 that the HPMEC adhered to the poly (NIPAm-co-NtBAm) coated surfaces better than the pNIPAm coated surfaces. The HPMEC formed better focal adhesions on the poly (NIPAm-co-NtBAm) films produced below the LCST confirming that the poly (NIPAm-co-NtBAm) films produced below the LCST are the best substrate for the culture of HPMEC in
this study. The positive focal adhesion staining for the HPMEC seen in figure 3.10 and 3.11 proves that HPMEC sheets which have been detached via temperature control and then transplanted are capable of adhering to another polymer coated surface, i.e. cell sheet transfer was successfully achieved and cell focal adhesions appeared to indicate cell health similar to cells detached and reseeded through conventional means. Furthermore, cell sheets detached via temperature control from poly (NIPAm-co-NtBAm) are capable of adhering to another surface similar to cells detached through trypsinization. It is apt to note that no non-specific binding was observed for either the primary or secondary antibody (figure 3.11 g and h) proving that the red staining seen in figures 3.10 and 3.11 is solely due to the presence of healthy focal adhesions.

3.4 CONCLUSIONS

The work outlined here-in shows that the simple physical adsorption process can be successfully adopted to produce thermoresponsive polymer surfaces capable of facilitating both cell adhesion and gentle cell detachment. To achieve this goal we used commercially available pNIPAm and a poly (NIPAm-co-NtBAm) copolymer and the coatings were prepared at temperatures below and above the LCST. We also show that control can be achieved over the structure and properties of the adsorbed films by the variation of adsorption temperature. The adsorption temperature was shown to have an effect on the thickness and roughness of the films and subsequently their ability to facilitate cell adhesion and proliferation. HPMEC proliferation and viability was the highest on poly (NIPAm-co-NtBAm) surfaces adsorbed below the LCST, similar to TCP and Thermanox™ positive controls. In fact cell growth was better on the more hydrophobic physically adsorbed films, which were produced below the polymers’ LCST. While the aim of this work was to prepare thermoresponsive coatings in as accessible a manner as possible, the commercially available pNIPAm films proved unsuitable for HPMEC cell culture as proliferation was significantly inhibited when compared with positive controls. As such, it is
advisable to copolymerize pNIPAm with a more hydrophobic monomer in order to achieve optimal cell growth on physically adsorbed films. Cell detachment was achieved by lowering the temperature below the polymers’ LCST. Detachment times ranged from twenty minutes for pNIPAm physically adsorbed films to sixty minutes for poly (NIPAm-co-NtBAm) films adsorbed above the LCST. The TEM, trypan blue and fluorescence analysis prove that the HPMEC were still viable after detachment from the poly (NIPAm-co-NtBAm) polymer films. TEM analysis confirms that HPMEC grown on poly (NIPAm-co-NtBAm) adsorbed below the LCST are similarly healthy to HPMEC grown on the Thermanox™ controls. Bright field and SEM microscopy indicated that the cell sheet maintains its structural integrity during the detachment process from poly (NIPAm-co-NtBAm) films produced below the LCST. A unique potential advantage of the physical adsorption method for preparing thermoresponsive coatings is that this approach could be used to coat materials with complicated geometric profiles, which may be useful for medical devices or microfluidic systems and future work will focus on exploring this potential. The main advantage of this approach is its simplicity and accessibility. Such developments are highly desirable to the general cell culture community which in-turn leads to the continued motivation to design systems that can replicate this attractive technology.
3.5 REFERENCES


4 FABRICATION AND APPLICATION OF PHOTOCROSSLINKED, NANOMETER-SCALE, PHYSICALLY ADSORBED HYDROGELS FOR TISSUE CULTURE

4.1 INTRODUCTION

Physical adsorption as a method of hydrogel preparation requires no specialist equipment or training and can be used to coat surfaces with non-planar geometries. Furthermore, it is a technique which is widely utilized in cell culture, for example in the creation of collagen and fibronectin coatings. To prepare thermoresponsive hydrogels with a view to cell culture and cell sheet regeneration, we adopted this adsorption approach using thermoresponsive polymers. The inclusion of a photocrosslinkable polymer moiety in the polymer mix allows for the fabrication of nanometer thick thermoresponsive polymer hydrogels which can be covalently bonded to the underlying tissue culture substrate mimicking the effect of grafted thermoresponsive polymer films.

Poly (N-isopropylacrylamide) (pNIPAm) is a much studied thermoresponsive polymer with a lower critical solution temperature (LCST) of 32 °C. Above the LCST pNIPAm exists in a hydrophobic state which, under the right conditions, is conducive to cell adhesion and proliferation. Below the LCST pNIPAm swells and becomes hydrophilic which renders the surface repellent to cells and cultured cells simply detach from the surface. Due to the physiological proximity of its transition temperature and the ease with which cells and cell sheets may be detached, pNIPAm and its derivatives have been investigated as possible cell culture substrates for over two decades [1-3]. From a tissue engineering viewpoint, detaching cells from thermoresponsive surfaces is highly attractive as a confluent cell sheet can be detached, unlike conventional cell culture protocols. Additionally, it is a gentle means of detachment compared to
traditional methods. The detached cell sheets can then be transplanted for further cultivation *in vitro* or used for *in vivo* applications. A wide range of cell types have been successfully grown on pNIPAm based thermoresponsive surfaces to date and the number of studies dedicated to the refinement and improvement of this approach to cell sheet detachment is a testament to the attractiveness of the outcome [3-6]. However, in spite of this, the wide-spread adoption of using thermoresponsive surfaces for the production of cell sheets with a view to practical, clinical applications has been comparatively low. We, the authors, believe that this can be attributed to the fact that the preparation of such surfaces can be highly technologically complex and can require significant investment in terms of equipment and skills.

With this in mind, the principal motivation behind this study was to eliminate such obstacles by adopting an operationally facile and inexpensive technique for thermoresponsive hydrogel preparation, with a view to increasing the accessibility of cell sheet regeneration to a wider research cohort. Therefore, nanometer thick thermoresponsive polymer hydrogels were prepared on tissue culture plastic surfaces using physical adsorption prior to crosslinking via UV exposure. Both steps are accessible to most cell culture laboratories. The inclusion of a crosslinkable moiety in the polymer ensures that post crosslinking, when detaching cultured cells; the hydrogel does not dissolve into the cell culture matrix upon temperature reduction.

A study carried out by Nash *et al.* [7] described the fabrication of thermoresponsive polymer hydrogels of nanometer thickness using spin coating as the hydrogel preparation method. A novel crosslinkable copolymer of NIPAm with acrylamidebenzophenone (AcBzPh) was synthesized and polymer films were formed from an ethanolic solution of the polymer and exposed to UV irradiation [7]. The same photocrosslinkable monomer AcBzPh was used in this study due to its ability to covalently bond with polystyrene ensuring hydrogel immobilization.

Two crosslinkable polymer cousins were used in this study; poly (NIPAm-co-AcBzPh) (0.99 molar ratio of NIPAm – 0.01 molar ratio of AcBzPh) and poly (NIPAm-co-AcBzPh)-co-N-tertbutylacrylamide (NtBAm) (0.65 molar ratio of
NIPAm, 0.338 molar ratio of NtBAm and 0.012 molar ratio of AcBzPh). The introduction of the more hydrophobic NtBAm monomer comparatively decreases the polymers’ LCST from 31 °C for poly (NIPAm-co-AcBzPh) to 13 °C for poly (NIPAm-co-NtBAm-co-AcBzPh). The advantage of such a reduction in the LCST is the facilitation of routine cellular observation without the risk of unwanted cell detachment. Furthermore, it is thought that cells adhere and proliferate better on slightly hydrophobic pNIPAm based surfaces and thus this hypothesis is examined here [8, 9].

Hydrogels were prepared by the adsorption of the polymer from solution both above and below the polymers’ LCSTs to evaluate if the preparation temperature influenced the chemical and physical properties of the hydrogels produced and ultimately the cell culture response. The physical and chemical character of the hydrogels was assessed by AFM, XPS and advancing contact angle techniques. Human pulmonary microvascular cells (HPMEC) were seeded and incubated on the physically adsorbed hydrogels and subsequent cell detachment was initiated by ambient temperature depression. Cell growth, health and detachment were qualitatively assessed using microscopical imaging and the cell metabolic activity and viability was quantified. Cell and cell sheet re-adhesion post detachment was also assessed.
4.2 MATERIALS AND METHODS

4.2.1 Materials

Hanks balanced salts solution (HBSS), trypsin-EDTA, M199 cell culture medium, penicillin and streptomycin, sodium heparin, sodium cacodylate trihydrate, fetal calf serum (FCS), and trypan blue stain were purchased from Sigma-Aldrich and used as received. Glutamax and Quant-iT™ PicoGreen® dsDNA assay kit from Invitrogen, endothelial growth factor supplement from Becton Dickinson, alamarBlue™ assay from Biosciences, 2 % osmium tetroxide and agar low viscosity resin kit from Agar Scientific, Thermanox™ plastic 35 mm disks from Nunc™ and 25 % glutaraldehyde solution in water from AMSBIO were all used as received. All other plastic consumables were purchased from Sarstedt. An Ectotherm chilling and heating dry bath from Torrey Pines Scientific was used for careful temperature control. Human pulmonary microvascular endothelial cells (HPMEC) were kindly provided by Prof. James Kirkpatrick from the Institute of Pathology, Johannes-Gutenberg University.

4.2.2 Polymer Synthesis and Characterization

The synthesis of Poly (NIPAm-co-AcBzPh) (0.99 molar ratio of NIPAm – 0.01 molar ratio of AcBzPh) has been previously reported and thus it will not be discussed in detail here. [7]

The synthesis of poly (NIPAm-co-NtBAm-co-AcBzPh) (0.65 molar ratio of NIPAm, 0.338 molar ratio of NtBAm and 0.012 molar ratio of AcBzPh) was carried out according to the procedure for the synthesis of poly (NIPAm-co-AcBzPh) where a certain amount of NIPAm was replaced by NtBAm to obtain the desired composition.

GPC analyses were carried out with PL-gel mixed-D (300*7.8 mm, 5 μm nominal particle size) Polymer Labs columns. The mobile phase used was dimethylformamide (DMF) with 30 mM KBr. Separations were performed at 35 °C at a flow rate of 1 mL/min using a RI detector. Molecular weights of polymers were referenced to polystyrene standards [10].
2 mls of a 0.1 mg/ml polymer solution was added to the substrate of choice, which was either a tissue culture polystyrene (TCP Petri dish) (35 mm diameter) or a Thermax™ coverslip (30 mm diameter) (which was housed in a TCP Petri dish). Thermax™ coverslips were used as the under-layer substrate for atomic force microscopy (AFM), transmission electron microscopy (TEM), scanning electron microscopy (SEM) and advancing contact angle analyses. TCP Petri dishes were used for cell culture, qualitative cell assays (alamarBlue™ and PicoGreen®) and x-ray photoelectron spectroscopy (XPS). Ibidi™ 8 well slides with polymer bottoms were used as the underlayer substrates for fluorescence microscopy. The lids were removed from the TCP Petri dishes during deposition and drying. A chilling/heating dry bath was used to maintain the sample temperature at the desired deposition temperature, above or below the respective polymer LCST, 10 °C and 40 °C for poly (NIPAm-co-NtBAm-co-AcBzPh) and 20 °C and 40 °C for poly (NIPAm-co-AcBzPh), over a three hour incubation period. Any remaining solution was removed after two hours and the samples were rinsed twice with Millipore water and allowed to dry on the chilling/heating dry bath in ambient air for two hours. The physically adsorbed films were placed in a vacuum oven at 40 °C and 600 mbar for 24 hours. The dried uncrosslinked physically adsorbed films were crosslinked using a UV irradiation from medium pressure water cooled Hg vapor 400 W UV lamp (Photochemical Reactors Ltd.)
4.2.4 Physical Characterization

4.2.5 Atomic Form Microscopy (AFM)

Hydrogel roughness was determined using a Dimension 3100 AFM (Digital Instruments, Santa Barbara, CA, USA) AFM in tapping mode equipped with a Veeco 1-10 Ohm-cm phosphorus (n) doped Si tip using the following scan parameters: 10 µm × 10 µm scan, 1 Hz scan rate, a matrix of 512 × 512 points along the x-y plane were analyzed in a single scan. The route mean square (RMS) (RMS denotes the standard deviation of the Z-value along the reference line) roughness was taken as the hydrogel roughness.

4.2.6 X-Ray Photoelectron Spectroscopy (XPS)

XPS measurements were performed on a Kratos AXIS 165 X-ray photoelectron spectrometer using monochromatic Al Kα radiation (hν = 1486.58 eV) and a fixed analyzer pass energy of 20 eV; the binding energy scale was referenced against the C 1s line (284.8 eV).

4.2.7 Contact Angle

The procedure used to measure advancing contact angle in this work has been described previously [4, 7, 10]. Briefly, the samples were inserted in a temperature controlled attachment with windows based on a tilt stage. A thermocouple placed close to the sample was used to ensure that the temperature remained above the chosen polymer’s LCST during the course of the analysis. A drop of water was placed on the samples surface and a thin stainless steel needle was then introduced into the drop from above. The drop volume was slowly enlarged using a syringe pump. Every three seconds a new drop image was taken. DROPimage software developed by F. K. Hansen and marketed by Rame Hart was used to calculate the contact angles.
4.2.8 Thickness

For AFM thickness analysis the samples were scratched randomly in three areas and fifteen thickness measurements per scratch were taken. The AFM was operated in scratch mode and the following parameters were used; ramp size 1.229 µm, scan rate 1 Hz, trigger threshold 0.2 V, scan angle 90°, scratch length 1 µm and scan size 3 µm. The under-layer substrates used for thickness analysis were Thermanox™ disks [11, 12].

4.2.9 LCST measurements

A Cary 3 Bio UV-visible spectrophotometer equipped with a Cary temperature controller was used to determine the polymers’ LCSTs by turbidity measurements in aqueous solutions (1% w/v) (λ = 500 nm) [13].

4.2.10 Cell Culture

M199 medium supplemented with glutamax (2 mM), 20 % fetal calf serum (FCS), endothelial growth factor supplement (50 µg/ml), 1 % penicillin and streptomycin antibiotics, and sodium heparin (25 µg/ml) was used for culturing the HPMEC. HPMEC were seeded in triplicate at a density of 20,000 cell/cm² on either the physically adsorbed films or on TCP/Thermanox™ controls. Standard cell culture conditions of 5 % CO₂ and 37°C were maintained. The cell culture medium was replaced with fresh medium every second day. During all cell culture operations a working temperature above the chosen polymer’s LCST was maintained using a chilling/heating dry bath in order to prevent untimely cell detachment.

4.2.11 Cell Detachment and Imaging

An Olympus BX51 phase contrast microscope equipped with an Image Pro-Plus analysis system was used to observe cell growth and capture images of cell detachment. In order to initiate cell sheet detachment the old medium was removed and the samples were then rinsed twice with pre-warmed HBSS (37°C) after which cold HBSS was added (4 °C). A chilling heating dry bath was then used to ensure that the sample temperature remained at 4 °C throughout the detachment process. Bright field microscopy was used to monitor detachment.
Trypan blue stain was used to assess cell viability post cell detachment. Trypan blue staining was carried out according to the manufacturer’s instructions. The point at which all of the cells have detached from the surface in question is taken as the point of cell detachment in this work.

4.2.12 Cell Activity Assays

Cell metabolic activity for HPMECs grown on the physically adsorbed hydrogels and on the controls was determined using the alamarBlue™ assay. The Quant-iT™ PicoGreen® dsDNA assay kit was used to measure the total DNA content of the HPMECs grown on both the thermoresponsive polymer hydrogels and the controls. Both assays were performed according to the manufacturer’s instructions.

4.2.13 Transmission Electron Microscopy (TEM)

Junction structure, integrity and cell organelle health for HPMECs grown on both the physically adsorbed hydrogels and the controls, were assest using TEM. The samples were rinsed twice with pre-warmed HBSS (37 °C). Fixation with a 1 % glutaraldehyde solution in 0.1 M cacodylate buffer was then carried out at room temperature for 1 hour. Samples were then washed with cacodylate buffer and post fixed with 1 % osmium tetroxide for another hour at room temperature. A final cacodylate rinse was carried out and the samples were then dehydrated through a graded alcohol series. Finally the samples were embedded in low viscosity resin (R1078A Agar Scientific). Ultra-thin sections were cut on an ultramicrotome (Lecia Reichert Jung Ultracut) using a diamond knife. The sections were then stained with uranyl acetate and lead citrate and examined on a Hitachi H7000 TEM.

4.2.14 Scanning Electron Microscopy (SEM)

The integrity of the detached HPMEC sheets was investigated using SEM. The fixation and dehydration procedure is identical to that described for TEM. A critical point dryer was used to further dehydrate the samples after which they were sputter coated with gold. The coated samples were examined on a Hitachi
S2600N variable pressure SEM. Cell sheet detachment was initiated using temperature control prior to sample fixation and dehydration.

4.2.15 Fluorescence Microscopy

Cell transplantation to a fresh substrate after temperature controlled detachment was investigated using fluorescent microscopy. HPMECs were seeded at a density of 20,000 cell/cm² on either polymer coated 8 well chamber slides (Ibidi™) or, on glass bottomed Petri dishes (MatTek™) (controls). Once the HPMECs reached confluence they were detached and transferred to a new polymer coated surface, which had been coated with the same polymer as the original well. HPMECs grown on the controls were detached using trypsin and reseeded onto new uncoated dishes. The media was removed after a 24 hour re-adhesion period and the cells were then rinsed quickly with pre-warmed (37 °C) phosphate buffered saline (PBS), and were fixed with 4 % paraformaldehyde in PBS for 15 mins. After fixation the samples were permeabлизed with 0.1% Triton X-100 in PBS (3 mins) and blocked with 3 % Normal Goat Serum (NGS) in PBS (20 mins). The primary rabbit monoclonal anti-paxillin antibody (Abcam) was then added for an hour at room temperature (1:250 in block). The goat anti-rabbit secondary antibody (Alexa 594 nm at 1:200 in PBS) (Invitrogen) was incubated with the samples for 1 hour at room temperature. Samples were counterstained with phalloidin, for F-actin, (Alexa 488nm 1:200) and Hoechst, for nuclei, (1:1000) (both Invitrogen). Cells were stored in PBS and imaging was carried out less than 24 hours after staining. All samples were imaged using an Olympus IX81 microscope and Volocity™ software. Low magnification overviews were captured at 40x magnification, with detailed views captured using a 100x oil lens. The absence of non-specific binding was confirmed by the use of appropriate primary and secondary antibody controls.
4.3 RESULTS AND DISCUSSION

4.3.1 Physical Characterization

The molecular weights (Mn) of poly (NIPAm-co-AcBzPh) and poly (NIPAm-co-NtBAm-co-AcBzPh) were estimated by gel permeation chromatography and were 310,000 g/mol and 320,000 g/mol with polydispersities of 3.7 and 2.7, respectively.

The LCST of the polymer solutions was defined through UV-Vis turbidity measurements and were 31 °C for poly (NIPAm-co-AcBzPh) and 13 °C for poly (NIPAm-co-NtBAm-co-AcBzPh), respectively.

The RMS roughness values as measured by AFM are given in table 4.1.

Table 4.1: Roughness measurements for the physically adsorbed films measured using AFM in tapping mode. Three samples of each surface type were analyzed in three random areas.

<table>
<thead>
<tr>
<th>Surface Type</th>
<th>RMS Roughness (Mean ± SD) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theranox™</td>
<td>17.0 ± 2.3</td>
</tr>
<tr>
<td>Poly (NIPAm-co-AcBzPh) &lt;LCST</td>
<td>15.6 ± 4.3</td>
</tr>
<tr>
<td>Poly (NIPAm-co-AcBzPh) &gt;LCST</td>
<td>23.6 ± 2.4</td>
</tr>
<tr>
<td>Poly (NIPAm-co-NtBAm-co-AcBzPh) &lt;LCST</td>
<td>11.8 ± 3.5</td>
</tr>
<tr>
<td>Poly (NIPAm-co-NtBAm-co-AcBzPh) &gt;LCST</td>
<td>32.9 ± 7.2</td>
</tr>
</tbody>
</table>
The disparity in roughness of physically adsorbed thermoresponsive films produced above and below a polymer’s LCST is due to the conformation of the polymer in question which is a product of the temperature conditions the polymer is adsorbed at. Below the LCST the polymer is adsorbed in its Flory coil conformation which leads to the formation of a thinner, smoother film. Adsorption can proceed via one of two pathways above the polymers LCST, the pathway chosen depends on the molecular weight of the polymer in question. High molecular weight polymers undergo inter-chain collapse prior to individual polymer chain collapse above the LCST; this gives rise to a mesoglobule. For the purpose of this work we are interested in the pathway for high molecular weight polymers as both of the aforementioned thermoresponsive polymers fall into this category. The size of the mesoglobule formed depends on a number of factors including the kinetics of aggregation and polymer concentration. These mesoglobules are then adsorbed and this gives rise to the rougher thicker films produced when adsorption is carried out above a polymer’s LCST [14, 15]. Low molecular weight polymers undergo intra-chain collapse which takes place prior to aggregation giving rise to smaller polymer molecules which are then adsorbed; yielding smoother, thinner polymer films.

AFM was also used to assess the thickness of the prepared hydrogels and the results are detailed in table 4.2.
Table 4.2: Thickness of the physically adsorbed films measured using AFM in scratch mode. Three samples were scratched in three random areas and fifteen thickness measurements were taken from each scratch.

<table>
<thead>
<tr>
<th>Surface Type</th>
<th>Thickness ± SD (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly (NIPAm-co-AcBzPh) &lt; LCST</td>
<td>13.2 ± 3.3</td>
</tr>
<tr>
<td>Poly (NIPAm-co-AcBzPh) &gt; LCST</td>
<td>22.9 ± 3.8</td>
</tr>
<tr>
<td>Poly (NIPAm-co-AcBzPh-co-NtBAm) &lt; LCST</td>
<td>14.8 ± 1.4</td>
</tr>
<tr>
<td>Poly (NIPAm-co-AcBzPh-co-NtBAm) &gt; LCST</td>
<td>33.1 ± 4.6</td>
</tr>
</tbody>
</table>

The physically adsorbed films produced above the polymer’s LCST are thicker than those produced below the LCST as a greater amount of polymer is absorbed due to the fact that a monolayer of mesoglobules is formed, each containing many polymer chains. The roughness’s of the physically adsorbed films formed above and below the chosen polymer’s LCST are comparable to the thickness of corresponding film.

The advancing contact angle profiles observed for the physically adsorbed thermoresponsive hydrogels indicate stick and slip type behavior, which has been previously observed for other pNIPAm based coatings [4, 7, 16]. The stick angle has been shown to be close to the true equilibrium contact angle and as such the value of the stick angle can be used to estimate the average contact angle of the prepared hydrogels. Stick and slip behaviour results in a saw-tooth like contact angle behaviour (Figure 4.1) and is due to polymer film deformation under the influence of vertical component of surface tension in the advancing drop [10]. The stick angle is the angle formed after the advancing drop has slipped. The slip angle is the angle observed just before the drop slips. The magnitude of the stick and slip behavior is greater in the poly (NIPAm-co-AcBzPh) (Figure 4.1) hydrogels than for the poly (NIPAm-co-NtBAm-co-AcBzPh) (Figure 4.2) hydrogels. This reduction in stick and slip magnitude is

199
common when a large percentage of hydrophobic monomer is introduced to the NIPAm based thermoresponsive polymer [10].

Contact angle measurements were taken above the polymers’ LCST to mirror the wettability environment that the seeded cells are exposed to and to facilitate a direct comparison to other measurements made by this research group on different thermoresponsive polymer systems.

Table 4.3: Advancing water contact angles for physically adsorbed films taken at 40 °C. Three samples of each surface type were measured.

<table>
<thead>
<tr>
<th>Surface Type</th>
<th>Contact Angle (degrees) (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermanox™</td>
<td>58° ± 0.2</td>
</tr>
<tr>
<td>Poly (NIPAm-co-AcBzPh) &lt;LCST</td>
<td>77° ± 2a</td>
</tr>
<tr>
<td>Poly (NIPAm-co-AcBzPh) &gt;LCST</td>
<td>76° ± 3a</td>
</tr>
<tr>
<td>Poly (NIPAm-co-NtBAm-co-AcBzPh) &lt;LCST</td>
<td>79° ± 2a</td>
</tr>
<tr>
<td>Poly (NIPAm-co-NtBAm-co-AcBzPh) &gt;LCST</td>
<td>86° ± 2a</td>
</tr>
</tbody>
</table>

a) Stick angle

The contact angles of the poly (NIPAm-co-NtBAm-co-AcBzPh) hydrogels produced above the LCST are larger than for those produced below. This is most likely due to the significantly higher roughness of the films produced above the LCST. This hypothesis is corroborated by the work of both Cassie et al. [17] and Wenzel [18] who proved that larger contact angles are observed on rougher surfaces than on smoother surfaces [17, 18]. This hypothesis can also be applied to explain the negligible difference observed in the contact angles of the poly (NIPAm-co-AcBzPh) hydrogels, as the roughness reported for the hydrogels produced above and below the LCST is not significantly different. The incorporation of NtBAm is responsible for the larger contact angle observed for the poly (NIPAm-co-NtBAm-co-AcBzPh) hydrogels compared to the poly
(NIPAm-co-AcBzPh) hydrogels. The effect of introducing a hydrophobic monomer on the contact angle has been well documented [10, 19]. The stick angle for the poly (NIPAm-co-NtBAm-co-AcBzPh) produced above the LCST is in good agreement with previously reported stick angles by Nash et al. [4] and Gilcreest et al. [10] for poly (NIPAm-co-NtBAm) films prepared by solvent casting [4, 10]. Nash et al. [7] previously reported the contact angles for poly (NIPAm-co-AcBzPh) hydrogels prepared by the spin coating technique, the values of which were similar to the poly (NIPAm-co-AcBzPh) hydrogels produced above and below the LCST in this study [7].
Figure 4.1: Advancing water contact angle of physically adsorbed poly (NIPAm-co-AcBzPh) < LCST
Figure 4.2: Advancing water contact angle of physically adsorbed poly (NIPAm-co-NtBAm-AcBzPh) < LCST
Table 4.4: Relative atomic composition of physically adsorbed films compared with the stochiometrically calculated composition of the NIPAm-co-AcBzPh monomer and the NIPAm-co-NtBAm-co-AcBzPh monomer

<table>
<thead>
<tr>
<th>Surface Type</th>
<th>% Carbon</th>
<th>% Nitrogen</th>
<th>% Oxygen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncoated TCP</td>
<td>85.4</td>
<td>2.9</td>
<td>11.6</td>
</tr>
<tr>
<td>Poly (NIPAm-co-AcBzPh) monomer</td>
<td>75.0</td>
<td>12.5</td>
<td>12.5</td>
</tr>
<tr>
<td>Poly (NIPAm-co-NtBAm-co-AcBzPh) monomer</td>
<td>76.0</td>
<td>12.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Poly (NIPAm-co-AcBzPh) &lt;LCST</td>
<td>80.0</td>
<td>10.8</td>
<td>9.2</td>
</tr>
<tr>
<td>Poly (NIPAm-co-AcBzPh) &gt;LCST</td>
<td>79.5</td>
<td>10.5</td>
<td>9.8</td>
</tr>
<tr>
<td>Poly (NIPAm-co-NtBAm-co-AcBzPh) &lt;LCST</td>
<td>81.5</td>
<td>8.0</td>
<td>10.5</td>
</tr>
<tr>
<td>Poly (NIPAm-co-NtBAm-co-AcBzPh) &gt;LCST</td>
<td>81.3</td>
<td>7.08</td>
<td>11.1</td>
</tr>
</tbody>
</table>

a) Obtained from molecular formula

There is good agreement between the relative atomic composition of the films produced above and below the LCST and the theoretical stochiometric calculations, seen in table 4.4. The AcBzPh component was omitted from the theoretical calculation due to its minimal contribution. The amide peak in the
N1s at 399 eV and the carbonyl peak in the C1s at 287.5 eV were observed for all of the above mentioned physically adsorbed films, confirming that polymer adsorption was successful.

4.3.2 Biocompatibility Assessment

For the biocompatibility assessment and subsequent cell detachment experiments HPMECs were used as the human model cell line. HPMEC metabolic activity and cell viability were quantitatively assessed by the alamarBlue™ and PicoGreen® assays and qualitatively using bright field microscopy, SEM, TEM and fluorescence microscopies.
Figure 4.3: Percentage metabolic activity of HPMEC grown on the prepared thermoresponsive polymer films compared to HPMEC grown on TCP controls after 4 days of incubation. Percentage metabolic activity was measured using the alamarBlue™ assay kit. The error bars refer to standard deviation where 3 separate samples of each type of substrate were used and assays were performed in triplicate. The asterisk denotes statistical significance.
Figure 4.4: Percentage DNA of HPMEC grown on various thermoresponsive polymer films compared to HPMEC grown on the TCP control after 4 days of incubation. The percentage DNA content of HPMEC was determined using a Quant-iT™ PicoGreen® kit. The error bars refer to the standard deviation where 3 separate samples of each type of substrate were used and assays were performed in triplicate. The asterisk denotes statistical significance.
The results observed in figures 4.3 and 4.4 show that HPMECs grew better on the hydrogels prepared below the polymers’ LCSTs, for both poly (NIPAm-co-NtBAm-co-AcBzPh) and poly (NIPAm-co-AcBzPh) hydrogels. These hydrogels are comparatively smoother, thinner and more hydrophilic than those adsorbed above the polymers LCST. The hydrogels which were adsorbed below the polymers LCST are more conducive to cell adhesion and proliferation due to a combination of factors such as thickness and roughness. The differences in cell metabolic activity and dsDNA content observed for HPMEC grown on the poly (NIPAm-co-AcBzPh) and poly (NIPAm-co-NtBAm-co-AcBzPh) films adsorbed above the LCST, compared to the HPMEC grown on the TCP control, are statistically significant (p value ≤0.05). No statistically significant difference was observed in terms of cell metabolically activity or dsDNA when the HPMEC grown on the poly (NIPAm-co-AcBzPh) and poly (NIPAm-co-NtBAm-co-AcBzPh) films below the LCST were compared to the HPMEC grown on the control. This may indicate that the HPMEC grown on the films adsorbed below the LCST are comparable to those grown on the TCP control.

There are a number of studies which support our finding that endothelial cells display a preference for smoother cell culture surfaces [20, 21]. Endothelial cells grown on smoother surfaces form more focal adhesions and they display improved proliferation and function compared to endothelial cells cultured on rougher surfaces [21, 22]. The effect of surface roughness is cell type dependent and while smoother surfaces are preferential for the HPMEC used in this investigation this will not be the case for all cell types.

The fact that cell adhesion and proliferation decrease as pNIPAm film thickness increases has been well documented [23-25]. This appears to be true for HPMEC cultured on the hydrogels used in this investigation. The poly (NIPAm-co-NtBAm-co-AcBzPh) and poly (NIPAm-co-AcBzPh) films adsorbed above the LCST are thicker than the corresponding films
adsorbed below the LCST and this may be why cell adhesion is better on the films adsorbed below the polymer’s LCST. It is thought that thin thermoresponsive polymer films are subject to the influence of the underlayer substrate and this influence seems to be beneficial for cell adhesion and proliferation. As film thickness increases the influence of the underlying substrate diminishes. It is interesting to note that cell adhesion, on spin coated hydrogels formed from poly (NIPAm-co-AcBzPh) which are covalently bound to the underlayer substrate is also influenced by film thickness [7]. There are some notable exceptions to the influence of film thickness on cell adhesion, including pNIPAm films formed via plasma polymerization along with investigations carried out by Nash et al. [13] and Fan et al. [26] on spin coated and solvent cast films respectively. Whether or not thermoresponsive film thickness influences cell adhesion and proliferation appears to be dependent on the method of film preparation.

Previous work by Nash et al. [7] reported that cell growth on spin coated ultra-thin poly (NIPAm-co-AcBzPh) hydrogels is similar to cell growth on TCP and thermanox™ positive controls and the results reported here support this finding [7]. Numerous studies have demonstrated that cell growth is better on my hydrophobic NIPAm based polymers such as poly (NIPAm-co-NtBAm) and therefore it stands to reason that cell growth would also be enhanced on poly (NIPAm-co-NtBAm-co-AcBzPh) compared to poly (NIPAm-co-AcBzPh) [8, 9, 16]. This was not found to be the case as can be seen from figures 4.3 and 4.4. HPMEC viability was higher on the poly (NIPAm-co-AcBzPh) hydrogels produced below the LCST compared to the HPMEC grown on the poly (NIPAm-co-NtBAm-co-AcBzPh) hydrogels produced below the LCST.

To initiate cell detachment the warm cell culture media was removed from the culture wells and the cells were then rinsed with pre-warmed HBSS (37 °C) to remove any remaining traces of media serum. This in turn was removed and cold HBSS (4 °C) was then added and the dishes were
placed on a chilling/heating dry bath which had been set to 4 °C. The specific cell detachment times for each polymer system can be seen in table 4.5. The comparatively faster detachment times observed for HPMEC grown on the physically adsorbed hydrogels produced above the polymers LCST are most likely due to the fact that the hydrogels produced above the polymers LCST are rougher than those produced below the LCST. This increased roughness would facilitate faster polymer hydration which in turn should facilitate faster cell detachment times. The slower detachment times observed for the HPMEC detaching from the poly (NIPAm-co-NtBAm-co-AcBzPh) hydrogels compared to those detaching from the poly (NIPAm-co-AcBzPh) hydrogels, can be attributed to the fact that the transition from the condensed hydrophobic form of the polymer to the hydrated form of the polymer is slower for poly (NIPAm-co-NtBAm-co-AcBzPh) due to the inclusion of the non-thermoresponsive NtBAm monomer. Upon temperature reduction below the chosen polymers LCST the hydrogels will simply swell rather than dissolving, similar to thermoresponsive polymer coatings produced using grafting techniques such as electron beam polymerization, plasma polymerization, surface initiated atom transfer radical polymerization and reversible addition fragmentation chain transfer. This is advantageous as there will be no dissolved polymer interacting with the detached cell sheets

The HPMEC sheets were imaged using bright field microscopy before, figures 4.5 (a) and 4.6 (a), and during temperature mediated cell detachment, figures 4.5 (b) and 4.6 (b). Additionally, a SEM micrograph of a cell sheet detaching from poly (NIPAm-co-NtBAm-co-AcBzPh) (prepared below the LCST) was captured mid cell sheet detachment, figure 4.7; this image shows that the cell-cell junctions were successfully maintained during temperature controlled cell detachment. HPMEC were detached from the physically adsorbed thermoresponsive polymer films via temperature control and re-seeded on new substrates which had been coated with the same thermoresponsive polymer adsorbed at the same
temperature. The HPMEC sheets were then detached a second time via temperature control and analyzed using trypan blue; the re-seeded cells displayed nearly full viability >90%.

Table 4.5: Detachment times for HPMEC grown on various thermoresponsive polymer films. Cell detachment was initiated by lowering the temperature of the sample below the particular polymer’s LCST. Three samples of each surface type were measured

<table>
<thead>
<tr>
<th>Surface Type</th>
<th>Detachment Time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly (NIPAm-co-AcBzPh) &lt;LCST</td>
<td>50 ± 3</td>
</tr>
<tr>
<td>Poly (NIPAm-co-AcBzPh) &gt;LCST</td>
<td>40 ± 2</td>
</tr>
<tr>
<td>Poly (NIPAm-co-NtBAm-co-AcBzPh) &lt;LCST</td>
<td>210 ± 10</td>
</tr>
<tr>
<td>Poly (NIPAm-co-NtBAm-co-AcBzPh) &gt;LCST</td>
<td>120 ± 8</td>
</tr>
</tbody>
</table>
Figure 4.5: Monolayer of Human Pulmonary Microvascular Cells (HPMEC) growth on a) poly (NIPAm-co-NtBAm-co-AcBzPh) <LCST b) Cell detachment from poly (NIPAm-co-NtBAm-co-AcBzPh) <LCST. Seeding density of 20,000 cell/cm$^2$ and 4 days incubation. Scale bar 200 µm
Figure 4.6: Monolayer of Human Pulmonary Microvascular Cells (HPMEC) growth on a) poly (NIPAm-co-AcBzPh) <LCST b) Cell detachment from poly (NIPAm-co-AcBzPh) <LCST. Seeding density of 20,000 cell/cm² and 4 days incubation. Scale bar 200 µm
Figure 4.7: SEM image of a HPMEC cell sheet detaching from a poly (NIPAm-co-NtBAm-co-AcBzPh) < LCST film after 4 days of incubation. Cell detachment was initiated using temperature control prior to sample preparation for SEM viewing. Scale bar 200 µm.
Figure 4.8: TEM image of a) overview of HPMEC grown on poly (NIPAm-co-NtBAm-co-AcBzPh) < LCST scale bar 2 µm b) overview of HPMEC grown on poly (NIPAm-co-AcBzPh) < LCST scale bar 2 µm c) mitochondria in HPMEC grown on poly (NIPAm-co-NtBAm-co-AcBzPh) < LCST scale bar 500 nm and d) mitochondria in HPMEC grown on poly (NIPAm-co-AcBzPh) < LCST scale bar 500 nm. An incubation period of 4 days was used.
Figure 4.9: The black arrows in the above TEM images are highlighting the respective organelles in HPMEC a) lysosomes in HPMEC grown on poly (NIPAm-co-NtBAm-co-AcBzPh) < LCST, b) lysosomes in HPMEC grown on poly (NIPAm-co-AcBzPh) < LCST, c) Golgi apparatus in HPMEC grown on poly (NIPAm-co-NtBAm-co-AcBzPh) < LCST and d) Golgi apparatus in HPMEC grown on poly (NIPAm-co-AcBzPh) < LCST. The scale bar is 500 nm in all of the above images. An incubation period of 4 days was used.
Figure 4.10: The black arrows in the above TEM images are highlighting the respective organelles: a) caveolae in a HPMEC grown on poly (NIPAm-co-NtBAm-co-AcBzPh) <LCST, b) caveolae in a HPMEC grown on poly (NIPAm-co-AcBzPh) < LCST, c) cell junction in HPMEC grown on poly (NIPAm-co-NtBAm-co-AcBzPh) < LCST and d) cell junction in HPMEC grown on poly (NIPAm-co-AcBzPh) < LCST. The scale bar is 500 nm in all of the images above. An incubation period of 4 days was used.
Cell sheet integrity and cell organelles were examined using (TEM). As can be seen from figures 4.8-4.10, the HPMEC grown on both poly (NIPAm-co-AcBzPh) and poly (NIPAm-co-NtBAm-co-AcBzPh) hydrogels produced below the polymers LCST, possess all the hallmarks of healthy, metabolically active cells. The following organelles; mitochondria, Golgi apparatus, rough endoplasmic reticulum (RER) and ribosomes which are indicative of cellular health were observed in both HPMEC grown on the polymer hydrogels and on the control. A high level of metabolic activity is confirmed by the presence of large quantities of mitochondria. Caveolae which are infoldings of the cell membrane that are involved in signal transduction and transcytosis along with modulating response to the shear stress experienced by endothelial cells due to blood flow can be seen in the HPMEC cultured on both polymer substrates [27-29]. The HPMEC cultured on poly (NIPAm-co-NtBAm-co-AcBzPh) may be more active as there is a significantly larger quantity of caveolae present. The presence of intact junctional complexes shows that cell sheet integrity has been maintained during the detachment process.
Figure 4.11: HPMEC grown on a) poly (NIPAm-co-NtBAm-co-AcBzPh) < LCST X 40 Scale bar 56 µm, b) poly (NIPAm-co-NtBAm-co-AcBzPh) < LCST X100 Scale bar 22 µm, c) poly (NIPAm-co-AcBzPh) < LCST X40 Scale bar 56 µm and d) poly (NIPAm-co-AcBzPh) < LCST X 100 Scale bar 22 µm. Red – paxillin staining, green- F-actin staining and blue- nuclear staining. The initial incubation period was 4 days after which the cells were detached and transferred to a new polymer coated substrate and an incubation period of 1 day was used.
The re-adhesion of cell sheets which had been grown, detached and reseeded on fresh hydrogels was investigated using fluorescence microscopy, **figure 4.11**. The cell sheets were transferred carefully from one well to another post detachment using a 1000 µl micropipette tip. Cells were detached from the control via trypsinization before re-seeding. Focal adhesions of the HPMEC sheet grown on both poly (NIPAm-co-NtBAm-co-AcBzPh) and poly (NIPAm-co-AcBzPh) hydrogels were stained with rabbit anti paxillin antibodies (red staining in **figure 4.11**) to visualize and confirm cell re-adhesion. For this analysis both polymer coatings were produced below the LCST as these surfaces had been shown to facilitate more optimal cell growth. Focal adhesions connect the actin cytoskeleton to the ECM and they also play a role in cell growth and gene expression and for these reasons they were chosen as markers of successful cell re-adhesion here [30-32]. **Figure 4.11 b**) shows the HPMEC which were detached and re-seeded on poly (NIPAm-co-NtBAm-co-AcBzPh) hydrogel produced below the LCST, undergoing cell division (blue nuclear staining); this proves that the detached and re-seeded cell sheets are metabolically active. The focal adhesion staining observed in **figure 4.11** indicates that HPMEC sheets grown and detached from poly (NIPAm-co-NtBAm-co-AcBzPh) < LCST and poly (NIPAm-co-AcBzPh) < LCST are capable for re-adhering to another polymer coated surface. No non-specific binding of either the primary or secondary antibodies was observed, as can be seen from the absence of red staining in **figure 8** in the appendix; this proves that the red staining observed in **figure 4.11** is due to the presence of healthy functional focal adhesions. The density and intensity of the green F-actin staining seen in **figure 4.11** above proves that the detached and re-seeded HPMEC are possess a healthy cytoskeleton.
4.4 CONCLUSIONS

The results and analyses presented herein indicate that a simple adsorption procedure produces ultra-thin crosslinkable thermoresponsive hydrogels based on the well-known pNIPAm polymer which successfully support cell adhesion and temperature controlled cell sheet detachment. The polymer hydrogels, prepared from poly (NIPAm-co-AcBzPh) and poly (NIPAm-co-NtBAm-co-AcBzPh), were adsorbed both above and below the polymers LCST and crosslinked post deposition. The adsorption temperature affects the hydrogels physical characteristics such as roughness, wettability and thickness; which in turn affect the hydrogels cell adhesion potential. Cell proliferation and viability were better on hydrogels produced below the polymers' LCST for both polymer types used. The introduction of a hydrophobic monomer did not appear to have a beneficial effect in terms of cell proliferation and viability as HPMEC grew and proliferated to a similar level on both of the polymer hydrogels produced below the LCST, comparable to TCP positive controls. Lowering the media temperature below the polymers' LCST resulted in complete cell sheet detachment. Cell detachment times ranged from 40 minutes for the poly (NIPAm-co-AcBzPh) hydrogel produced above the LCST to 210 minutes for the poly (NIPAm-co-NtBAm-co-AcBzPh) hydrogel produced below the LCST. Cell sheet integrity was maintained during the detachment process as is evident from the bright field and SEM images above. TEM, trypan blue and fluorescence analysis indicates that HPMEC grown and detached from the crosslinkable thermoresponsive polymer hydrogels produced below the LCST are as healthy as HPMEC grown on the TCP and Thermax™ positive controls. In terms of routine cell culture use, the poly (NIPAm-co-AcBzPh) polymer hydrogels produced below the LCST are the best candidates for the desired application due to the shorter cell sheet detachment times although the comparably depressed LCST of the poly (NIPAm-co-NtBAm-co-AcBzPh) polymer hydrogels presents advantages in terms of routine cellular observation without the risk of pre-mature cell detachment. The crosslinkable physically adsorbed hydrogels offer a viable and operationally simple alternative to other thermoresponsive hydrogels prepared via grafting techniques.


5 DEVELOPMENT AND CHARACTERIZATION OF POLY (NIPAM-CO-ODMA) AND POLY (NIPAM-CO-NTBAM-CO-ODMA) THERMORESPONSIVE FILMS AND DETERMINATION OF THEIR BIO-COMPATIBILITY

5.1 INTRODUCTION

Poly N-isopropylacrylamide (pNIPAm) is a thermoresponsive polymer which undergoes a hydrophilic to hydrophobic transition at 32 °C in aqueous solution. The temperature at which this phase transition takes place is known as the lower critical solution temperature (LCST). The proximity of pNIPAm’s LCST to physiological temperature has led to the use of pNIPAm in cell culture. Cells can be seeded on pNIPAm films above 32 °C, when the polymer is in its hydrophobic or condensed conformation. Once the desired level of cell growth has been attained the temperature is reduced below the LCST of the polymer and the cells detach. Depending on the method of film preparation the film may either dissolve or swell once the temperature has been reduced below the polymers LCST. Temperature controlled cell detachment from thermoresponsive polymer films possesses several advantages over traditional cell detachment methods such as enzymatic digestion and mechanical scrapping [1-4]. These advantages included the fact that intact cell sheets can be harvested as cell-cell junctions remain intact, the synthesized extracellular matrix is detached with the cells and this form of cell detachment is gentler than other methods [5-8].

Grafting methods such as electron beam polymerization, (EBP) plasma polymerization, surface initiated atom transfer radical polymerization (SI-ATRP) and reversible addition fragmentation chain transfer (RAFT) yield thermoresponsive polymer films where the polymer layer is covalently bound to the underlayer substrate [9-13]. These grafted films will swell rather than dissolving at temperatures below the polymer’s LCST. Non-grafted or physical methods of film preparation such as; spin coating, solvent casting, dip coating,
spray coating and physical adsorption produce films which are not bound to the underlayer substrate and consequently the polymer film will dissolve at temperatures below the LCST \([14-16]\). It is possible to produce thermoresponsive polymer coatings which are covalently bound to the underlayer substrate using non-grafting methods by the incorporation of a photocrosslinkable monomer into a pNIPAm polymer \([17]\). This process adds another step to film preparation. A number of studies have shown that pNIPAm is non-cytotoxic in much higher concentrations than those used in cell culture and consequently polymer dissolution upon temperature reduction will not harm the detached cells \([18-20]\). While this is true it would be advantageous to be able to produce thermoresponsive polymer films which swell rather than dissolving using physical deposition/non grafting methods without the need for an additional step such as photo-crosslinking.

In this study the biocompatibility and cell culture potential of a series of novel thermoresponsive polymers was investigated. Three different polymers were utilized in this work; poly (N-(isopropylacrylamide) (pNIPAm) (99.5 \%) -co-octadecyl methacrylate (ODMA) (0.5 \%), poly (NIPAm (99.25 \%) -co-ODMA (0.75 \%) and poly (NIPAm-co-ODMA-co-N-tert butylacrylamide (NtBAm)). The poly (NIPAm-co-ODMA) polymers were synthesized in order to determine whether or not it is possible to produce a thermoresponsive polymer that is capable of swelling rather than dissolving below the LCST. The ODMA monomer should prevent dissolution of the thermoresponsive polymer upon temperature reduction. NtBAm was added to the poly (NIPAm-co-ODMA) as numerous studies have shown that the addition of a more hydrophobic monomer increases cell adhesion and proliferation and this hypothesis will be tested here for this series of polymers. Spin coating was used as the film deposition method as it is a simple film preparation method which is capable of producing nanometre thick uniform polymer films reproducibly.

The polymer films were characterized using the following techniques; atomic force microscopy (AFM) (roughness), profilometry (thickness) and advancing contact angle (surface wettability). 3T3 cells were seeded on the thermoresponsive polymer films and cell viability was assessed quantitatively
using cell viability assays and qualitatively using light microscopy. FT-IR was used to confirm successful films formation.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Dulbecco’s modified eagle medium (DMEM), fetal bovine serum (FBS), trypsin, trypan blue stain, penicillin and streptomycin, phosphate buffered saline (PBS), hanks balanced salts solution (HBSS), human fibronectin, collagen and poly-L-lysine were purchased from Sigma Aldrich and used as received. The Quant-iT™ PicoGreen® assay from Biosciences, alamarBlue™ assay from Invitrogen, Thermanox™ disks from Nunc™ were all used as received. All other plastic consumables were purchased from Sarstedt. An Ectotherm chilling and heating dry bath from Torrey Pines Scientific was used for careful temperature control.

5.2.2 Polymerization

The synthesis of copolymer of octadecyl methacrylate (ODMA) and N-isopropylacrylamide (NIPAm) was carried out according to the procedure outlined below. The ODMA from Merck was used as received. NIPAm from Aldrich was recrystallized three times from n-hexane. 2, 2′-Azobis (2-methylpropionitrile) (AIBN) was used as the initiator at a concentration of 1 mol % of the monomers. 1, 4-dioxane was used as the solvent for the polymerization solution. The concentration of monomers was 20 % (w/v). Oxygen was removed by passing argon for 40 minutes. The reaction was started by placing the reaction vessel fitted with condenser in an oil bath at 63 ºC. The polymerization was carried out for 22 hours under argon atmosphere. The resulting polymer was purified by a precipitation from acetone to n-hexane and the dried in a vacuum oven at 60 ºC.
The synthesis of poly (NIPAm-co-NtBAm-co-ODMA) (0.65 molar ratio of NIPAm, 0.3425 molar ratio of NtBAm and 0.0075 molar ratio of ODMA) was carried out according to the procedure identical to the synthesis of poly (NIPAm-co-ODMA) where a certain amount of NIPAm was replaced by NtBAm to obtain the desired composition.

5.2.3 Spin Coating

Spin coated poly (NIPAm-co-ODMA) and poly (NIPAm-co-NtBAm-co-ODMA) films were fabricated by initially depositing a 150 μl aliquot of an ethanolic polymer solution onto a slowly spinning substrate, (150 RPM) on a Laurell Technologies WS-400B-6NPP/LITE spin coater. The following parameters were used; concentration 2 % w/v pNIPAm in ethanol, final spin speed 6000 RPM, total spin time 30 s. Films were sterilized by exposure to UV light for two hours prior to cell culture in order to sterilize the polymer films. Thermanox™ disks were used as the underlayer substrate for AFM and contact angle analysis, aluminium coupons were used for FT-IR, quartz glass was used for profilometry and tissue culture polystyrene was used for cell culture [14].

5.2.4 Atomic Force Microscopy (AFM)

AFM was used to assess the roughness of the thermoresponsive polymer films. 10 μm × 10 μm scans with a scan rate of 1 Hz were carried out and images were taken with a Dimension 3100 AFM (Digital Instruments, Santa Barbara, CA, USA) Veeco using a 1-10 Ohm-cm phosphorus (n) doped Si tip. Matrixes of 512 × 512 points along the x-y plane were analysed in a single scan. Film roughness was recorded as the route mean square (RMS) roughness, where RMS denotes the standard deviation of the Z-value along the reference line.
5.2.5 Advancing Contact Angle

Advancing contact angle measurements were carried out using a home built goniometer. The goniometer was assembled on an optical rail from Newport Optics with opto-mechanical components from Newport Optics and Edmund Optics. Samples were placed on a tilt stage in a temperature controlled chamber and the temperature was monitored using a thermocouple. A drop was allowed to form on the surface of the sample after which a thin stainless steel needle was inserted in the centre of the drop. Liquid was then pumped into the drop via a syringe pump in order to increase the volume of the drop. Drop images were taken every three seconds. DROPimage software developed by F. K. Hansen and marketed by Rame Hart was used to calculate the contact angles [21].

5.2.6 FT-IR

FT-IR analysis was carried out on a Shimadzu FT-IR 830 Fourier transform infrared spectrophotometer equipped with a golden gate diamond ATR accessory. Polymer samples were prepared on aluminium coupons to avoid the noise associated with a non-metallic substrate. Prior to sample analysis a baseline scan was taken to account for any environmental interference.

5.2.7 Profilometry

Films were prepared according to the spin coating procedure outlined in section 5.2.3 on high quality quartz glass. A Zygo Newview 100 surface profiler was used to determine the thickness of the deposited films. The films were scratched with a 1000 µl pipette tip prior to being placed on the profilometer stage. Scans of three randomly selected areas along each scratch were recorded on three different samples in order to insure statistical accuracy. The objective used for the measurements was a 20X Mirau, with zoom set at 0.5X. The profile outlines the step height and is obtained from the interference patterns produced as the objective scans through the vertical range. The variation of the interference patterns is recorded by a CCD camera, the data is then analysed by fast Fourier
transform and the height distance is measured from the generated surface profile.

5.2.8 Cell Culture

3T3 were cultured in DMEM supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin and streptomycin antibiotics. For experimentation 3T3 were seeded in triplicate on the poly (NIPAm-co-ODMA) and poly (NIPAm-co-NtBAm-co-ODMA) polymer films at a density of 40,000 cell/cm² and incubated for 48 hours. A chilling heating dry bath was used to maintain a working temperature above the polymers LCST in all cases. Care was taken when changing the medium and handling the samples to ensure that the temperature did not fall below the LCST and initiate premature cell detachment.

5.2.9 Cell adhesion promoter coating

The thermoresponsive polymer films were kept on a chilling heating dry bath set to 37 °C during the protein deposition step. The thermoresponsive polymer films were coated with collagen, poly-L-lysine or fibronectin. The coating protocols are as follows. The procedure described here is suitable for 6 well plates or 35 mm Petri dishes.

- Rat tail collagen (type 1) (3 mg/ml in 0.1M acetic acid) was diluted to 200 μg/ml in PBS and 1 ml of the solution was then used to coat the thermoresponsive polymer films and the films were then dried in the laminar flow hood. The coated dishes were stored in an incubator overnight at 37 °C. Hanks balanced salt solution containing phenol red was heated to 37 °C and then used to rinse the dishes until the surface was neutral.
- Poly-L-lysine (500 μl) was added to each dish. The solution was spread evenly across the surface and allowed to dry in the laminar flow hood for three hours at 37 °C. The dishes were then rinsed twice with pre-warmed HBSS (37 °C).
A 6 μl/ml dilution of fibronectin in HBSS was prepared and 1ml of the solution was used to coat the dishes. The dishes were placed in an incubator overnight at 37 °C and then rinsed twice with pre-warmed HBSS (37 °C) [22].

5.2.10 Cell Assays

The alamarBlue™ assay was used to assess cell metabolic activity of the 3T3 grown on both the thermoresponsive polymer films and the tissue culture polystyrene (TCP) controls. Total DNA content of the 3T3 grown on both the thermoresponsive polymer films and the controls was determined using the Quant-iT™ PicoGreen® dsDNA assay kit. Both assays were performed as per the manufacturer’s instructions.

5.3 RESULTS AND DISCUSSION

5.3.1 Physical Characterization

Thermoresponsive ODMA polymer films were formed via spin coating using the procedure outlined in the materials and methods section above. Atomic force microscopy was used to measure the roughness of the ODMA polymer films and the comparative root mean square (RMS) roughness of the films is given in table 5.1; where RMS denotes the standard deviation of the Z-values along the reference line. The spin coated ODMA polymer films possess roughness less than 10 nm and as such they are relatively smooth. It is unlikely that film roughness will affect cell adhesion due to the films relative smoothness. The ODMA polymer films possess roughness values in the nanoscale roughness range (roughness values less than 100 nm). Nanoscale roughness is thought to increase cell adhesion, proliferation, and viability. It has been proposed that nanoscale roughness emulates the nano-architecture of the extracellular matrix (ECM) and consequently promotes the adsorption of cell adhesion promoting proteins in a conformation suitable for cell adhesion [23, 24]. Film thickness was determined by optical profilometry and the ODMA polymer films possess
thickness in the range of 115-125 nm (Table 5.2). FT-IR confirmed successful film formation with the characteristic C=O amide stretching frequencies being observed at 1650 cm$^{-1}$ and the N-H stretching frequency at 1550 cm$^{-1}$ [25, 26].

Table 5.1: Roughness measurements for the thermoresponsive polymer films measured using atomic force microscopy (AFM). Three samples of each surface type were analyzed in three random areas.

<table>
<thead>
<tr>
<th>Surface Type</th>
<th>RMS Roughness ± SD (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermanox™</td>
<td>17.0 ± 2.3</td>
</tr>
<tr>
<td>Poly (NIPAm-co-ODMA (0.5 %))</td>
<td>4.1 ± 2.1</td>
</tr>
<tr>
<td>Poly (NIPAm-co-ODMA (0.75 %))</td>
<td>10.1 ± 1.8</td>
</tr>
<tr>
<td>Poly (NIPAm-co-ODMA-co-NtBAm)</td>
<td>5.8 ± 1.4</td>
</tr>
</tbody>
</table>

Table 5.2: Thickness measurements for the thermoresponsive polymer films measured using profilometry. Three samples were scratched in three random areas and three thickness measurements were taken from each scratch.

<table>
<thead>
<tr>
<th>Surface Type</th>
<th>Mean Thickness ± SD (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly (NIPAm-co-ODMA (0.5 %))</td>
<td>119 ± 4.5</td>
</tr>
<tr>
<td>Poly (NIPAm-co-ODMA (0.75 %))</td>
<td>125.5 ± 2.6</td>
</tr>
<tr>
<td>Poly (NIPAm-co-ODMA-co-NtBAm)</td>
<td>115.3 ± 0.6</td>
</tr>
</tbody>
</table>
Advancing contact angles for the ODMA polymer films were measured at 40 °C on a home built goniometer, the results can be seen in table 5.3 below. The measured contact angles for poly (NIPAm-co-ODMA (0.5 %)) and poly (NIPAm-co-ODMA (0.75 %)) are in agreement with previously reported contact angles for pNIPAm films of similar thickness [17]. The introduction of a hydrophobic monomer will increase the contact angle of a polymer and this was observed for the poly (NIPAm-co-NtBAm-co-ODMA) polymer films [21]. The measured contact angle for the poly (NIPAm-co-NtBAm-co-ODMA) films is in agreement with previously reported contact angles for poly (NIPAm-co-NtBAm) spin coated films [15].

Table 5.3: Measured advancing contact angle data for the thermoresponsive polymer films. Contact angles were reordered at 40 °C. Three samples of each surface type were measured.

<table>
<thead>
<tr>
<th>Surface Type</th>
<th>Contact Angle (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermanox™</td>
<td>58.0° ± 0.2</td>
</tr>
<tr>
<td>Poly (NIPAm-co-ODMA (0.5 %))</td>
<td>72.4° ± 2</td>
</tr>
<tr>
<td>Poly (NIPAm-co-ODMA (0.75 %))</td>
<td>79.4° ± 3</td>
</tr>
<tr>
<td>Poly (NIPAm-co-ODMA-co-NtBAm)</td>
<td>83.8° ± 1</td>
</tr>
</tbody>
</table>

* a: stick angle

Stick and slip behaviour was observed for all of the ODMA polymer films in this investigation. This behaviour is attributed to film deformation by the vertical component of liquid surface tension leading to the pinning of the moving contact line. Water is fed to the droplet on the sample surface and the contact angle increases until it “slips”. The “stick” angle is the contact angle formed after the drop has slipped and the “slip” angle is the angle prior to the drop slipping. It was shown by Gilcreest et al. [21] that the stick angle should be close to the “true” equilibrium contact angle [21]. Therefore we can compare stick angles to contact angles obtained on the systems where stick and slip behaviour is not
observed. As can be seen from figures 5.1, 5.2 and 5.3 below the magnitude of stick and slip is less for the poly (NIPAm-co-NtBAm-co-ODMA) films than the other ODMA polymer films. This is due to the introduction of the hydrophobic monomer [21].

Figure 5.1: Advancing water contact angle of poly (NIPAm-co-ODMA (0.5 %))
Figure 5.2: Advancing water contact angle poly (NIPAm-co-ODMA (0.75 %))
Figure 5.3: Advancing water contact angle poly (NIPAm-co-NtBAm-co-ODMA)
This investigation sought to determine the cell culture potential of spin coated ODMA polymer films. Mice fibroblasts (3T3) were seeded on the ODMA polymer films and TCP controls. Cell growth was assessed qualitatively via bright-field microscopy and quantitatively via cellular assays such as alamarBlue™ and PicoGreen® after 48 hours of incubation. The assay results summarized in figures 5.4 and 5.5 show that the ODMA polymer films are unsuitable for cell adhesion without the addition of a cell adhesion promoter (CAP) layer. The poly (NIPAm-co-ODMA (0.5 %)) polymer proved to be water soluble and as a result it was not utilized in cell studies as the main advantage of this polymer is its ability to swell upon temperature reduction below the polymers LCST without dissolving.
Figure 5.4: Percentage metabolic activity of 3T3 grown on various thermoresponsive polymer films compared to 3T3 grown on the TCP (tissue culture polystyrene) control after 48 hours of incubation. The error bars refer to standard deviation where 3 separate samples of each type of substrate were used and assays were performed in triplicate. The astrix denotes statistical significance.
Figure 5.5: Percentage DNA of 3T3 grown on various thermoresponsive polymer films compared to 3T3 grown on the TCP control after 48 hours of incubation. The percentage DNA content of the 3T3 was determined using a Quant-iT™ PicoGreen® kit. The error bars refer to the standard deviation where 3 separate samples of each type of substrate were used and assays were performed in triplicate. The astrix denotes statistical significance.
Collagen, fibronectin and poly-L-lysine were investigated as possible CAPs and a detailed description of the coating deposition methods can be found in the materials and methods section. It was found that collagen coated poly (NIPAm-co-ODMA (0.75 %)) films were capable of facilitating both cell adhesion and detachment upon temperature reduction. As can been from figures 5.4 and 5.5 above the 3T3 grown on the collagen coated poly (NIPAm-co-ODMA (0.75 %)) polymer films are comparable to 3T3 grown on the TCP control. Cell detachment was initiated by removing the warm media and then rinsing the cells with pre-warmed Hanks balanced salt solution (HBSS) (37 °C). Cold HBSS (4 °C) was then added to the cells and the dishes were placed on a chilling heating dry bath set to 4 °C. Cells detached from the collagen coated poly (NIPAm-co-ODMA (0.75 %)) films with twenty minutes of cold HBSS introduction (figure 5.6).

The NtBAm monomer was added to the poly (NIPAm-co-ODMA) polymer in order to try and improve cell adhesion as previous studies have shown that the introduction of a hydrophobic monomer can improve cell adhesion [15, 16, 27]. While the addition of NtBAm did improve cell adhesion compared to the uncoated poly (NIPAm-co-ODMA (0.75 %)) films, cell growth was still not comparable to the TCP control. The introduction of a CAP coating to the poly (NIPAm-co-NtBAm-co-ODMA) films improved cell adhesion and proliferation as can be seen in figures 5.4 and 5.5. The addition of the CAP coating prevented successful cell detachment from the poly (NIPAm-co-NtBAm-co-ODMA) polymer films upon temperature reduction.

The differences in cell metabolic activity and dsDNA content observed for 3T3 grown on the uncoated poly (NIPAm-co-ODMA), uncoated poly (NIPAm-co-NtBAm-co-ODMA) and poly-L-lysine coated poly (NIPAm-co-NtBAm-co-ODMA) films, compared to the 3T3 grown on the TCP control are statistically significant (p value ≤0.05). No statistically significant difference was observed in terms of cell metabolically activity or dsDNA when the 3T3 grown on the collagen poly (NIPAm-co-ODMA), collagen coated poly (NIPAm-co-NtBAm-co-ODMA) and fibronectin coated poly (NIPAm-co-NtBAm-co-ODMA) films below the LCST were compared to the 3T3 grown on the control. This shows that the 3T3 grown on the films collagen coated poly (NIPAm-co-ODMA) and poly (NIPAm-co-
NtBAm-co-ODMA) and fibronectin coated poly (NIPAm-co-NtBAm-co-ODMA) films are comparable to those grown on the TCP control.

The ability of a biomaterial to adsorb cell adhesion promoting proteins in a suitable conformation determines whether or not the material in question is cell adhesive. Studies have shown that hydrophobic polymer surfaces adsorb greater quantities of protein than hydrophilic surfaces as the substrate surface must undergo at least partial dehydration before protein adsorption can take place [28]. The dehydration of hydrophobic surfaces is thermodynamically favourable and this causes larger quantities of protein to be adsorbed which should result in improved cell adhesion compared to hydrophilic surfaces [29, 30]. This may be why cell adhesion is slightly better on the uncoated poly (NIPAm-co-NtBAm-co-ODMA) films compared to the uncoated poly (NIPAm-co-ODMA) films. Surface chemistry is another important property which influences protein adsorption and cell adhesion. Numerous investigations have shown that while larger quantities of protein are adsorbed on CH₃ terminated surfaces, cell adhesion and proliferation are better on surfaces which possess oxygen containing groups; this is because the conformation the protein is adsorbed in matters more than the quantity of protein adsorbed [31, 32]. It is thought that proteins which have adsorbed on a CH₃ containing surfaces may have undergone deformation. Proteins undergo less conformational change upon adsorption on surfaces with oxygen containing groups and this is why surfaces with oxygen containing groups may be better in terms of cell adhesion despite the fact that they adsorb less protein [33]. ODMA possess a long hydrocarbon chain and it is possible that during the film deposition procedure these hydrocarbon chains become orientated on the films extremity and this would lead to the adsorption of cell adhesion promoting proteins in a conformation which is unsuitable for cell adhesion. The deposition of a cell adhesion promoter layer such as collagen has been shown to improve cell adhesion on thermoresponsive polymer films and this is the case for the ODMA polymer films [34]. The lack of cell detachment form the poly (NIPAm-co-NtBAm-co-ODMA) polymer films may be due to the strength of the attractive forces between the adhered cells and the cell adhesion promoting layer and the polymer underlayer. Successful cell adhesion and detachment form thermoresponsive polymer
surfaces is a balance; if the cells do not adhere strongly enough, the sample with be cell repulsive but if the cells adhere too strongly, detachment will not take place.

Thickness is another factor which may be influencing the cell adhesion capability of the ODMA polymer films. It has been well documented that there is a thickness threshold for pNIPAm based films above which cells will not adhere and proliferate on polymer film [35-37]. pNIPAm films thicker than 30 nm are thought to be incapable of supporting cell adhesion. There are some exceptions to this thickness threshold namely films prepared via plasma polymerization, non-grafted spin coated films and some solvent cast films [14, 15, 38]. While the ODMA polymer films are indeed non-grafted it appears as if they obey this thickness threshold.
Figure 5.6: 3T3 grown on (a) uncoated poly (NIPAm-co-ODMA (0.75 %)), (b), collagen coated poly (NIPAm-co-ODMA (0.75 %)), (c) uncoated poly (NIPAm-co-NtBAm-co-ODMA) and (d) collagen coated poly (NIPAm-co-NtBAm-co-ODMA). Seeding density of 40,000 cell/cm² and an incubation period of 48 hours were used. Scale bar 500 µm.
5.4 CONCLUSION

A novel class of thermoresponsive polymers were synthesized; poly (NIPAm-co-ODMA (0.5 and 0.75 %)) and poly (NIPAm-co-NtBAm-co-ODMA). These polymers can undergo temperature mediated reversible swelling/deswelling in aqueous solution without dissolving. The nano-meter thick thermoresponsive polymer films formed from these polymers via spin coating proved unable to facilitate cell adhesion and proliferation without the addition of a cell adhesion promoter layer. Fibronectin, collagen and poly L lysine were tested as potential cell adhesion promoters and collagen was the best in terms of cell adhesion and detachment. 3T3 cells detached form the collagen coated poly (NIPAm-co-ODMA (0.75 %)) polymer films within 20 minutes of temperature reduction. Cell viability assays confirmed that the cells grown on the collagen coated poly (NIPAm-co-ODMA (0.75 %)) films are comparable to those grown on the TCP controls. Cell adhesion on the uncoated poly (NIPAm-co-NtBAm-co-ODMA) films was better than the uncoated poly (NIPAm-co-ODMA (0.75 %)) film but it was much less than on the TCP control. The addition of a collagen coating improved cell adhesion on the more hydrophobic poly (NIPAm-co-NtBAm-co-ODMA) films but this prevented cell detachment upon temperature reduction. Future work will focus on the modification of poly (NIPAm-co-ODMA (0.75 %)) and poly (NIPAm-co-NtBAm-co-ODMA) in order to produce a polymer which is capable of supporting cell adhesion and detachment without the need for a CAP layer.
5.5 REFERENCES


pNIPAm and its various copolymer derivatives have been investigated as potential cell culture substrates for over two decades. During this time numerous methods of film preparation have been explored. Film preparation methods can be divided into grafted and non-grafted. Grafted films are covalently bound to the underlayer substrate and as a result they will swell rather than dissolving upon temperature reduction. Grafting methods included electron beam polymerization, plasma polymerization, SI-ATRP and RAFT. Non-grafted films are not covalently bound to the underlayer substrate and as such they dissolve upon temperature reduction.

This thesis describes the use of physical adsorption as a method of thermoresponsive film preparation. The main aim in this investigation was to determine whether or not physical adsorption could be used to create thermoresponsive films suitable for cell culture applications. Four different polymers were utilized pNIPAm, poly (NIPAm-co-NtBAm), poly (NIPAm-co-NtBAm-co-AcBzPh) and poly (NIPAm-co-AcBzPh). Films were formed above and below the different polymers LCST’s in order to determine whether or not adsorption temperature influences the physical and chemical properties of the films. It was found that the roughness, thickness and wettability of the resultant polymer films can be controlled by changing the temperature at which polymer adsorption is carried out. The films adsorbed below the polymers LCST were found to be smoother, thinner and more hydrophilic than those adsorbed above the polymers LCST.

The differences observed between the crosslinked and non-crosslinked films are most likely due to combination of factors, in particular molecular weight when considering pNIPAm and poly (NIPAm-co-AcBzPh). The pNIPAm used in this investigation has a much lower molecular weight 20,000-25,000 g/mol compared to 310,000 g/mol for poly (NIPAm-co-AcBzPh). Consequently the adsorption of pNIPAm takes place via the intra-chain collapse pathway for low
molecular weight polymers, while the adsorption of poly (NIPAm-AcBzPh) occurs via inter-chain collapse. The differences in wettability between poly (NIPAm-co-NtBAm) and poly (NIPAm-co-NtBAm-co-AcBzPh) are probably due to the introduction of AcBzPh which is a hydrophobic monomer.

Human pulmonary microvascular endothelial cells (HPMEC) were seeded on the prepared films and the HPMEC grew better on the smoother, thinner, more hydrophilic films produced below the polymers LCST. It was found that cell proliferation and viability are optimal on surfaces produced below the polymer’s LCST with contact angles in the range of 77°-79°, roughness of 4.5-15.6 nm and thickness of 10.8–15.9 nm. Quantitative analysis showed that HPMEC grown on poly (NIPAm-co-NtBAm), poly (NIPAm-co-AcBzPh) and poly (NIPAm-co-NtBAm-co-AcBzPh) films produced below the LCST are comparable to those grown on the TCP controls. Cell detachment was achieved by lowering the temperature below the particular polymer’s LCST. Detachment times ranged from 20 minutes for the pNIPAm homopolymer to 3.5 hours for poly (NIPAm-co-NtBAm-co-AcBzPh). The introduction of the photocrosslinkable monomer AcBzPh increased cell detachment times. This is thought to be due to the fact that the polymer films will swell upon temperature reduction rather than dissolving.

SEM analysis proved that HPMEC sheets grown and detached from the thermoresponsive films adsorbed below the LCST maintained structural integrity during detachment. TEM and fluorescence analysis of HPMEC sheets which had been detached via temperature controlled cell detachment from the thermoresponsive substrates adsorbed below the LCST and then transplanted to new thermoresponsive substrates proved that the cell sheets possessed intact cell-cell junctions, healthy organelles (mitochondria, lysosomes, rough endoplasmic reticulum (RER) and ribosomal structures) and were capable of forming focal adhesions on new thermoresponsive substrates.

The poly (NIPAm-co-AcBzPh) film adsorbed below the LCST is the most attractive in terms of potential cell applications as cells detached from these films within 50 minutes of temperature reduction, also the introduction of the AcBzPh monomer means that the polymer film is covalently bound to the
underlayer substrate and it will swell rather than dissolving upon temperature reduction.

This work proved that the simple film preparation method, physical adsorption is a valuable addition to the arsenal of thermoresponsive film preparation techniques. Future work will focus on using physical adsorption to coat substrates with non-planar geometries. The cell lines grown on physically adsorbed films will also be expanded. It would be interesting to study protein adsorption on the physically adsorbed films adsorbed at different temperature.

A new class of thermoresponsive polymers which swell rather than dissolving upon temperature reduction were investigated as possible thermoresponsive cell culture substrates. Three ODMA copolymers were investigated; poly (NIPAm-co-ODMA (0.5 %)), poly (NIPAm-co-ODMA (0.75 %)) and poly (NIPAm-co-NtBAm-co-ODMA). The films were formed via spin coating and 3T3 cells were seeded on the prepared films. The resultant films could not support sufficient cell adhesion without the addition of a cell adhesion promoter layer (CAP). After the addition of a (CAP) layer the poly (NIPAm-co-ODMA (0.75%)) copolymer proved capable of supporting both cell adhesion and detachment. The 3T3 cells detached from the CAP coated poly (NIPAm-co-ODMA (0.75%)) polymer films within 20 minutes of temperature reduction. Collagen was found to be the most suitable CAP in terms of cell adhesion and detachment. Qualitative and quantitative analysis proved that 3T3 cells grown and detached from the collagen coated poly (NIPAm-co-ODMA (0.75 %)) films are comparable to those grown on the TCP controls. The collagen coated poly (NIPAm-co-NtBAm-co-ODMA) polymer films proved suitable for cell adhesion but the adhered cells failed to detach upon temperature reduction.

Future work will focus on improving the biocompatibility of the ODMA copolymers films. This may be achieved by copolymerizing with a biological component such as collagen. Different quantities of NtBAm monomer will also be investigated as varying the amount of NtBAm may improve cell detachment upon temperature reduction.
Scratch AFM

Figure 1: a) Scratch to determine the thickness of poly (NIPAm-co-NtBAm) > LCST and b) scratch to determine the thickness of pNIPAm > LCST. Mesoglobules can clearly be seen on poly (NIPAm-co-NtBAm) film adsorbed above the polymers LCST (figure a) above). AFM scratch measurements were taken with a Veeco Dimension 3100 AFM using a 1-10 Ohm-cm phosphorus (n) doped Si tip and a matrix of 512 × 512 points along the x-y plane were analyzed in a single scan. Samples were scratched in three random areas and fifteen thickness measurements were taken from each scratch. Thickness measurements were taken parallel to the scratch and the visible pile up on either side of the scratch was not included in the thickness measurement.
Profilometry

Figure 2: Screen shot of the thickness measurement for the poly (NIPAm-co-NtBAm-co-ODMA) film, taken using the Zygo Newview 100 surface profiler. The poly (NIPAm-co-NtBAm-co-ODMA) film was prepared on high quality quartz glass via spin coating and then scratched using a micropipette tip prior to profilometry analysis. Scans of three randomly selected areas along each scratch were recorded on three different samples. Thickness measurements were taken parallel to the scratch as can be seen in the figure above. The pile up which formed on either side of the scratch was not included in the thickness calculation.
Figure 3: Screen shot of the C1s XPS spectra for the physically adsorbed polymer films produced above the LCST. The XPS spectra were obtained using a Kratos ULTRA DLD spectrometer using monochromatic Al Kα radiation \((hν = 1486.58 \text{ eV})\) and a fixed analyzer pass energy of 20 eV; the binding energy scale was referenced against the C 1s line (284.8 eV).
Figure 4: Screen shot of the N1s XPS spectra for the physically adsorbed films produced above the polymers LCST.
Figure 5: Screen shot of the O1s spectra for the physically adsorbed films produced above the polymers LCST.
Figure 6: Gel permeation chromatography analysis of the poly (NIPAm-co-NtBAm) copolymer. GPC was used to determine the molecular weight of the polymers and their polydispersity index. GPC analysis was carried out with PL-gel mixed-D (300*7.8 mm, 5μm nominal particle size) Polymer Labs columns. The mobile phase used was dimethylformamide (DMF) with 30 mM KBr. Separations were performed at 35°C at a flow rate of 1 mL/min using a RI detector. Molecular weights of polymers were referenced to polystyrene standards.
Figure 7: 3D view of physically adsorbed films obtained using AFM. AFM images were taken with a Veeco Dimension 3100 AFM in tapping mode using a 1-10 Ohm-cm phosphorus (n) doped Si tip and a matrix of 512 × 512 points along the x-y plane were analyzed in a single scan. Top left to right: A) poly (NIPAm-co-NtBAm) adsorbed on Thermanox™ at 10 °C, B) poly (NIPAm-co-NtBAm) adsorbed on Thermanox™ at 40 °C. Bottom left to right: C) pNIPAm adsorbed on Thermanox™ at 20 °C and D) pNIPAm adsorbed on Thermanox™ at 40 °C.
Fluorescent Microscopy

Figure 8: HPMEC grown on e) poly (NIPAm-co-AcBzPh) < LCST (primary antibody control) X40 Scale bar 56 µm and f) poly (NIPAm-co-AcBzPh) <LCST (secondary antibody control) X40 Scale bar 56 µm.
List of Conference Attendance

- 65th Irish Universities Chemistry Research Colloquium, presentation entitled; *Ultra-thin physically adsorbed thermoresponsive films for cell culture – An alternative to grafting techniques?*
- 3rd RSC Young Members Symposium, presentation entitled; *Development and Characterization of Physically Adsorbed Thermoresponsive Films for Cell Culture*
- 25th European Biomaterials Conference, poster entitled; *Physically adsorbed thermoresponsive films for cell culture*
- 5th Advanced Functional Polymers for Medicine, poster entitled; *An Investigation into the Cell Culture Potential of Ultra-Thin Thermoresponsive Polymer Films*

List of Publications

- D. Healy, M. E. Nash, A. Gorelov, K. Thompson, P. Dockery, S. Beloshapkin, J. Madden and Y. A. Rochev, Nanometer scale physically adsorbed thermoresponsive films for cell culture. Accepted to the International Journal of Polymeric Materials and Polymeric Biomaterials in May 2016. DOI: 10.1080/00914037.2016.1201765