I, Sandip U Argekar, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Materials Science.

It is entitled:
Influence of biologically relevant thin-film morphology on protein immobilization and cell-adhesion

Student’s name: Sandip U Argekar

This work and its defense approved by:

Committee chair: Dale Schaefer, Ph.D.
Committee member: Nell Ayres, Ph.D.
Committee member: Gregory Beaucage, Ph.D.
Committee member: Jude Irch, Ph.D.
Committee member: Yi-Gang Wang, M.D., Ph.D.
Influence of biologically relevant thin-film morphology on protein immobilization and cell-adhesion

A Dissertation Submitted To

The Division of Research and Advanced Studies

UNIVERSITY OF CINCINNATI

In partial fulfillment of the Requirements for the degree of

DOCTOR OF PHILOSOPHY

In the Materials Science and Engineering Program, School of Energy, Environmental, Biological and Medical Engineering of the College of Engineering and Applied Science

By

Sandip Argekar

Committee Chair: Dr. Dale W. Schaefer
Abstract

Thin-films show enormous potential in altering interfacial behavior. Thin films are ubiquitous in the fields of electronics and optics. More recently, thin-film strategies have also been applied to biological systems in an effort to elicit specific desirable responses. Generally however, systematic studies of thin-film morphology in biological systems are lacking in literature.

An investigation of influence of thin-film morphology in two unique systems, protein immobilization and cell adhesion, is presented in this dissertation.

**Protein immobilization.** Cardiac stents were implanted in an estimated 560,000 Americans in 2007 in an attempt to re-open clogged arteries and prevent heart attacks and strokes. Common shortcomings of stents include inflammation, thrombosis and restenosis (the re-narrowing of the treated blood vessel), which occurs in up to 25% of implanted stents, potentially invalidating the applicability of stent implantations.

Soluble calcium activated nucleotidase (SCAN) is a human protein, that can be engineered via site-directed mutagenesis to exhibit an enhanced ability to hydrolyze adenosine diphosphate (ADP). ADP is a platelet agonist so hydrolysis of ADP limits platelet activation.

In this work, engineered SCAN was immobilized on stainless steel substrates using a 3-aminopropyltriethoxysilane (APTES) interfacial-coupling strategy. In order to optimize SCAN loading and improve hydrolytic stability under *in vivo* conditions, the APTES thin-film morphology was studied using X-ray reflectivity (XRR). APTES films are smooth and uniform irrespective of deposition conditions, although some larger aggregated structures are present on the film. The films grow from a sparse structure (density < 1 g / cm$^3$) when less than two-
molecule-thick to a dense structure (1.26 g/cm$^3$) as the film thickens with increasing deposition time. The density of the deposited films limits accessibility to amine groups embedded in the APTES film. Therefore SCAN immobilization only occurs through the amine groups present on the external surface. This density is calculated to be \(\sim 2\) amine groups/nm$^2$. Finally, after evaluating multiple curing conditions, a high temperature cure (120°C) was found to be essential to assure hydrolytic stability.

The morphology of immobilized SCAN films as determined by XRR showed a dense, single layer (37 Å, 0.94 g/cm$^3$) of the protein assembles at the SS316L substrate. Surface immobilization somewhat reduces the selectivity of SCAN (~44% reduction). However the protein is still effective in hydrolyzing ADP. The ADPase activity was determined on SS316L coupons (over 30 days) and SS316L stents (~250 days) confirming excellent stability of the thin-films after immobilization at the substrates. Finally, the immobilized single layer of SCAN was effective in down-regulating platelet activation through ADPase activity as determined under \textit{ex vivo} conditions. The presented results show that an optimized reaction scheme for SCAN immobilization can be applied to stents and other medical devices to attenuate the negative consequences of these interventions via increased biocompatibility.

**Cell-adhesion.** Standard cell-harvesting protocols utilize proteolytic enzymes to degrade the extracellular matrix (ECM) between cells. Utilization of cells harvested via such protocols can result in atypical cell-cell junctions and cell-damage. Cell-sheet technology is an alternative to proteolytic-enzyme based harvesting.

Poly (\(N\)-isopropylacrylamide) (PNIPAm) is a well-known thermally responsive polymer exhibiting an aqueous lower critical solution temperature (LCST) around 32°C. The convenient
LCST is used as a thermally activated switch facilitating the recovery of entire cell-sheets with intact extracellular junctions. The mechanism of cell release and the effect of PNIPAm thin-film morphology however are not clear. Release of cell sheets from the thin-films can be unpredictable and slow, affecting cell-sheet integrity. Understanding the physico-chemical basis for cell-release is important to optimize the behavior of thermo-responsive thin-films.

Multiple free radical and atom transfer radical polymerization (ATRP) strategies were developed for the synthesis of PNIPAm thin-films. From the synthesis protocols, activators generated by electron transfer atom transfer radical polymerization (AGET ATRP) approach was the most facile, and allowed good control of polymer growth characteristics. The synthesis protocol allows for rapid synthesis of surface-grafted PNIPAm thin films (between 0 – 155 nm), with varying graft densities.

The effectiveness of cell-adhesion and release from PNIPAm thin-films was evaluated. For the first time, the cell-adhesion behavior was directly related to the swelling behavior of PNIPAm using XRR and neutron reflectivity (NR). The density of PNIPAm thin-films as determined from the XRR is $0.98 \pm 0.04$ g / cm$^3$. NR results show a relationship between extent of hydration of PNIPAm and cell-adhesion (film hydration $< 32 \pm 4$ vol % promotes cell-adhesion while hydration $> 39 \pm 2$ vol % prevents cell-adhesion). The effect of varying molecular weight and grafting density on acceleration of cell-release was also established.
Acknowledgements

This dissertation is the product of two multi-disciplinary projects, and would not have been possible without the help, advice and support of so many friends, colleagues and professors. My most sincere gratitude goes to my advisor Dr. Dale W. Schaefer, who provided guidance throughout my PhD while encouraging independent and critical thought. I hope to draw inspiration from Dr. Schaefer’s work ethic and his devotion to scientific research.

My strongest personal supporter and a continuous source of encouragement is Lisa Kain. Through thick and thin, her presence has given me the motivation to move forward despite any adversities.

I have to thank Dr. Yi-Gang Wang, and Dr. Terence L. Kirley. Indeed without their support and most helpful advice my research would have been left wanting. I was a chemical engineer and it was Dr. Dale L. Huber who made me a chemist. I will eternally be thankful for your advice and guidance during my internship at Sandia National Laboratories.

A special thanks goes to Yan Zhang, Naiping Hu and Oliver Rogers. I enjoyed having supportive colleagues such as yourselves.

So many others have helped my research and my personal life is discrete ways - Mike Starr, Aniruddha Palsule, Pravahan Salunkhe, Judith Lavith, Abhishek Telang, Huang Wei, Ramnath Ramachandran, Jason Robinson, Anna Hartsock, Chaitanya Kane, Anshuman Sowani and Swetha Subramanian. I thank all of you.

This dissertation is dedicated to my parents and my brother Mayur for supporting my dream and providing the love and inspiration I sought.
Table of Contents

Abstract ............................................................................................................................................ i

Acknowledgements ......................................................................................................................... v

Table of Contents ........................................................................................................................... vi

List of Figures ................................................................................................................................ xi

1. General Introduction ............................................................................................................... 1
   1.1. Influence of thin-film morphology on protein immobilization ........................................ 1
   1.2. Influence of responsive thin-film morphology on cell adhesion and release ................... 3
   1.3. Statement of objectives .................................................................................................... 6

2. Determination of Structure-Property Relationships for 3-aminopropyltriethoxysilane (APTES) Films................................................................................................................................ 8
   2.1. Introduction ...................................................................................................................... 8
   2.2. Experimental section ...................................................................................................... 11
       2.2.1. Materials ................................................................................................................. 11
       2.2.2. Pretreatment of silicon wafers ................................................................................ 11
       2.2.3. APTES film deposition on silicon .......................................................................... 11
       2.2.4. Curing of deposited APTES film ............................................................................ 12
       2.2.5. Stability of APTES film under neutral aqueous conditions .................................... 12
       2.2.6. X-ray reflectivity measurements ............................................................................. 12
       2.2.7. AFM measurements ................................................................................................ 13
2.2.8. Chemical composition ................................................................. 13

2.3. Results and discussion ................................................................. 14
  2.3.1. Time dependent evolution of the APTES film structure. ........... 14
  2.3.2. Stability of uncured APTES films ............................................ 20
  2.3.3. Effect of curing on the stability of APTES films ....................... 22
  2.3.4. Accessibility of amine functionality in APTES films after curing ... 27

2.4. Conclusions .................................................................................. 30

3. Feasibility of engineered human soluble calcium activated Nucleotidase as an anti-
thrombotic coating for stent applications ............................................. 32
  3.1. Introduction .................................................................................. 32
  3.2. Materials and Methods ................................................................. 35
    3.2.1. Substrate pretreatment and amine functionalization ................. 35
    3.2.2. Maleimide functionalization of the substrate ............................ 36
    3.2.3. Site-directed mutagenesis of SCAN to allow sulfhydryl-based enzyme coupling 36
    3.2.4. Engineered SCAN coupling on maleimide functionalized substrates .......... 38
    3.2.5. X-ray reflectivity measurements ............................................ 38
    3.2.6. Nucleotidase assays .............................................................. 39
    3.2.7. Platelet adhesion assays ....................................................... 40
    3.2.8. Platelet depletion assays ...................................................... 41
    3.2.9. Statistical analysis ............................................................... 41
3.3. Results .................................................................................................................................................. 42

3.3.1. Structure of covalently bound SCAN .................................................................................. 42

3.3.2. Evaluation of SCAN immobilization strategy and nucleotidase activity .................. 45

3.3.3. Platelet adhesion ................................................................................................................ 48

3.4. Discussion ........................................................................................................................................... 51

3.5. Conclusions ....................................................................................................................................... 54

4. Interactions of water with poly (N-isopropylacrylamide) ......................................................... 55

4.1. Introduction ........................................................................................................................................ 55

4.2. Thermodynamics of mixing in polymer-solvent systems .................................................... 56

4.3. LCST behavior in PNIPAm ............................................................................................................ 58

4.3.1. Simulation studies ................................................................................................................ 59

4.4. Effect of molecular weight on the LCST of PNIPAm ............................................................. 62

4.5. Effect of co-solutes on the LCST of PNIPAm ............................................................................ 64

4.6. Effect of co-polymers on the LCST of PNIPAm ........................................................................ 65

4.7. Conclusions ....................................................................................................................................... 66

5. Synthesis and control of PNIPAm thin-film morphology .............................................................. 68

5.1. Introduction ...................................................................................................................................... 68

5.2. Experimental section ..................................................................................................................... 72

5.2.1. Substrate preparation .............................................................................................................. 72

5.2.2. Physical (e-beam) polymerization ......................................................................................... 72
6.2.1. Synthesis of PNIPAm thin-films using AGET ATRP……………………………………99
6.2.2. X-ray reflectivity…………………………………………………………………………………99
6.2.3. Neutron reflectivity………………………………………………………………………………100
6.2.4. Cell cultures ……………………………………………………………………………………..100
6.2.5. Statistical analysis………………………………………………………………………………..101
6.3. Results and Discussion…………………………………………………………………………102
6.3.1. Effect of PNIPAm thin-film morphology on LCST behavior ……………………102
6.3.2. Influence of PNIPAm molecular weight on cell-adhesion and release …………105
6.3.3. Hydration of the PNIPAm film inhibits cell-adhesion ………………………………110
6.3.4. Effect of PNIPAm molecular weight and graft density on cell-adhesion and release 112
6.4. Conclusions…………………………………………………………………………………………118
7. References…………………………………………………………………………………………..120
List of Figures

Figure 1.1 An example of a coronary stent. The mesh-like structure is usually comprised of low carbon stainless steel (SS316L), blown up using a balloon to its final form within the artery. ..... 1

Figure 1.2 SCAN protein structure. SCAN protein in its soluble form exists as a dimer. The circles denote the sites playing a role in dimerization. ADP hydrolysis occurs at the active sites shown in the image.\textsuperscript{9}.............................................................................................................................. 2

Figure 2.1 X-ray reflectivity data (colored solid dots) and corresponding fits (black lines) showing evolution of APTES film on silicon under anhydrous deposition conditions. The curves are shifted from each other by two orders of magnitude for clarity. ................................................. 14

Figure 2.2 SLD profile for a representative 24-h deposited APTES as compared to a bare silicon wafer. The APTES layer is modeled as a single film of material over the native oxide film on the bare wafer...................................................................................................................................... 15

Figure 2.3 Thickness (red dots) and density (blue dots) evolution of APTES layers under anhydrous deposition conditions.......................................................................................................................... 15

Figure 2.4 Evolution of APTES-air interfacial roughness for anhydrous deposition............ 16

Figure 2.5 Schematic structure of APTES films under anhydrous deposition conditions. Some condensation may occur between APTES molecules and the native interface. However, the APTES molecules are held at the substrate by hydrogen bonds. Red circles denote bonding. .... 16

Figure 2.6 Calculated density profiles for island-based deposition mechanism. As islands grow, the layer roughens until coalescence starts, after which the layer tends to smooth out. Such profiles are not observed in our experiments. .................................................................................................................. 18

Figure 2.7 Tapping mode AFM images for 24-h deposited APTES film (10 µm × 10 µm). Aggregated structures are seen studding underlying the smooth APTES film observed by XRR. The AFM roughness is 270 Å. ........................................................................................................................ 20

Figure 2.8 Tapping mode AFM images for a 24-h deposited APTES film, showing a close up section (500 nm × 500 nm) of the substrate without aggregate structures. The calculated roughness is 5.5 Å. ........................................................................................................................ 20

Figure 2.9 Stability of uncured APTES films exposed to neutral phosphate buffer. The reflectivity data (colored dots) and best fits (solid black lines) have been shifted for clarity. The uncured sample was measured immediately after deposition and not subjected to any post deposition curing......................................................... 21

Figure 2.10 SLD profiles for uncured APTES exposed to neutral phosphate buffer. The detailed shape of the curves for times greater than 60 min is less reliable due to the featureless nature of the reflectivity profiles in Figure 8a............................................................................. 21
Figure 2.11 Stability of RT-cured APTES films exposed to neutral phosphate buffer. The reflectivity data (colored dots) and best fits (solid black lines) have been shifted vertically for clarity. The uncured sample was measured immediately after deposition, followed by RT curing for 24 h.

Figure 2.12 SLD profiles for RT-cured APTES exposed to a pH 7 buffer for increasing times. Both uncured and RT cured profiles are identical, indicating no changes in the morphology of the APTES film due to the cure.

Figure 2.13 Stability of HT-cured APTES films exposed to neutral phosphate buffer. The reflectivity data (colored dots) and best fits (solid black lines) have been shifted vertically for clarity. The uncured sample was measured immediately after deposition, followed by HT curing (120 ºC, 1 h).

Figure 2.14 SLD profiles for HT-cured APTES exposed to a pH 7 buffer for increasing times. HT cure results in a reduction in film thickness. The film remains stable over long time durations when exposed to pH 7 buffer.

Figure 2.15 Effect of curing on the normalized total mass of APTES exposed to a pH 7 buffer. The mass increase observed for the HT cure may be due to initial water uptake.

Figure 2.16 Comparison of accessible amine functionality (determined by Coomassie blue dye adsorption) and total surface APTES molecule concentration (determined by XRR) as a function of APTES deposition time in HT cured APTES films.

Figure 3.1 Sites for mutations to introduce cysteine for coupling of SCAN. Amino acids before the start of the soluble SCAN protein sequence are italicized, the thrombin recognition sequence is bolded and underlined, the amino acids encoded by the NheI restriction site are underlined, and the three sites of cysteine substitution mutations for coupling are colored red, green and blue for mutants 1-3, respectively.

Figure 3.2 Schematic showing substrate pretreatment steps prior to covalent deposition of SCAN.

Figure 3.3 XRR data and best-fits showing evolution of film structure during substrate pretreatment and covalent binding of SCAN. The curves have been shifted by two orders of magnitude for clarity.

Figure 3.4 SLD profiles comparing bare Si interface to surface bound SCAN structure. The effect of pretreatment steps – APTES deposition, EMCS modification can be seen in the figure. SCAN forms a dense single layered structure with broadened interface.

Figure 3.5 XRR data for SCAN protein bound to maleimide functionalized surfaces following non-specific HSA adsorption. Deposition conditions are the same for all 3 depositions.

Figure 3.6 SLD profiles for SCAN protein bound to pretreated surfaces following non-specific HSA adsorption. The differences represent the statistical variability due to deposition and fitting.
Figure 3.7 Enzymatic ADPase activity of covalently bound SCAN on SS316L and silicon wafers as compared to bare untreated SS 316L. Immobilized SCAN showed substantial activity over the entire measurement duration.................................................................45

Figure 3.8 Enzymatic ATPase activity of covalently bound SCAN on SS316L as compared to bare untreated SS 316L. Immobilized SCAN showed substantial activity over the entire measurement duration ..................................................................................................................45

Figure 3.9 ADPase activity of surface bound SCAN on a stent surface. The ADPase activity was measurable over 250 days supporting the robustness of SCAN protein and immobilization strategy.........................................................................................................................................46

Figure 3.10 ADPase : ATPase ratio for surface bound SCAN as compared to SCAN activity in solution. Immobilized protein activity was constant over the duration of activity measurement, although its mean (~ 3.7) was lesser than activity of the protein in solution. ........................................47

Figure 3.11 SEM image (350 ×) showing platelet adhesion on bare SS316L coupons after incubation in PRP at 37˚C for 60 minutes. Some spreading and clumping of the adhered platelets is observed (identified by red arrows). .................................................................................................................................48

Figure 3.12 SEM image (350 ×) showing platelet adhesion on SCAN coated coupon after incubation in PRP at 37˚C for 60 minutes. The number of clumps are lower (identified by red arrows), however platelet density and spreading are both reduced as compared to the bare control coupons ........................................................................................................................................48

Figure 3.13 SEM image (350 ×) showing platelet adhesion and extensive thrombus formation on HSA coated coupon after incubation at 37˚C for 60 minutes. Platelet density is significantly higher than observed on bare and SCAN coated coupons ........................................................................................................................................48

Figure 3.14 Percentage area of bare, SCAN coated and HSA coated coupons covered with platelets and clotting after incubation in PRP at 37˚C for 60 minutes (p < 0.05). Error bars represent the standard error of the mean (SEM). ..................................................................................................................49

Figure 3.15 Platelet adhesion on SCAN immobilized SS316L as compared to bare SS316L after 60 minutes of incubation in PRP at 37˚C; p < 0.05 against control ..........................................................49

Figure 3.16 Average platelet counts in PRP solution after incubation at 37˚C for 60 minutes....50

The LCST for non-polar polymer-solvent systems generally occurs between the boiling point and the critical temperature of the solvent. However aqueous solutions of many polar polymers exhibit LCST behavior well below the boiling point.93 Polymer-water phase diagrams can be distinctly different from non-polar polymer solutions. For instance poly (ethylene oxide)-water systems show a ‘closed miscibility gap’ with a lower LCST than its corresponding UCST (Figure 4.2).94 These observations hint at stronger interactions with water in polymer-water binary systems. The role of water and its specific interactions with the polymer must be understood to obtain a physical interpretation of polymer-water systems. .................................................................................................57

Figure 4.1 Closed loop miscibility observed in PEG-water systems ........................................57
Figure 4.3 Single unit of PNIPAm. The structure optimized through molecular simulations using GAUSSIAN software (DFT-B3LYP).

Figure 4.4 LJ and WCA force fields used to describe attractive forces and lack of attraction respectively.

Figure 4.5 Gibbs free energy for polymer unfolding. A positive value indicates the folded state is favorable. The top and bottom figures show the free energy for WCA and LJ force fields respectively. The term ‘x’ in the images indicates chain length.

Figure 4.6 Residence rate $P_{res}(t)$ of water at 300 K near amide linkage and hydrophobic region in PNIPAm.

Figure 4.7 Difference in free energy of hydration contribution to unfolding for varying polymer sizes. The colors denote chain length. Filled and unfilled circles represent results obtained from LJ and WCA force field applied polymers.

Figure 4.8 LCST depression due to increasing molecular weights of PNIPAm. Slight variations observed are due to the end-group effects on the LCST.

Figure 4.9 LCST depression in PNIPAm-water system in the presence of anions.

Figure 4.10 Surface tension dependence on concentration of anions and monovalent cations.

Figure 5.1 IR spectra comparing untreated polystyrene dishes to e-beam polymerized PNIPAm in polystyrene dishes. While a peak was observed at 1650 cm$^{-1}$, indicative of amide I, characteristic amide II and isopropyl doublets were not observable. IR spectra are inconclusive and grafting of PNIPAm to polystyrene substrates cannot be confirmed.

Figure 5.2 Cell-culture on commercially available PNIPAm grafted tissue culture polystyrene dishes. The red arrow points to the initiation of cell-release.

Figure 5.3 Schematic for chain-transfer agent based PNIPAm thin-film synthesis.

Figure 5.4 IR spectra confirming the polymerization of PNIPAm over thiol functionalized substrates. Characteristic amide I and amide II peaks are visible at 1650 cm$^{-1}$ and 1550 cm$^{-1}$ and the isopropyl doublet is seen at 1388 cm$^{-1}$ and 1370 cm$^{-1}$. The spectra were obtained using a Bruker IFS 66v/s FT-IR spectrometer.

Figure 5.5 Red-dye conjugated BSA adsorption observed at 37 °C to chain-transfer immobilized PNIPAm. The absence of the red color at room temperature indicates successful protein release behavior.

Figure 5.6 Red-dye conjugated BSA is released as the temperature shifts from 37 °C to room temperature for chain-transfer immobilized PNIPAm. The absence of the red color at room temperature indicates successful protein release behavior.
Figure 5.7 Cell-adhesion on chain-transfer agent based PNIPAm thin-film. A tear was induced to encourage cell-release (red arrow). However no release behavior was observed. ....................... 81

Figure 5.8 Schematic showing the mechanism of an ATRP reaction. X and Y in this representation are halogens. M is the monomer. ................................................................. 82

Figure 5.9 PNIPAm growth kinetics while varying monomer concentration. ......................... 83

Figure 5.10 PNIPAm film thickness after 10 minutes of polymerization while varying monomer concentration. ........................................................................................................ 83

Figure 5.11 Schematic for synthesis of surface initiated PNIPAm thin-films over hydroxylated substrates ........................................................................................................ 84

Figure 5.12 Reflection absorption IR spectra for PNIPAm grafted on silicon wafer substrate. 85

Figure 5.13 Effect of varying ascorbic acid content, while using PMDETA as the ligand. Poor control of polymerization is observed, with rapid termination in under 10 minutes of polymerization. .............................................................. 86

Figure 5.14 Effect of varying ascorbic acid content while using HMTETA as the ligand. At higher ascorbic acid content, rapid termination is observed. However better control over PNIPAm polymerization was obtained by controlling the ascorbic acid content. The ideal reaction conditions that allowed synthesis of PNIPAm films from 0 – 80 nm (ascorbic acid content 1.6 mM) was chosen to prepare substrates for tissue cultures. ........................................ 86

Figure 5.15 Loss in effective graft density due to surface initiated PNIPAm growth from small nanoparticles. (The black dots represent ATRP initiators. As the polymerization proceeds the spacing between individual ATRP initiators increases and the corresponding grafted density decreases. ................................................................. 88

Figure 5.16 SEM image showing 321 nm silica nanoparticles synthesized using the Ströber process. ........................................................................................................ 88

Figure 5.17 SEM image showing the spherical shape and smooth interfaces of the synthesized silica nanoparticles ........................................................................................................ 88

Figure 5.18 TGA curves for initiator functionalized and PNIPAm functionalized silica. ........... 89

Figure 5.19 Incompatibility in dodecyltriethoxysilane and (N (3-triethoxysilylpropyl hydroxybutyramide)) results in phase separation at the substrate. PNIPAm polymerization from such a substrate resulted in magnification of the inhomogeneity. The image presented here is a delta map taken using an imaging ellipsometer. The variations in delta crudely approximate thickness variations of the PNIPAm film. ......................................................................................... 90

Figure 5.20 Static contact angles for varying fractions of ATRP initiator – dummy initiator after reaction with the underlying silane layer. The linear trend indicates perfect mixing. .................. 91
Figure 5.21 Effect of varying initiator fraction on the thickness of PNIPAm films. A linear and direct relationship is observed. ................................................................. 91

Figure 5.22 XRR profile (dots) and corresponding best-fit (solid line) for a representative pre-cleaned Fisherfinest premium glass slide. .................................................. 93

Figure 5.23 SLD profile for a representative Fisherfinest premium glass slide from fitting shown in Figure 4. The dotted lines signify the layered structure utilized during fitting. ................. 93

Figure 5.24 XRR data (colored dots) and corresponding fits (black lines) comparing PNIPAm films prepared over silicon wafer and Fisherfinest premium glass. The curves have been shifted by two orders of magnitude for clarity. ................................................................. 94

Figure 5.25 SLD profiles comparing PNIPAm film structure over silicon wafer and Fisherfinest premium glass (solid colored lines). The dotted line represents the SLD profile for a pre-cleaned glass slide shown in Figure 5. ................................................................. 94

Figure 6.1 (top) Neutron reflectivity raw data (colored dots) and best fits (solid lines) for intermediate graft density PNIPAm thin-films swollen under D$_2$O at varying temperatures..... 103

Figure 6.2 (top) Neutron reflectivity raw data (colored dots) and best fits (solid lines) for low graft density PNIPAm thin-films swollen under D$_2$O at varying temperatures............... 103

Figure 6.3 XRR data (colored dots) and corresponding best-fits (solid black line) for PNIPAm thin-films synthesized on fisherfinest glass slides. The profiles have been shifted by two orders of magnitude for clarity. .................................................. 106

Figure 6.4 SLD profiles corresponding to the best-fits for PNIPAm thin-films. ............... 106

Figure 6.5 hSBMC adhesion over PNIPAm grafted substrates after 1 day of incubation at 37 °C. ............................................................................................................. 107

Figure 6.6 Cell-adhesion to and release from thin PNIPAm films (1GD0.5MW and 1GD1MW) after 1 week incubation at 37 °C and 25 minute incubation at 20 °C. The scale bar represents 500 µm. ............................................................................................................. 109

Figure 6.7 NR profiles (colored solid dots) and corresponding best-fits (solid lines) for representative PNIPAm films exposed to PBS-D$_2$O at 37 °C.................................................. 111

Figure 6.8 SLD profiles corresponding to the best-fits for representative PNIPAm films exposed to PBS-D$_2$O at 37 °C. The thicker PNIPAm film shows significantly greater D$_2$O penetration and may explain the lack of cell-adhesion previously observed. .................................................. 111

Figure 6.9 Rat endothelial cells adhered to a PNIPAm grafted substrate following 5 minutes incubation at 20 °C................................................................................................. 113

Figure 6.10 ImageJ processed Figure 6.9, showing clearly defined cell spreading and boundaries. These modified images were used for quantification of cell-coverage areas. .................. 113
Figure 6.11 Cell-release kinetics (represented by the loss of areal coverage of cells) from PNIPAm thin-films maintained at a constant graft density while varying molecular weights. The dotted lines are meant to highlight the trends in cell-release behavior. ......................................................... 113

Figure 6.12 Cell-release kinetics (represented by the loss of areal coverage of cells) from PNIPAm thin-films maintained at a constant, low graft density (0.2GD) while varying molecular weights. The dotted lines are meant to guide the reader to follow the cell-release behavior. .... 115

Figure 6.13 Cell-release kinetics (represented by loss in areal coverage of cells) from PNIPAm thin-films maintained at a constant molecular weight while systematically varying graft density. The dotted solid black line indicates a time point wherein the differences in cell-release kinetics are clearly visible. ............................................................................................................................... 116
1. General Introduction

Interfaces between synthetic and biological materials, also called “bio-interfaces” are one of the factors that induce biological responses. Commonly observed biological phenomena, such as membrane fouling, tissue-growth, cell-differentiation, protein adsorption, biological rejection all depend on physical and chemical properties of the interfaces.

Thin films (typically 0 – 100 nm) serve as interfacial modifiers. An interfacial film has the advantage of retaining the mechanical properties of the substrate while providing properties at the interface suitable to the biological system. By varying the physicochemical properties of thin-films, interfacial behavior can be modified to induce the necessary biological responses. This dissertation presents an investigation of influence of thin films morphology on protein immobilization and cell-adhesion in two unique systems.

1.1. Influence of thin-film morphology on protein immobilization

A stent can be described as an expandable meshwork of stainless steel / metal alloy, deployed by a balloon catheter. Stents are used during percutaneous coronary interventions (also known as angioplasty), as a long-term strategy to permanently open clogged arteries, thereby preventing myocardial infarctions.

Considering the prevalent usage of coronary stents, the high incidence rate of unwanted complications is somewhat surprising. For instance,
restenosis (the re-narrowing of the treated artery), can occur in up to 10% of stent applications. Immunological responses such as thrombosis (clotting) and inflammation also occur at the site of stent placement, further increasing complications associated with coronary stents.

Drug eluting stents (DES), are stents coated with anti-platelet drugs. The drugs are slowly released into the blood stream resulting in reduced incidences of restenosis. However, DES are only effective in preventing short-term restenosis, and may actually promote late-stage thrombosis events. A coating strategy that can provide both short-term and long-term, complication-free stent deployment is currently not available.

A recent approach to address complications in stents involves preventing thrombosis by hydrolysis of platelet activators. Platelet activators such as adenosine diphosphate (ADP) are released by platelets upon activation at the stent surface, resulting in a cascading effect that promotes further platelet activation, eventually leading to restenosis and thrombosis. Soluble calcium activated nucleotidase (SCAN, a human nucleotidase modified via site directed mutagenesis, Figure 1.2) is expected to prevent the cascading effect by hydrolyzing ADP, thereby limiting platelet activation. Similar strategies using other hydrolyzing enzymes were found to reduce inflammation and thrombosis. SCAN is highly stable, and its surface structure can be modified without loss of activity, making it an ideal replacement for typical bare-metal stent based strategies.

Figure 1.2 SCAN protein structure. SCAN protein in its soluble form exists as a dimer. The circles denote the sites playing a role in dimerization. ADP hydrolysis occurs at the active sites shown in the image.
In our work, engineered SCAN protein, with optimized ADPase activity was covalently bound to SS316L substrates and silicon wafers using a novel immobilization scheme designed to maintain enzymatic activity. The immobilization strategy utilizes 3-aminopropyltriethoxysilane (APTES) in conjunction with specifically engineered SCAN, in order to covalently attach the protein with minimal loss of activity at the stainless steel substrate. Chapter 2 describes the evolution and stability of APTES films studied using X-ray reflectivity, complimented by atomic force microscopy (AFM) and chemical analysis.

Chapter 3 describes the SCAN immobilization strategy and its effectiveness as an anti-platelet strategy. Utilizing X-ray reflectivity, we find that a single, dense, smooth layer of SCAN assembles at the interface. The immobilized protein maintained enzymatic activity for almost a year, making SCAN a viable candidate for therapeutic use as an anti-thrombotic stent coating. Furthermore, we find through in vitro experiments that immobilized SCAN successfully reduces platelet activation and spreading on stainless steel substrates.

This work demonstrates the viability of SCAN as an anti-thrombotic coating, showing enzymatic activity over time scales necessary for re-endothelialization, while potentially overcoming later-stage thrombosis events observed using drug-eluting stents.

1.2. Influence of responsive thin-film morphology on cell adhesion and release

The most widely adopted technique for tissue engineering is the use of tissue scaffolds. Cells are deposited on a porous biodegradable polymer; and under appropriate conditions, the cells proliferate taking the shape of the scaffold. However, low cell density and atypical extracellular matrix (ECM) deposition can limit scaffolding strategies. Cell-necrosis due to slow
diffusion of nutrients and inefficient removal of metabolic wastes can also occur, which affects the tissue structure. The scaffolding approach is not suitable for tissue engineering applications where dense cellular coverage and intact cell-cell junctions are necessary, for instance skin-grafts.

Typically the cells used in scaffolds are harvested from tissue culture polystyrene dishes (TCPS) using proteolytic enzymes like trypsin to degrade cell adhesion molecules (CAM’s) and ECM networks. Treatment with these enzymes however also affects growth factor receptors and breaks down cell-to-cell junction proteins that are vital for the differentiated functions of many cell types.\(^{11}\)

A non-invasive cell recovery technology without the use of damaging enzymes can lead to stronger and denser cell-cell junctions. This approach was first explored by Okano et al.\(^ {12}\) Thermally responsive substrates were synthesized using poly (\(N\)-isopropylacrylamide) (PNIPAm) to actuate the release of entire cell-sheets with intact ECM’s and cell-cell junctions.

PNIPAm is a thermally responsive polymer exhibiting lower critical solution temperature (LCST) behavior between 31 and 35 °C in water.\(^ {13-17}\) At temperatures below the LCST, PNIPAm exists in a solvated chain-like configuration (radius of gyration \(R_g\) \(\propto\) number of repeating units\(N^{0.5}\)). Above the LCST, the PNIPAm chain collapses to a globule \(R_g \propto N^{0.33}\).\(^ {14}\) Present-day understanding of this transition phenomena is discussed in chapter 4.

Surface grafted PNIPAm exhibits a hydrophobic – hydrophilic transition coincident with the chain - globule transition. Tissue cultures are typically incubated at 37°C. At this temperature, PNIPAm grafted substrates support cell-adhesion and proliferation. At temperatures below the LCST, following transition to a hydrophilic state, cells are released from the substrate.\(^ {18}\)
A disadvantage of cell-sheet release technology is unpredictability in the release of cell-sheets. Cell-sheet release from PNIPAm-grafted substrates has been reported to take as long as a few hours.\textsuperscript{19, 20} Since release of cells occurs below normal metabolic temperatures, tissue viability and cell-sheet integrity can be compromised. There is a need to accelerate the release of cells from PNIPAm grafts.

Our intent is to identify the factors controlling cell-adhesion and release from model thermo-responsive PNIPAm grafted substrates. For this purpose, multiple chemical and physical polymerization schemes were developed and evaluated for best control of PNIPAm thin-film morphology. Chapter 5 discusses the various strategies used for the synthesis of PNIPAm grafted substrates. Activators generated by electron transfer atom transfer radical polymerization (ATEG ATRP) provided the best control over polymer grafting density and molecular weight.

Utilizing AGET ATRP, PNIPAm thin-films were synthesized over multiple substrates. Chapter 6 presents the effect of PNIPAm film thickness, grafting density and molecular weight on cell-adhesion and release. Using neutron reflectivity (NR), we established a correlation between the extent of hydration of a PNIPAm thin-film and its cell-adhesion behavior. Neutron reflectivity was used to identify the effect of grafting density and molecular weight on the transition behavior of PNIPAm.
1.3. Statement of objectives

The objective of this research was to develop structure-property relationships between thin-film structure and its influence on protein immobilization and cell-adhesion. The objective was achieved through the completion of three tasks:

1. Development of thin-film synthesis approaches for both protein immobilization and cell-adhesion studies.
2. Characterization of the synthesized thin-films using specular reflectivity.
3. Assessment of the influence of thin-film morphology on protein adsorption and cell-adhesion characteristics.

Both protein immobilization and cell-adhesion aspects of this dissertation involved collaborative, multi-disciplinary efforts. The protein immobilization aspect was carried out in collaboration with Yan Zhang (Department of Chemistry, University of Cincinnati), Oliver Rogers and Dr. Terence L. Kirley (Department of Pharmacology and Cell Biophysics, University of Cincinnati).

The cell-adhesion and release aspect of the dissertation was performed in collaboration with Dr. Huang Wei and Dr. Yi-Gang Wang of the Department of Pathology and Laboratory Medicine, University of Cincinnati and Girish Kumar (NIST). Dr. Ron Millard (Pharmacology and Cell Biophysics, University of Cincinnati) played a significant role in identifying the project goals and coordinating the collaborating groups. A roadmap of the experimental strategy to reach the objective is presented below.
Tissue culture

Weight variation

Thickness, graft density and molecular

Reactivity

Neutron

ATRP, AGET ATRP

Polymerization

Controlled radical

PNIPAM thin-film synthesis

Release

Cell adhesion and

Effect of thin-film morphology

Protein immobilization

APTES-synthies

Relationships – APTES structure

Covariant SCAN

Immobiliation

SCAN stability & activity

determination

Effect of immobilized

SCAN thin-film on
2. Determination of Structure-Property Relationships for 3-aminopropyltriethoxysilane (APTES) Films

2.1. Introduction

3-aminopropyltriethoxysilane (APTES) is a widely used silane that allows for facile amine functionalization of hydroxylated inorganic substrates. The amine functionality of APTES has been used to immobilize polymers\textsuperscript{21} and proteins,\textsuperscript{22} as well as to promote cell adhesion.\textsuperscript{23, 24} Owing to its ease of surface modification, APTES was used as the preliminary step for immobilization of SCAN on stainless steel stents.

The deposition mechanism of APTES, is more complicated as compared to standard alkoxysilanes film formation. Typically, alkoxysilane deposition is preceded by hydrolysis of the alkoxy groups. The hydrolyzed silanes condense with hydroxylated substrates, forming siloxane bonds. The basic nature of the amine functionality in APTES however promotes self-condensation, driving multi-layer formation. The self-condensation behavior also affects APTES molecular orientation during film formation.\textsuperscript{25}

As a coupling-agent for SCAN immobilization, understanding APTES thin-film structure – property relationships becomes relevant. For instance, an uneven APTES film may result in non-uniform SCAN immobilization. Similarly, changing the APTES film deposition conditions can alter accessible amine density, thereby affecting SCAN loading. Finally, wear resistance and long – term hydrolytic stability of generated APTES films must be excellent, in order to maintain activity of the immobilized protein in the blood stream.
The effect of deposition and curing conditions on the surface morphology of APTES films have been previously studied. However, the methods used do not probe the internal structure of the films. There is some evidence to suggest that APTES films deposited from toluene and heptane, for example, are over two orders of magnitude harder than those prepared from polar or hydrated solvent systems. This hardening effect has not been related to thin-film structure. Similarly, multi-layered APTES films are shown to be wear resistant, hinting that multi-layered APTES films prepared from non-polar solvents may be structurally unique.

In this chapter, we study the relationship between reported properties of anhydrous APTES thin-films and their internal film structure using specular x-ray reflectivity (XRR), complemented by atomic force microscopy (AFM) and dye-binding analysis of the surface-active amine functionality. This is the first study of evolution and degradation of the internal structure of multilayer APTES films.

XRR provides structural information perpendicular to the substrate, extending from a few Ångström up to a micrometer. Structure is revealed through the scattering length density (SLD) profile perpendicular to the surface. In most cases the X-ray SLD is proportional to the electron density, which in turn depends on mass density and chemical composition. The SLD profile is obtained by inverting the XRR data using numerical techniques. For a typical coating system the SLD profile reveals layer density, layer thickness and interfacial roughness.

XRR analysis shows the formation of homogenous and smooth APTES films. The film density varies markedly as a multi-layer structure evolves with multi-layered films being denser (~1.26 g / cm$^3$), than sub-bilayer counterparts (~1 g / cm$^3$) and films prepared in the presence of water. The observed densification effect is unusual and explains previously reported hardness and wear...
resistance improvements. High temperature curing does not change film density. However it is necessary for hydrolytic stability.

Significantly roughened, aggregated structures have been observed at the APTES-air interface.\textsuperscript{27-29, 31, 34, 35} A roughened interface may be unsuitable for protein deposition. However XRR indicates that these aggregates do not significantly affect the underlying film structure.

Evidence is also presented regarding the nature of the amine functionality in APTES films. A majority of amine groups remain embedded and unreactive in the film. Only amine groups at the interface of the APTES film can contribute to SCAN immobilization. Protein immobilization therefore is independent of APTES film thickness.

To our knowledge this chapter is the first analysis of the relationship between the internal structure of APTES films and its effect on film reactivity, stability, hardness and tribological behavior.
2.2. Experimental section

2.2.1. Materials

N-doped single-side polished silicon (1 0 0) wafers (0.5 mm thick) were obtained from College Wafers, Inc. 3-aminopropyltriethoxysilane (APTES, 99 %) was purchased from Aldrich, purified using vacuum distillation and stored refrigerated in a desiccator until used. Toluene (ACS grade, Pharmaco-AAPER) was dried over calcium hydride (Aldrich), distilled, and stored in a desiccator. Acetone (ACS grade, Pharmaco-AAPER), sulfuric acid and hydrogen peroxide (Fisher) were used as supplied. All glassware was rinsed using deionized water and acetone, and dried for at least 24 hours in a drying oven at 120 °C and cooled in air prior to use.

2.2.2. Pretreatment of silicon wafers

The received silicon wafers were cut into approximately 70 × 15 mm coupons. In order to facilitate the removal of organic contaminants, the coupons were initially rinsed using acetone, ethanol and deionized water before immersion in piranha solution (3:1 (v/v)) sulfuric acid: hydrogen peroxide) for at least 2 hours. The coupons were then rinsed with copious amounts of deionized water, dried under a stream of nitrogen and heated briefly (120 °C, 1 min) to remove residual water. The APTES deposition was carried out immediately after cleaning and drying of the silicon wafers.

2.2.3. APTES film deposition on silicon

A 1% (v/v) solution of APTES in anhydrous toluene was prepared in 40 ml glass vials. Pretreated silicon coupons were immersed in the APTES solution; the vials were flushed with nitrogen and sealed during deposition. After deposition, the APTES-coated coupons were rinsed using toluene and acetone, and dried under a stream of nitrogen.
2.2.4. Curing of deposited APTES film

We explored two curing protocols; an extended room temperature (RT) cure, and a brief high temperature (HT) cure. The RT cure was carried out by placing the treated APTES deposited silicon coupons in nitrogen flushed sealed 40-ml glass vials for 24 hours. For the HT cure, the APTES coated coupons were cured at 120 °C in an oven for one hour.

2.2.5. Stability of APTES film under neutral aqueous conditions

In order to determine the hydrolytic stability of APTES films, the as-is and cured APTES-coated coupons were exposed to neutral phosphate buffer (7.0 ± 0.1) for varying lengths of time. The samples were then removed from the buffer solution, rinsed with deionized water, and dried under a stream of nitrogen. The structure of the films was observed using XRR. Following this step, the samples were again exposed to the phosphate buffer.

2.2.6. X-ray reflectivity measurements

XRR was performed using a PANalytical X’pert Pro Materials Research Diffractometer at the Advanced Materials and Characterization Center (University of Cincinnati). The x-ray source (Cu Kα=1.542 Å) was operated at 1.8 kW (45 mV, 40 mA). A typical alignment and reflectivity scan takes approximately one hour. The normalized reflectivity data were analyzed using PANalytical X’Pert reflectivity software version 1.1. The simplest model (involving the least number of layers) that adequately fits the collected data was accepted. Based on the model obtained, the SLD profile (SLD dependence on perpendicular distance from the interface) was generated using Irena Macros 2.38 for Igor Pro 6.10.36
During fitting, the silicon substrate roughness was allowed to vary between 3 – 7 Å. We set the native oxide layer density at 2.2 g / cm$^3$. To account for substrate variability, the native oxide layer thickness was allowed to vary slightly (13 ± 3 Å).

2.2.7. **AFM measurements**

AFM images were gathered using a Digital Instrument Veeco Metrology Dimension 3100 AFM with a Nanoscope IIIa controller in the tapping mode. The scans were performed on 10 × 10 µm and 0.5 × 0.5 µm areas on 24-h deposited APTES thin-films and roughness analysis was performed using Nanoscope 6.13b28.

2.2.8. **Chemical composition**

The surface density of accessible amine groups was measured using a staining method.$^{37}$ Silicon coupons of known surface area were masked on the unpolished side with electrical tape (3M 1739-7 tape). Masked coupons were stained with 0.5 mg / mL Coomassie Brilliant Blue G-250 (TLC purified) for 5 minutes at room temperature in 10% methanol / 5% acetic acid with gentle shaking. After 5 washes in 0.3 ml of 10 % methanol /5% acetic acid, the bound dye was stripped off using a solution of methanol and sodium carbonate (100 µL of a 1:1 mixture of 100% methanol: 0.25 M Na$_2$CO$_3$) for one minute at room temperature with gentle shaking. After re-acidification of the extracts with 2 µL of 6.85 M HCl (to maximize the blue color and absorbance at 610 nm), the concentration of dye in solution was calculated using visible light spectrophotometry at 610 nm. An extinction coefficient of 83,100 cm$^{-1}$M$^{-1}$ was used to calculate the number of moles of dye, which is equivalent to the number of moles of amino groups, from which the surface density of amino groups was calculated. This aspect of APTES chemical characterization was performed by Dr. Terence L. Kirley at the department of pharmacology at UC.
2.3. Results and discussion

2.3.1. Time dependent evolution of the APTES film structure.

Typically the deposition of APTES films from non-polar solvents is carried out utilizing 0.2 – 2 % (v/v) concentration of APTES.\textsuperscript{22,32,35,38} We chose a concentration of 1% (v/v) to study the evolution of APTES film structure. XRR data and corresponding best-fits for APTES-coated samples at different stages of film deposition are shown in Figure 2.1. A discernible change to the XRR profile occurs in one hour and the film continues to evolve over the entire 24-h deposition period.

A two-layer structure (APTES over the native oxide) adequately fits the reflectivity data (Figure 2.2). The film structure deviates from models used for long-chain silanes, which are typically fit using the expected profile based on the structure of the molecules.\textsuperscript{39} Such detailed molecular structures are not required for short-chain silanes, which are adequately represented by a uniform layer with a broadened interface.
Under the assumption that APTES is uncured, the 24-h film SLD implies a density of $1.26 \pm 0.13 \text{ g/cm}^3$.

Partial curing (formation of Si – O – Si bonds) may occur during anhydrous deposition, especially at the oxide layer interface,\textsuperscript{26} potentially compromising the density measurement. Since cured and uncured APTES have nearly the same SLD (±4%) however, the value obtained from the XRR fits reliably represents APTES film density.

The evolution of APTES layer thickness and density with time is shown in Figure 2.3. After one hour of deposition the layer thickness ($10 \pm 4 \text{ Å}$) is close to that expected for an APTES monolayer. At two hours, a two-molecule-thick layer accumulates. Over the entire deposition period, the APTES layer thickness increases linearly at ~4.2 Å/h. In contrast to the linear thickness growth, the layer density shows two distinct stages. During the initial stage (from 0 – 4 hours) the density increases rapidly but stabilizes after 4 h at 1.26 g/cm$^3$. At 1 h the layer density is $0.72 \pm 0.16 \text{ g/cm}^3$, somewhat less than the neat silane ($0.94 \text{ g/cm}^3$).

The evolution of APTES – air interfacial roughness is shown in Figure 2.4. After one hour of deposition, the single-layer film shows a roughness of ~3 Å, which increases to ~7 Å after 24 hours. Compared to the thickness of the 24-h deposited APTES layer (94 Å) however this
roughness remains small. The increase in roughness is not proportional to the film thickness, indicating the films become relatively smoother with increasing deposition times.

The observed APTES deposition profile can be explained by adsorbed water on the silicon wafer interface. Deposition is strongly affected by adsorbed water\(^\text{27}\) since APTES hydrolyzes and condenses rapidly in the presence of water.\(^\text{40}\) In the initial stages of deposition, surface-adsorbed water contributes to the partial hydrolysis and condensation of APTES at the interface, resulting in covalent binding of APTES to the silicon substrate. Irreversible bonding produces poorly packed molecules with substantial unoccupied volume, accounting for the observed sparse initial coverage. This view is supported by the fact that APTES films generated in the presence of water exhibit lower density.\(^\text{34}\)

After consumption of the surface adsorbed water, hydrogen bonding increasingly influences deposition. Spectroscopic studies indicate that hydrogen bonding\(^\text{27, 30, 38}\) is the key mechanism involved in APTES deposition under anhydrous conditions. Hydrogen bonding can result in unhydrolyzed APTES molecules with intact ethoxy groups adsorbing on the initial covalently bound
APTES layer. A schematic of a partially hydrolyzed APTES molecules deposited at the native oxide interface is shown in Figure 2.5.

The film continues to increase in density as further deposition ensues through hydrogen bonding with further APTES deposition compressing the layers closest to the substrate. The film density stabilizes after 4 hours of deposition to a level 27 % denser than the neat silane (0.942 g / cm$^3$). This densification effect has not been observed for other silanes. Richter et al., for example, report an SLD of $(8.0 \pm 0.3) \times 10^{-6}$ Å$^{-2}$ for octadecyltri-chlorosilane monolayers from heptane, which is slightly less than that of the neat silane, $9.11 \times 10^{-6}$ Å$^{-2}$. Wasserman et al. report similar SLD’s for long-chain silane monolayers.

The densification effect is also not observed for APTES films deposited from aqueous or dilute solutions. For instance, Heiney et al. deposited pre-hydrolyzed APTES on Si wafers in the presence of water, and reported that layer densities are comparable to the neat silane. Zhang et al. used a low concentration (0.24% v / v) of APTES in toluene, and report multilayer formation with sparse layer density (0.46 g / cm$^3$). Only Birkholz et al. report a dense APTES film (1.21 g / cm$^3$) using chloroform as the solvent, at a significantly higher silane concentration (6.6% v/v). It appears that dense APTES film formation is limited by the concentration of the silane.

We believe the observed high density of APTES films is due to partial curing of APTES films. In the cured state, the APTES structure is smaller than the uncured molecule due to absence of ethoxy groups. The density of uncured APTES is also sensitive to the nature of the alkoxy groups. For instance, 3-aminopropyltrimethoxysilane (APTMS) with its three methoxy is significantly denser (1.027 g / cm$^3$) than APTES, which has three ethoxy groups. The extreme limit due to (Si – O – Si) linkages at the interface is SiO$_2$, whose density is twice that of APTES.
Indeed, Vallée et al. find a 100% increase in density on condensation of tetraethyl orthosilicate (TEOS) films. Given these facts, a 27% increase observed in APTES films is credible. The absence of ethoxy groups and increased hydrogen bonding within the partially cured APTES molecule may account for the observed increase in film density.

Tribological studies have shown that APTES films formed using anhydrous deposition are harder, more stable and show greater wear resistance than those generated from aqueous deposition. The observed densification effect accounts for these observations.

The measured interfacial roughness at the APTES-air interface also remains small during deposition. Silane deposition may occur due to either island growth or a gradual buildup at the interface, without recognizable island-like structures.

The island growth model predicts significantly rougher interfaces during island nucleation, followed by a smoothening of the interface during coalescence. Theoretical density profiles for hemispherical island growth demonstrate this phenomenon (Figure 2.6). These profiles differ significantly from the profile shown in Figure 2.2 leading us to reject island type growth.

Instead, the interfacial roughness increases rapidly in the initial stages of deposition and leveling out as deposition continues (roughness ~ 6 Å). We believe partial condensation of APTES molecules in the initial stages of deposition may result in poor packing, resulting in a lower surface density and slightly elevated roughness. Further APTES deposition, driven by hydrogen...
bonding, allows for some restructuring of the film resulting in a slower rate of increase in roughness. XRR data are consistent with a gradually accumulating APTES layer, in contrast to previously reported island-based growth.\textsuperscript{32}

Our observation of smooth films seems to conflict with AFM and SEM studies on multi-layer APTES films generated under similar deposition conditions,\textsuperscript{27,29,31,34,35} which report aggregate formation during deposition leading to much rougher film surfaces. For instance, Howarter et al. find a rougher (r.m.s. roughness 118 Å), and thinner film (50 Å) with multiple island formations.\textsuperscript{29} These authors claim that partially hydrolyzed APTES can perpetrate self-condensation locally, which leads to aggregated structures formed in solution that deposit on the surface.

We performed AFM analysis on dry, 24-h deposited APTES films (Figure 2.7) that confirms a much higher value of surface roughness (~ 270 Å). The apparent inconsistency in roughness can be explained by the differences in the surface characterization techniques.
Figure 2.7 Tapping mode AFM images for 24-h deposited APTES film (10 µm × 10 µm). Aggregated structures are seen studding underlying the smooth APTES film observed by XRR. The AFM roughness is 270 Å.

XRR is sensitive to variations in roughness of the APTES – air interface. Sparsely placed large-scale structures show little scattering and are nearly invisible to XRR. In contrast, roughness values from AFM include the aggregated structures deposited over the APTES film. Our reflectivity measurements imply that these aggregates do not alter the homogeneity of the underlying APTES film. AFM roughness values (5.5 Å) for regions without aggregates correlate well to XRR measured interfacial roughness (~ 6 Å) (Figure 2.8). Our XRR studies and complementary AFM analysis indicate that the APTES film is deposited smoothly over the silicon substrate, occasionally studded with aggregated structures. Importantly, however, the underlying film structure is uniform.

2.3.2. Stability of uncured APTES films

The evolution of APTES multilayers under anhydrous conditions indicates that a majority of molecules are uncured and held together by hydrogen bonds. Hydrogen-bonded APTES can be washed away by protic, polar solvents. In order to determine the extent of loosely bound APTES, we exposed 24-h freshly deposited coupons to a pH-neutral aqueous phosphate buffer. The
uncured coupons are not subjected to any post-deposition cure. Minimal curing may have occurred, however, during sample preparation and XRR measurements (~1 h).

The XRR data and corresponding fits for uncured APTES films before and after exposure to neutral buffer solution are shown in Figure 2.9. After only 30 minutes, both thickness and density of the APTES layer decreased substantially, with an over 70 Å thickness reduction (Figure 2.10).

The layer-structure can be described as a sparse, rough (0.7 g / cm$^3$) top layer with a denser region closer to the interface (1.0 g / cm$^3$), whose density is approximately that of neat APTES. Continued exposure to pH 7 buffer further reduces layer density and thickness. After 360 minutes, a diffuse APTES layer structure remains, with a SLD of $2 \times 10^{-6}$ Å$^{-2}$, corresponding to a layer density of 0.22 g / cm$^3$ and layer thickness of 20 Å, with over 90 mass % of APTES lost from the film.

Figure 2.9 Stability of uncured APTES films exposed to neutral phosphate buffer. The reflectivity data (colored dots) and best fits (solid black lines) have been shifted for clarity. The uncured sample was measured immediately after deposition and not subjected to any post deposition curing.

Figure 2.10 SLD profiles for uncured APTES exposed to neutral phosphate buffer. The detailed shape of the curves for times greater than 60 min is less reliable due to the featureless nature of the reflectivity profiles in Figure 8a.
The thickness of the remaining APTES corresponds well to that of a 4-h deposited APTES layer, indicating that APTES deposited during the initial stages is covalently bound to the substrate, and is not washed away by aqueous buffer. Thus, the morphology of the film may be described as an easily removed “carpet” of hydrogen bonded APTES over the covalently bound surface layer. These results imply that curing is essential to stabilize APTES films, especially for applications where long term stability of the film under aqueous conditions is necessary. There may be partial curing of APTES throughout the layer. The degree of curing however is not sufficient to prevent dissociation from the surface.

2.3.3. Effect of curing on the stability of APTES films

We investigated the effect of time and temperature of curing on the stability of the multi-layer APTES film. Specifically, room temperature (RT, 24 h) and high temperature (HT, 120 °C, 1 h) cures were employed (details described in experimental section). The XRR data for uncured APTES, followed by post-deposition RT cure and subsequent exposure to pH 7 buffer are shown in Figure 2.11. The corresponding SLD profiles are shown in Figure 2.12. Figure 2.13 shows XRR data for HT-cured films subsequently exposed to pH-7 buffer, while Figure 2.14 shows the corresponding SLD profiles.

The effect of RT cure on the morphology of the APTES layers was inconsistent (not shown). Some samples showed small increases in the thickness (up to 5 Å), while others showed almost no change in the layer morphology as seen in Figure 2.11. The small morphological changes are attributed to partial APTES condensation, as the Si – O – Si bonds formed during condensation are flexible and may allow for layer restructuring.
A 24-h RT cure leads to some improvement in stability under neutral aqueous conditions. Thirty-minute exposure to the neutral buffer results in a decrease in both SLD ($8.64 \times 10^{-6} \text{Å}^{-2}$) and thickness (74 Å) of the layer, following a similar trend to the uncured APTES films (Figure 2.10). However the magnitude of material loss is less for the RT-cured films. The film surface stabilizes, with a slight decrease in layer thickness from 30 to 60 minutes. Longer exposure to the neutral buffer further decreases the film thickness, and a two-layer structure is formed, with the denser layer closer to the substrate and a sparse outer layer. After 360 minutes of exposure, the inner layer and outer layer exhibit SLD’s of $8.64 \times 10^{-6} \text{Å}^{-2}$ and $3.21 \times 10^{-6} \text{Å}^{-2}$ respectively. We did observe slight roughening at the native-oxide and APTES interface, which may occur due to re-organization of the remaining APTES molecules.
RT curing increases the APTES layer stability, but only marginally (compare Figure 2.10 to Figure 2.12). This observation agrees with curing studies by Vandenberg et al. After 30 minutes of exposure to the neutral buffer, the amount of material remaining in the RT cured film is three times greater than seen remaining in uncured APTES films. The two-layer structure with a denser layer at the native oxide interface implies that degradation in the neutral buffer solution proceeds inward from the film-solution interface. The two-layered structure also suggests that greater APTES condensation and stronger bonds are formed closer to the native oxide layer.

In contrast to RT curing, where no loss of material is seen during the cure (Figure 2.12), HT curing results in some loss of material from the APTES films (Figure 2.13). On HT curing the APTES layer SLD remains almost constant at $11 \times 10^{-6}$ Å$^{-2}$ while the thickness decreases by 7 Å (Figure 2.14), corresponding to a ~9% loss of mass.

![Figure 2.13](image1.png)  
Figure 2.13. Stability of HT-cured APTES films exposed to neutral phosphate buffer. The reflectivity data (colored dots) and best fits (solid black lines) have been shifted vertically for clarity. The uncured sample was measured immediately after deposition, followed by HT curing (120 °C, 1 h).

![Figure 2.14](image2.png)  
Figure 2.14. SLD profiles for HT-cured APTES exposed to a pH 7 buffer for increasing times. HT cure results in a reduction in film thickness. The film remains stable over long time durations when exposed to pH 7 buffer.
The HT curing substantially increases film stability. Exposure to neutral buffer results in minimal change in SLD compared to the uncured state. Some roughening of the air-APTES interface is seen, but the internal structure (i.e., SLD) is stable. Interestingly, one hour after immersion of the film in water, some swelling is observed (~17 % increase in mass), along with roughening of the air-APTES interface. The swelling of amine-containing silane films is not unique to APTES. Previous vapor swelling neutron reflectivity and FTIR analyses have shown up to 33 volume % swelling of bis-amino silane films upon exposure to D$_2$O vapor.$^{46}$

APTES films are known to show swelling behavior.$^{47}$ It is possible that some water is trapped in the APTES film after exposure to the buffer solution. Neutron reflectivity studies will be needed to generate the necessary contrast to determine water distribution in the APTES film. XRR cannot accurately identify the composition of the APTES film after exposure to the buffer.

The area under an SLD profile is quantitatively related to the total mass in the film. We calculated total material in the APTES films before and after exposure to the neutral buffer. The resulting mass-loss curves were normalized to show the effect of curing on the relative stability. The effect of curing on total mass is summarized in Figure 2.15.

Two important observations can be made regarding the observed mass loss. 1) A majority of material is lost from the films in 90 minutes of exposure to the neutral buffer. 2) HT cured films are very stable in neutral aqueous solution. After 360 minutes of exposure to the neutral buffer, the HT
Cured films retain an order of magnitude more APTES than uncured films. The initial increase in film mass reflects an initial 17% water swelling effect.

An approximate calculation of the extent of condensation shows that the HT curing accounts for 20% of the total Si – O – Si crosslinks. Silane curing occurs in two steps: a) hydrolysis of the alkoxy silane resulting in released ethanol; b) condensation between silanol groups, and between silanol and alkoxy silane groups resulting in release of water. Complete curing results in the incorporation of three water and release of six ethanol molecules for every two APTES molecules in the film. However, care was taken to prevent hydrolysis of APTES film prior to anhydrous deposition. Some molecules necessary for the hydrolysis of the alkoxy silane therefore may be generated from APTES molecules themselves, following initial hydrolysis and condensation at the silicon substrate. Water necessary for hydrolysis may also be absorbed from moisture in the air after anhydrous deposition. For the purpose of this calculation we assumed water is already present in the film, and only ethanol is released upon HT cure. This calculation shows that approximately 15 molecules/nm² of ethanol are released from 34 molecules/nm² of uncured APTES corresponding to ~ 1 molecule of ethanol for every two APTES molecules. A complete cure of APTES from the unhydrolyzed or condensed state would result in the release of 6 ethanol molecules. Therefore, the HT cure results only ~ 20 % curing of the layer. Assuming that a complete cure is obtained after HT cure, the uncured APTES film is roughly 80 % crosslinked. The partial curing of APTES films can explain the increase in density observed during deposition of the film.

We must point however that this analysis can only identify maximum degree of curing in APTES films prior to the HT cure. Also, XRR analysis of the APTES layer cannot identify the structure of the released material. Future studies to determine extent of water and ethanol loss may provide
more accurate information regarding the extent of pre-curing and water absorption prior to a HT cure. Nonetheless, the information obtained reveals that some condensation occurs in APTES films prior to curing. Whatever the level of crosslinking, it is insufficient to prevent penetration of water and hydrolysis of the APTES film. HT cure drives covalent bonding in the APTES layer, while maintaining its density with some shrinkage. Curing of the APTES film maintains its dense layer morphology, while improving hydrolytic stability of the film.

These observations show that curing of APTES films is essential for the stability needed for most applications. Uncured films rapidly lose integrity. RT curing helps somewhat, but such films still hydrolyze in a matter of hours. HT curing is essential in order to assure long-term stability. Even with HT curing, some water penetration and degradation of the APTES - air interface is observed, indicated by its roughening after an extended time; however the HT cure is superior to other curing approaches. Our results are in qualitative agreement with previous reports on the effect of curing.27, 38

2.3.4. Accessibility of amine functionality in APTES films after curing

In order to ascertain the impact of dense APTES film structure on reactivity of the amine functionality the accessible amine concentration from HT-cured APTES films was determined on the basis of Coomassie blue dye adsorption. Coomassie blue primarily binds to the positive charges contributed by the amine groups that are unhindered, so dye concentration is proportional to accessible amine groups.37 The surface areal coverage of APTES was calculated from XRR and compared to the accessible amine density calculated from dye binding. The results are shown in Figure 2.16.
XRR shows that APTES surface concentration continually increases as a function of APTES deposition time. After one hour a surface areal coverage of 2 molecules of APTES molecules / nm$^2$ is obtained, increasing to 3 molecules / nm$^2$ in two hours as multilayers accumulate. APTES surface density monotonically increases to 33 ± 4 molecules / nm$^2$ in 24-h. It must be noted that quantification of surface area coverage obtained from XRR is based on the assumption of uncured APTES films. If we assume curing within the film, surface area coverage values would be considerably higher.

The measurement of the dye-accessible amine groups shows a different trend. After one hour of deposition, the accessible amine concentration is the same as the APTES molecules accumulated on the surface (~ 2 amine groups / nm$^2$). However the accessible amine concentration does not increase as APTES multilayers are formed. The accessible amine concentration remains constant independent of deposition time. Jang et al. report similar deposition time-independent immobilization of fluorescein isothiocyanate (FITC) on APTES thin films. However the amine density was not measured. Interestingly, our dye-binding amine concentration is an order of magnitude higher than reported by Vaughn et al (~0.25 amines / nm$^2$). Although thick APTES layers are formed at longer deposition times, the amine groups within the layer are masked and inaccessible. This sequestration is due to the density of the cured APTES layer. A dense layer prevents penetration of dye. Our results indicate that only amine groups at
the APTES-air interface are chemically reactive. Kim et al. have recently reported that curing reorganizes anhydrously deposited APTES and forms well-ordered reactive amino groups. However, the current results indicate that, while reorganization of APTES molecules may occur during curing, a majority of amine groups within the film remain inaccessible.
2.4. Conclusions

XRR was used to study anhydrous deposition of APTES, complemented by AFM and chemical analysis. We studied the evolution of the APTES film structure, noted the effect of curing on film stability and identified the accessibility of amine groups in the multi-layered films. Our significant conclusions are:

1. The deposition of APTES over inorganic substrates from non-polar solvents occurs in two stages: Initially, a less dense (≈ 1 g / cm$^3$) covalently bound APTES film is formed at the interface, followed by a denser multi-layer film (≈ 1.26 g / cm$^3$), held at the interface through hydrogen bonding. The densification effect is due to partial curing of the APTES film during deposition.

2. The interfacial roughness also increases rapidly during the initial stage of APTES deposition, and stabilizes as the layer thickens (roughness ≈ 6 Å). XRR data indicate that APTES is uniformly deposited during film evolution, as opposed to the island growth.

3. Aggregated structures previously reported to form on APTES films were confirmed using AFM. However XRR data show the underlying film structure remains is uniform. Therefore the topology of a multi-layer APTES film can be described as a smooth interface occasionally studded with large aggregated structures.

4. The film density is higher than the native silane and is maintained as the APTES film grows. Densification above the precursor silane has not been observed in other silane films and explains the tribological observations by Gu et al.$^{32}$ and Simon et al.,$^{35}$ and hardness enhancements reported by Amemiya et al.$^{31}$
5. Although a dense multi-layer film is formed during anhydrous deposition, APTES molecules at the air-film interface are hydrolytically unstable and easily washed away after exposure to a neutral buffer. Over 90% mass of the film is lost. After exposure only a thin, covalently bound APTES film remains at the substrate interface.

6. The density of the APTES film also affects the accessibility of amine groups. Although multilayers of APTES are readily formed by anhydrous deposition, most amine groups are trapped within the dense APTES layer and are chemically inactive. Independent of the deposition time and APTES film thickness, the reactive amine accessibility remains constant. Therefore, for the purposes of interfacial modification, single-layer films are just as effective as thick layers.

7. A high temperature cure is essential for the stability of APTES films. A 120 °C cure results in covalent binding of the APTES molecules both to themselves and to the surface-bound APTES layer, thereby dramatically improving layer stability.
3. Feasibility of engineered human soluble calcium activated Nucleotidase as an anti-thrombotic coating for stent applications

3.1. Introduction

Hundreds of thousands of cardiac and cranial stents are implanted every year in an attempt to re-open clogged arteries and prevent future heart attacks and strokes. These stents are typically made of an expandable meshwork of stainless steel, which is deployed by a balloon catheter to open constriction of flow in vessels. The implantation of stents however introduces a foreign body into the blood stream and triggers a myriad of unwanted responses: inflammation, thrombosis, and often restenosis, resulting in the therapeutic failure. Stent surfaces may be rendered hemocompatible by the addition of coatings with slow-release anti-proliferative drugs (drug-eluting stents) in conjunction with oral anti-platelet therapy. This strategy has substantially decreased the incidence of restenosis compared to bare metal stents. However, late-stage thrombosis events are more likely with the drug-eluting stents, especially if anti-platelet therapy is discontinued. The thrombosis events occur due to the delayed endothelialization, necessitating long-term anti-platelet therapy after stent deployment. In light of multiple complications, it has been recently argued that stents may not offer a substantial improvement over stand-alone medical therapies. Thus, there remains an unmet need for a material or coating that adequately alleviates all three complications of stents: thrombosis, restenosis, and inflammation.

Enzymes that hydrolyze extracellular nucleotides (apyrases and ecto-nucleoside triphosphate diphosphohydrolase (NTPDase1 or CD39) )may serve to greatly attenuate or eliminate issues
associated with stents via modulation of purinergic receptors (a family of plasma membrane receptors which among other things also affect platelet aggregation). Apyrases \(^{56}\) and CD39\(^{55}\) function to maintain hemostasis by hydrolyzing adenosine diphosphate (ADP). ADP is an agonist released by platelets upon activation resulting in local cascading events that aid rapid thrombus formation. By suppressing the ADP trigger to the platelet cascade, CD39 nucleotidase treatments were found to suppress thrombosis, neointimal hyperplasia (thickening of blood vessels), and inflammation, and eliminated the need for administration of anti-platelet (anti-coagulant) drugs after stent placement. \(^{57,58}\) The studies cited above demonstrate the potential utility of nucleotidases for cardiovascular therapeutic applications. However, Prof. Terence Kirley’s Group at UC and other researchers have demonstrated that soluble forms of NTPDases used in some of these previous studies are less active and less stable than the natural enzymes, as well as being difficult to produce on a therapeutic scale.

These difficulties are in stark contrast to engineered human soluble calcium activated nucleotidase (SCAN), which can be efficiently produced in bacteria.\(^{59}\) After simple and near quantitative refolding SCAN has higher nucleotidase activity than soluble NTPDases, and is stable for years when stored refrigerated. In addition, SCAN’s surface structure is easily mutated and subsequently chemically modified, without loss of activity, making it ideal for covalent coupling to coated stent surfaces.

We have assessed the feasibility of engineered human SCAN as a stent coating to prevent platelet aggregation and eventual clotting. Stainless steel (type 316L, SS316L) is typically used for stents and other implants due to its excellent mechanical properties and corrosion resistance. In this study engineered SCAN protein, with optimized ADPase activity was covalently and specifically bound to SS316L substrates. The immobilization methodology involved reaction of a
heterobifunctional agent (N-(e-maleimidocaproyloxy)-N-hydroxysuccinimide ester (EMCS) to APTES modified interfaces. EMCS utilizes the N-hydroxysuccinimide ester to react with amine functionality of APTES forming an amide linkage. The reaction also end-functionalizes the APTES film with maleimide groups. Utilizing the maleimide group, SCAN was immobilized covalently on SS316L substrates.

Utilizing X-ray reflectivity, we find that a single, dense and smooth layer of SCAN assembles at the interface. The single layer was effective in down-regulating platelet activation through ADPase activity. Furthermore, the immobilized protein maintained enzymatic activity over five months, making SCAN a viable candidate for therapeutic use as an anti-thrombotic stent coating. Incorporation of a SCAN coating will prevent platelet aggregation over time scales necessary for re-endothelialization, while overcoming later-stage thrombosis necessary with drug-eluting stents.
3.2. Materials and Methods

3.2.1. Substrate pretreatment and amine functionalization

The immobilization strategy was evaluated on two substrates: stainless steel coupons and silicon wafers. Stainless steel (SS) 316L coupons (1/8 × 1/8” and 1 × 1” sizes, 0.5 mm thick) were obtained from Stainless Supply Inc. (Monroe, NC). N-doped single side-polished 0.5 mm thick silicon wafers (1 0 0) were purchased from College Wafers Inc. (South Boston, MA) and cut to the required dimensions. Both polished SS316L and silicon surfaces were washed with multiple solvents to remove any soluble contaminants. The order of solvent washes was water, ethanol, acetone, toluene and chloroform. Following solvent washes, the SS316L coupons were placed in a Harrick plasma cleaner (Ithaca, NY) for at least 15 minutes to oxidize any remaining surface bound organic matter. A fifteen-minute air plasma treatment adequately removes organic contaminants (determined by water contact angles tending to zero). Silicon wafers were cleaned by immersion in a 3:1 concentrated sulfuric acid: hydrogen peroxide (piranha solution). The wafers were rinsed with water, dried initially using a stream of nitrogen and finally dried in an oven at 120 °C for 5 minutes. Both SS and silicon wafer substrates were functionalized with amines immediately after pretreatment.

APTES was used to provide amine functionality at the silicon and the stainless steel substrates. APTES (99%) obtained from Sigma-Aldrich Co. (St. Louis, MO) was vacuum distilled and stored refrigerated in a desiccator prior to use. Toluene was dried over calcium hydride, distilled, and stored in a desiccator. The SS and silicon coupons were functionalized under anhydrous conditions. Two APTES deposition and curing conditions were explored: SS coupons and silicon wafers were placed in either 1 (v / v) %, 2 h or 10 (v / v) %, 8 h solutions of APTES in toluene at room temperature under gentle agitation. Following APTES deposition, the substrates were
rinsed using toluene and acetone and dried in a nitrogen stream. The APTES-coated coupons were completely cured at 120°C for 1 h in order to stabilize the APTES coating.

### 3.2.2. Maleimide functionalization of the substrate

A maleimide functionality was introduced over APTES modified substrates to facilitate SCAN immobilization. A buffered solution of the heterobifunctional linking agent, \([N-e\text{-maleimidocaproyloxy}]-N\text{-hydroxysuccinimide}\) (EMCS) ester (5 mM EMCS, in 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS) containing 10% (v/v) dimethyl sulfoxide (DMSO)) was prepared fresh and used immediately. The EMCS was first dissolved in DMSO, due to its limited solubility in water. Roughly 100-μL aliquots of the buffered EMCS solution (enough to submerge the metal coupons in 1.5 mL Eppendorf tubes) was added to APTES-coated SS and silicon coupons. The reaction was allowed to proceed at room temperature for 1 hour. Following maleimide functionalization, the coupons were washed two times with 10 % DMSO, 50 mM MOPS and two times with 50 mM MOPS buffer. The maleimide-functionalized coupons were used immediately for coupling to SCAN.

### 3.2.3. Site-directed mutagenesis of SCAN to allow sulfhydryl-based enzyme coupling

For the purpose of linking SCAN to the maleimide functionalized coupons, a single cysteine residue was introduced in the ADPase-enhanced, engineered human SCAN enzyme (containing 5 mutations to increase stability and ADPase activity, “Mutant B”). The 5-mutation SCAN ADPase-enhanced construct prevents platelet coagulation *in vitro* and thrombosis *in vivo* in a ferric chloride-induced murine model of thrombosis. This aspect of SCAN immobilization methodology was carried out exclusively by Dr. Terence L. Kirley at the Department of Pharmacology, UC.
The cysteine residue was introduced near the N-terminus, according to available crystal structures. The N-terminal portion of the molecule is unstructured, and unrelated to the activity of the enzyme, being distant from both the active site and the dimerization site, both of which are important for maximal activity. A single cysteine molecule was introduced by substitution of specific amino acids in order to generate three unique mutants (Figure 3.1); one at the first amino acid in the sequence after thrombin cleavage of the N-terminal hexa-his purification tag (as per Liu et al.), and termed (“Thrombin G-C”, Mutant 1), one disrupting an NheI restriction site used for cloning, just before the beginning of the SCAN protein sequence (“NheI S-C”, Mutant 2) and one at the 3rd residue of the soluble part of the SCAN molecule (“T41C”, Mutant 3).

None of the mutations interfered with bacterial expression or refolding of active nucleotidase enzyme from bacterial inclusion bodies. In addition, none of the mutations substantially decreased the specific nucleotidase activities of the refolded enzymes, indicating the feasibility of the proposed approach and studies.

SCAN is more stable as a dimer, but dimerization leads to intermolecular disulfide formation in SCAN mutants containing cysteine near the N-terminus. Therefore, in order to promote covalent binding to the substrate; the introduced cysteine residue was reduced using dithiothreitol (DTT) immediately before coupling to surfaces. During a typical reduction, 23 µL (1.33 mg / ml) stock

![Figure 3.1 Sites for mutations to introduce cysteine for coupling of SCAN. Amino acids before the start of the soluble SCAN protein sequence are italicized, the thrombin recognition sequence is bolded and underlined, the amino acids encoded by the NheI restriction site are underlined, and the three sites of cysteine substitution mutations for coupling are colored red, green and blue for mutants 1-3, respectively.](image-url)
solution of SCAN was added to 2 µL of 17mM DTT in 750 µL of 50 mM MOPS containing 2mM CaCl$_2$. The reduction was allowed to proceed for 15 minutes at 37 °C. The reduced SCAN protein was separated on an 800 µL Sephadex G-50 size exclusion column in 200 µL fractions. SCAN concentration in the second fraction was determined at 280 nm assuming an extinction coefficient of 81,400.

3.2.4. **Engineered SCAN coupling on maleimide functionalized substrates**

Maleimide-functionalized coupons were incubated in a 0.05 mg / ml solution of SCAN in 50 mM MOPS pH = 7.4 / 2mM CaCl$_2$ solution for 90 minutes at room temperature. The coupons were washed five times with 50 mM MOPS / 2 mM CaCl$_2$ solution. Following SCAN immobilization, the coupons were incubated in 1 mg / ml solution of human serum albumin (HSA, Sigma) overnight at room temperature. This step ensures that only covalently bound SCAN is present at the coupon surface. Physically adsorbed SCAN is displaced by HSA. The samples were again washed five times using 50mM MOPS / 2mM CaCl$_2$ buffer solution and stored in the same buffer prior to use.

3.2.5. **X-ray reflectivity measurements**

X-ray reflectivity (XRR) is a surface sensitive, non-destructive characterization technique wherein reflected X-ray intensities are analyzed in order to determine physical characteristics of ultra-thin films (0 – 1 µm). Layer structure, film thickness, scattering length density (SLD) and interfacial roughness can be obtained from a typical reflectivity scan and analysis. A limitation of the technique however is the necessity for ultra-smooth substrates (roughness < 7 Å). Since stainless steel coupons were not sufficiently smooth, the SCAN immobilization procedure and layer structure was evaluated on silicon coupons (roughness ~ 5 Å).
The PANalytical X’pert Pro Materials Research Diffractometer at the Advanced Materials and Characterization Center (University of Cincinnati) was used to obtain XRR results. The x-ray source (Cu Kα=1.542 Å) was operated at 1.8 kW (45 mV, 40 mA) and XRR analysis was carried out using PANalytical X’Pert reflectivity v. 1.1.

The number of layers chosen during fitting was based on the immobilization protocol. Based on the model obtained, the SLD profile (SLD as a function of the perpendicular distance from the substrate surface) was generated using Irena Macros 2.38 for Igor Pro 6.17. The SLD profile relates to material composition and density. Knowing the material composition, the mass density can be accurately determined. During fitting, the silicon substrate roughness was allowed to vary between 3 – 7 Å. We set the native oxide layer mass density at 2.2 g / cm³. To account for substrate variability, the native oxide layer thickness was allowed to vary slightly (13 ± 3 Å).

3.2.6. Nucleotidase assays

The enzymatic activity of surface-bound SCAN was determined by measuring the amount of inorganic phosphate (Pᵢ) released due to catalysis of either ADP or ATP from nucleotide substrates (Sigma) at 37°C using modifications of the technique Fiske and Subbarow as previously described. Briefly, SCAN-coated, bare and HSA coated stainless steel coupons were pre-incubated at 37°C for 3 minutes with shaking in 280 μL of 50mM MOPS / 2mM CaCl₂ buffer. 20 μL of 37.5 mM ADP / ATP (Sigma) was added to the buffer solution while shaking for a period of 12 minutes. The solution was removed from the coupons and transferred to 12 × 75 mm test tubes and 300 μL of ammonium molybdate, then 300 μL of amidol solution was added to the test tubes. The enzymatic activity was determined from the absorbance at 660 nm after 10 minutes at room temperature. Following the enzymatic activity measurement, the coupons were transferred to the buffer solution maintained at room temperature.
We also coated a SS316L stent surface with SCAN using competitive adsorption with HSA. The stent surface was continuously incubated at 37 °C, following storage at 4 °C. The enzymatic activity of the stent surface was determined using the identical procedure as described for the stainless steel coupons. This aspect of SCAN immobilization methodology was carried out exclusively by Oliver Rogers and Dr. Terence L. Kirley at the Department of Pharmacology, UC.

3.2.7. Platelet adhesion assays

Platelet adhesion and activation behavior was assessed using citrate-anticoagulated platelet-rich plasma (PRP) obtained from Hoxworth's Blood Center (platelet count ~ 3 × 10^5 platelets / μL, prepared by centrifugation of citrated whole human blood at 140 g for 10 minutes at 22 °C). CaCl_2 was added to the PRP to a final concentration of 2mM. In order to eliminate the effect of surface roughness (which can influence platelet adhesion), the stainless steel substrates were polished to a mirror finish. Bare (Bare-SS), SCAN-coated (SCAN-SS), and HSA-coated (HSA-SS) SS316L coupons were incubated in the platelet suspension at 37°C in sterile 96-well microtiter plates under gentle agitation.

The platelet fixation procedure was adopted from Shin et al. Following platelet adhesion, the coupons were incubated in 2 % glutaraldehyde solution in tris-buffered saline (TBS) for 30 minutes at room temperature. After incubation, the coupons were rinsed using TBS. The coupons were then dehydrated using increasing concentrations of ethanol in water (50, 70, 90, 100 (v/v) % for 5 minutes respectively). Finally, the coupons were dried in air for 5 minutes, followed by vacuum drying for one hour.

For scanning electron microscopy (SEM) image acquisition, the coupons were sputtered with a palladium-gold alloy coating and analyzed using the FEI XL30 ESEM operated at 15 kV. The numbers of platelets per unit area was determined by acquiring at least nine images (~10^5 μm^2 /
image) from spots chosen at random on each coupon with a minimum of three coupons per experimental condition.

### 3.2.8. Platelet depletion assays.

75 μL of the PRP solution was collected from each microtiter plate well following incubation at 37 °C. 10 μL of 50 mM EDTA was added to the collected platelet rich plasma and centrifuged at 400 × g for ten minutes at room temperature. 50 μL of the resulting platelet suspension was diluted in 950 μL TBS. The platelets were counted using a hemacytometer to measure platelet depletion due to incubation with coupons.

### 3.2.9. Statistical analysis

All data except platelet adhesion results are represented using mean ± standard deviation. Statistical significance was calculated for comparisons of platelet adhesion to bare-SS, SCAN-SS and HSA-SS coupons. Platelet adhesion experiments are represented using mean ± standard error of mean. Statistically significant differences were established using the two-sample student $t$-test assuming unequal variances. A $p$-value of less than 0.05 was considered statistically significant.
3.3. Results

3.3.1. Structure of covalently bound SCAN

XRR was used to observe the evolution of interfacial thin-film structure during covalent binding of SCAN protein on silicon substrates. The silicon substrates are highly polished (roughness $\sim 5$ Å), which facilitates XRR data collection and interpretation.

A thin film of APTES was anhydrously deposited on the native oxide layer on a silicon wafer. After APTES deposition, EMCS was bound to the amine groups via the N-hydroxysuccinimide ester (Figure 3.2). Finally, SCAN protein was immobilized to the EMCS bound substrate via the maleimide functionality of the surface and the introduced N-terminal thiol group in the enzyme. This deposition protocol justifies the application of a four-layer model to fit the XRR curves (silicon wafer followed by native SiO$_2$, APTES, EMCS and SCAN protein in that order). The XRR data and corresponding best-fits are shown in Figure 3.3 and Figure 3.4.

A two-hour deposition of APTES results in $13 \pm 3$ Å thick silane film, with a previously reported accessible amine density of 2 amine groups / nm$^2$. Following EMCS modification (layer

Figure 3.2 Schematic showing substrate pretreatment steps prior to covalent deposition of SCAN
density $0.83 \pm 0.1 \text{ g / cm}^3$), film thickness increases by $9 \pm 1 \text{ Å}$, which is comparable to the cross-linker arm length (9.4 Å).

XRR confirms a single layer of SCAN is immobilized on the silicon substrate. SCAN protein immobilization (layer 4) with the maleimide results in a 37-Å thick protein layer with a broadened interface. The density of immobilized SCAN is $0.94 \text{ g / cm}^3$. In its stable form in solution, SCAN exists as a dimer with the dimensions of 86 Å × 50 Å. Since XRR measurements are performed in the dried state, some denaturation of the protein may occur resulting in a thinner layer than the native protein dimensions. Also, some SCAN may non-specifically, non-covalently adsorb to the substrate.

In order to minimize non-covalently bound SCAN on the substrate, a competitive adsorption strategy using HSA was employed. Following SCAN adsorption, the substrates were exposed to
a concentrated solution of HSA. XRR data and corresponding best fits for SCAN immobilization performed under competitive adsorption conditions (triplicate samples) are shown Figure 3.5 and Figure 3.6. The deposited SCAN layer is substantially thinner (27 Å) and smoother as compared to pure SCAN deposited without HSA competition.

While the protein layer SLD is comparable to that obtained during pure SCAN immobilization (an expected result since most proteins would have similar density and composition), the SLD values obtained from fitting cannot be directly related to protein density due to the presence of both proteins at the substrate. The film properties for triplicate SCAN films deposited in the presence of HSA are summarized in Table 3.1.

Table 3.1. Film thickness, SLD and roughness for SCAN protein immobilized on EMCS modified substrates following HSA adsorption.

<table>
<thead>
<tr>
<th></th>
<th>Thickness $t$ (Å)</th>
<th>SLD ($Å^{-2}$)</th>
<th>Roughness (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$27 \pm 1$</td>
<td>$(9.07 \pm 0.17) \times 10^{-6}$</td>
<td>$4 \pm 2$</td>
</tr>
</tbody>
</table>

Figure 3.5 XRR data for SCAN protein bound to maleimide functionalized surfaces following non-specific HSA adsorption. Deposition conditions are the same for all 3 depositions.

Figure 3.6 SLD profiles for SCAN protein bound to pretreated surfaces following non-specific HSA adsorption. The differences represent the statistical variability due to deposition and fitting.
3.3.2. Evaluation of SCAN immobilization strategy and nucleotidase activity

The enzymatic activity of surface-immobilized SCAN was determined by measuring inorganic phosphate concentration due to the hydrolysis of ADP and ATP solutions.

Ideally, the engineered SCAN is more suited to hydrolyze ADP as compared to ATP, showing a high selectivity (~7). By evaluating both ADPase and ATPase activities, the effect of long time durations on the selectivity of SCAN can be determined. Figure 3.7 and Figure 3.8 show the ADPase and ATPase activity of surface immobilized SCAN compared to physically adsorbed SCAN (on SS316L and silicon wafers) over a period of 40 days. The intent of this experimental protocol was threefold: a) To evaluate effect of varying underlying APTES deposition conditions on SCAN immobilization; b) To determine stability of covalent binding protocol over time and multiple washings; c) To determine ADPase : ATPase ratio for surface immobilized SCAN.
Varying the underlying APTES deposition conditions (1 %, 2 h vs. 10 %, 8 h) or substrate (SS316L vs. silicon) does not affect SCAN coating stability. After 15 days, ADPase activity is similar for all deposition conditions. The APTES deposition conditions chosen represented two extremes: APTES deposited at low concentration and short deposition time vs. at high concentration and long deposition time. Morphologically, the structure of APTES deposited from both conditions is different. However on the basis of XRR results, a dense layer of SCAN is formed when low concentration, short deposition time conditions for APTES functionalization were used. Therefore further loading of SCAN is limited by the lack of binding sites due to complete coverage of the protein at the interface.

Initial loading of SCAN shows some dependence on nature of the substrate. SCAN deposited on silicon wafers shows a higher ADPase activity as compared to SS316L substrates, indicating greater loading. The rapid loss in activity in the initial few days of ADPase determination suggests possible physical adsorption due to accompanied by loss of the immobilized SCAN.

The immobilization protocol results in a robust, stable layer of SCAN at the stainless steel interface. Throughout the 40-day duration of activity measurements, SCAN immobilized coupons were incubated in 50 mM MOPS / 2 mM CaCl₂ and washed at least six times during each enzymatic activity measurement. A gradual decay was observed in the activity of SCAN,
potentially due to enzymatic deactivation and release of weakly bound protein. However ADPase and ATPase activities were maintained.

We repeated the immobilization protocol on a stainless steel stent substrate (Figure 3.9 ADPase activity of surface bound SCAN on a stent surface. The ADPase activity was measurable over 250 days supporting the robustness of SCAN protein and immobilization strategy.), maintained at 37 °C and evaluated its nucleotidase activity over 250 days in an effort to mimic in vitro conditions and determine the stability of SCAN. As before, after each ADPase measurement, the SCAN immobilized stents were subjected to multiple washings. ADPase activity was measurable over 150 days, confirming the long-term stability of immobilized SCAN.

Figure 3.10 shows the selectivity of SCAN in hydrolyzing ADP as compared to ATP. ADPase : ATPase ratios were determined from Figure 3.7 and Figure 3.8 over the duration of enzymatic activity measurements. When immobilized, SCAN shows a selectivity of ~3.9. This is significantly lower than ADPase : ATPase activity in solution (~7). Limitations in the degrees of freedom of bound SCAN may account for the reduced selectivity. The selectivity however does not change with time, indicating the immobilized SCAN structure is stable.
3.3.3. Platelet adhesion

To determine the effectiveness of immobilized SCAN as an anti-thrombotic coating, bare (Bare-SS), HSA-coated (HSA-SS), and SCAN-coated (SCAN-SS) SS316L coupons were incubated in PRP at 37°C for 60 minutes under gentle agitation. While platelet binding was observed on all coupons, irrespective of interfacial modification strategy, significant differences in platelet morphology and densities were observed (Figure 3.14 vs. Figure 3.12 vs. Figure 3.16).

On bare-SS substrates, some thrombus formation and spreading of platelets was observed with significant clustering of platelets. Platelets were seen to bind individually at the SCAN-SS interfaces for the most part, although significant platelet clumping was observed. HSA-SS surfaces promoted platelet adhesion and clotting, in contrast to previous reports. HSA-SS substrates were covered with multi-layers of platelets showing significant spreading, and dense fibrin formation. Surface-adsorbed platelet density determination was difficult due to this effect. Therefore, the areal coverage of platelets and thrombus formation on coated and uncoated SS316L substrates was compared as a percentage of the total area observed using SEM and shown in Figure 3.14. Bare-SS and SCAN-SS coupons show markedly less % area covered by
platelets and thrombi, as compared to the HSA-SS coupons \( p < 0.05 \). Furthermore, the % area covered by platelets on SCAN coated coupons is significantly lower than on bare control coupons \( p < 0.05 \).

Quantification of platelet density (between bare-SS and SCAN-SS) confirms the area coverage trends (Figure 3.15). A statistically significant lowering of platelet density over SCAN-SS \((0.626 \pm 0.047) \times 10^6 \) platelets / cm\(^2\) as compared to Bare-SS \((1.072 \pm 0.074) \times 10^6 \) platelets / cm\(^2\) was observed with platelet concentration at the interface almost halved.

Platelet depletion in platelet-rich plasma following platelet adhesion to, and removal of, the coupons was expected to show trends consistent with the platelet density and % area coverage measurements. Figure 3.16 shows platelet depletion results i.e. concentration of platelets remaining in solution from bare-SS, SCAN-SS, and HSA-SS coupons compared vs. platelets put through the same conditions in the absence
of any coupon substrate (untreated).

The SCAN coated coupons show the lowest platelet depletion, which is consistent with the least platelet density measurements made directly from the coupons. HSA coated coupons show the lowest platelet count remaining in solution, which agrees with the largest percentage platelet adhesion observed for these coupons. However, the trends of differences in the means of the platelet counts remaining in solution after exposure to the different substrates do not reach statistical significance.

Figure 3.16 Average platelet counts in PRP solution after incubation at 37°C for 60 minutes.
3.4. Discussion

We have successfully immobilized human SCAN on stainless steel 316L substrates utilizing a novel, covalent, specific binding protocol. This immobilization strategy represents the first application of ‘site directed mutagenesis’ to improve the activity of immobilized nucleotidases. In this instance, site-directed mutagenesis refers to the incorporation of a cysteine molecule at an enzymatically inactive region of SCAN. The cysteine molecule presents an easily modifiable thiol group on the SCAN protein.

The immobilization strategy involves three steps. Firstly, using 3-aminopropyltriethoxysilane (APTES), stable amine functionalized films were prepared on stainless steel and silicon substrates. Alkoxy silanes form robust, chemically bound organic thin films over inorganic substrates, acting as a coupling between the inorganic metal substrate and organic matter.\(^{70-72}\) The stainless steel interface has a native iron and chromium oxide layer,\(^{73}\) which facilitates APTES deposition at the substrate.

The APTES layer was then reacted with a bi-functional linking group – EMCS. EMCS reacts to the primary amine moiety via the N-hydroxysuccinimide ester functionality resulting in an amide linkage. This modification presents an immobilized maleimide functional group at the air – metal interface.

Finally, the maleimide group reacts to the thiol moiety introduced on SCAN via a michael addition type reaction. This reaction scheme facilitates the deposition of a single layer of SCAN (0.94 g / cc, 37 Å thick determined using XRR) at the stainless steel interface.

The specific, covalent bonding strategy employed is superior to other more commonly used protein immobilization approaches. Physical adsorption, for instance, is a simple technique to
immobilize proteins on a substrate. The extent and reversibility of protein adsorption however are dependent on the surface properties i.e. surface charges and hydrophobicity. Furthermore, substantial loss in activity of the protein occurs due to conformational changes during adsorption. Proteins immobilized using physical adsorption may also leach into the blood stream, resulting in an unfavorable immunological reaction. Other strategies involve non-specific covalent binding to immobilize proteins. However, non-specific adsorption may hinder the conformations of the protein and substantially reduce activity.

Following successful SCAN immobilization, we attempted to increase the loading of SCAN by means of modifying APTES deposition conditions. However, this effort was unsuccessful. SCAN loading is independent of APTES deposition conditions, presumably due to the entrapped amine groups within the APTES film. This result therefore validates chemical quantification of APTES functionalized substrates presented in section 2.2.8.

As a precaution, to eliminate physically adsorbed SCAN from the stainless steel surfaces, a co-adsorption strategy was employed (SCAN immobilization followed by exposure to HSA solution). This procedure reduced the amount of protein at the substrate. The SCAN layer thickness reduced by ~10 Å, indicating some loss of material, presumably the adsorbed SCAN. This co-adsorption strategy stabilizes the immobilization protocol, and rapid loss of activity seen for SCAN only films (data not shown) was eliminated.

The ability of SCAN to hydrolyze ADP was ascertained via nucleotidase assays. Some loss in selectivity of the immobilized SCAN was observed. ADPase activity, however, was observed using stainless steel coupons and modified stent surfaces. The activity reduced gradually over a few months. However even after 250 days, SCAN immobilized substrates continued to
demonstrate ADPase activity. It must be noted that in the blood vessels, shear forces may also be active on the stent surfaces. Shear forces at a stent site can serve to further accelerate thrombosis and restenosis.\textsuperscript{76} The effect of such forces on coating stability is not known at this stage. \textit{In vivo} placement of SCAN immobilized substrates will be carried out in order to assess coating stability under real-world conditions.

The immobilized SCAN was also successful in deterring platelet adhesion, demonstrating potential of SCAN and the immobilization strategy. Platelet adhesion tests were performed using citrate anti-coagulated PRP. 2mM solution of CaCl$_2$ was added to the PRP as a platelet activator, and to provide Ca$^{+2}$ ions in the solution, which are essential for the ADPase activity of SCAN.\textsuperscript{77} Platelet adhesion results support the anti-thrombogenicity of SCAN immobilized on SS316L. A platelet density of (1.072 ± 0.074) × 10$^6$ platelets / cm$^2$ was observed on SCAN immobilized substrates as compared to bare SS316L (0.626 ± 0.047) × 10$^6$ platelets / cm$^2$. This result is statistically significant ($p < 0.05$). Corresponding to platelet counts, a morphological effect was also seen via SEM images, with lesser platelet spreading and clumping on the SCAN coated coupons.

Excellent stability both in solution, and in the immobilized state coupled with proven ADPase activity in conjunction with reduced platelet adhesion make SCAN an viable candidate for therapeutic anti-thrombotic stent coatings.
3.5. Conclusions

This is the first report showing successful covalent immobilization of engineered human SCAN on SS316L substrates for the generation of an anti-thrombotic surface. The strategy used for optimization of immobilized SCAN activity is also unique, giving a single (~27 Å, SLD), specifically bound layer of SCAN. SCAN-bound SS316L surfaces maintained significant ADPase activity over 250 days, demonstrating the robustness of our coating approach.

The immobilized SCAN reduced platelet activation on the surfaces exposed to PRP, by preventing secondary activation of platelets due to the release of ADP. Our results suggest that SCAN and the optimized immobilization procedure can be applied to stents and other medical devices, attenuating the negative consequences of these interventions by increasing the biocompatibility of the implanted device.
4. Interactions of water with poly (N-isopropylacrylamide)

4.1. Introduction

PNIPAm is a thermally responsive polymer exhibiting lower critical solution temperature (LCST) behavior between 31 and 35 °C in water. At temperatures below the LCST, PNIPAm exists in a solvated chain-like configuration (radius of gyration \( R_g \) \( \propto \) number of repeating units \( (N^{0.5}) \)). Above the LCST, the PNIPAm chain collapses to a globule \( R_g \propto N^{0.33} \). Surface grafted PNIPAm exhibits a hydrophobic – hydrophilic transition corresponding to the chain-globule transition. The physiologically convenient LCST temperature has led to many applications in microfluidics, drug delivery, selectivity, and tissue engineering.

Although temperature is the stimuli for the collapse transition in PNIPAm, external environmental and structural variations can impact the LCST and the characteristics of the collapse. For instance, varying polymer concentration has a very small effect on the LCST, however end-group and molecular weight effects are substantial. Similarly, ionic solutes in water can substantially lower the LCST. The tacticity and co-polymerization of PNIPAm with different monomers are also known to affect transition behavior and temperature. Utilizing this knowledge, PNIPAm transition behavior can be fine-tuned to optimize its impact on physiological systems. However current understanding of the LCST transition especially in polymer – water systems is still incomplete.

The PNIPAm LCST transition is conventionally viewed as occurring due to a competition of favorable enthalpic intermolecular hydrogen bonding and entropic effects due to unfavorable water structuring. In large part, the transition phenomena can be attributed to the anomalous
behavior of water. Water, a polar solvent exhibits a strong self–association.\textsuperscript{87} Unusually high boiling and melting points are properties affected by this self-association tendency. Forces between water and organic and inorganic solutes are the key to the molecular organization and phase behavior in colloidal and biological systems.\textsuperscript{88, 89}

This chapter presents a short, theoretical discussion of the LCST phenomena in PNIPAm – water system. Also the influence of PNIPAm structure, concentration and solvent quality on LCST transition are presented.

4.2. Thermodynamics of mixing in polymer-solvent systems

Polymer mixing thermodynamics are understood on the basis of lattice theories developed by Flory and Huggins.\textsuperscript{90} The incorporation of a solute in a solvent increases its configurational entropy. From the Gibbs free energy equation, it follows that increasing temperature should also increase the entropic contribution, resulting in dissolution of the solute even if enthalpic contributions are unfavorable.\textsuperscript{91} The Flory-Huggins expression assigns enthalpic contributions to the interaction parameter $\chi$. (Eq 4.1)

$$\frac{\Delta G}{RT} = n_1 \ln \phi_1 + n_2 \ln \phi_2 + \chi \phi_1 \phi_2$$  \hspace{1cm} \text{Eq 4.1}

The Flory-Huggins interaction parameter is a measure of polymer-solvent immiscibility. Greater values indicate increased tendency for phase separation. This interaction parameter has temperature dependence $\chi = \alpha / T + \beta$. However the physical origin of the constants remains unclear.\textsuperscript{92} The Flory-Huggins expression provides a satisfactory description for the UCST, i.e. phase separation at low temperatures. This theory however, provides no explanation for phase separation due to increasing the temperature i.e. the LCST.
Modifications to the Flory-Huggins theory, account for differences in volume of the solvent and monomer units. These modifications preserve the Flory-Huggins expression while incorporating volume-of-mixing effects into the interaction parameter:

\[ \chi = \frac{\alpha}{T} + \beta + \gamma T + \delta \ln T \]  

Eq 4.2

The modified interaction parameter in Eq 4.2 shows both a direct and inverse dependence on temperature. Thus depending on the values of constants \( \alpha \) and \( \gamma \), the presence of both LCST and UCST can be explained through this model. However the nature of the constants \( \alpha, \beta, \gamma, \delta \) is once again poorly understood. These theories do not account for highly specific, orientation-dependent polar interactions.

The LCST for non-polar polymer-solvent systems generally occurs between the boiling point and the critical temperature of the solvent. However aqueous solutions of many polar polymers exhibit LCST behavior well below the boiling point. Polymer-water phase diagrams can be distinctly different from non-polar polymer solutions. For instance poly (ethylene oxide)-water systems show a ‘closed miscibility gap’ with a lower LCST than its corresponding UCST (Figure 4.2). These observations hint at stronger interactions with water in polymer-water binary systems. The role of water and its specific interactions with the polymer must be understood to obtain a physical interpretation of polymer-water systems.

Figure 4.1 Closed loop miscibility observed in PEG-water systems.
4.3. LCST behavior in PNIPAm

PNIPAm is an amphiphilic polymer with a hydrophobic backbone [-CH₂-CH₂-] and both hydrophobic (isopropyl-) and hydrophilic (amide-) sidegroups (Figure 4.3). PNIPAm solvation behavior occurs due to a balance of favorable and unfavorable forces between the polymer segments and water. However, there is some conflict regarding the driving mechanism for the collapse transition.

The LCST behavior of PNIPAm was first reported through visual cloud point observation by Heskins and Guillet. They attempted to explain the LCST behavior using lattice theory models, concluding that the phase separation occurs at the LCST due to greater entropy in the two phase system. The solvation behavior of PNIPAm was attributed to a combination of hydrogen bonding effect and “structuring of water” around hydrophobic groups. Since the original study, many other explanations to the LCST behavior have been reported.

Tanaka et al. explain the PNIPAm transition purely on the basis of preferences in hydrogen bonding. According to this theory, below the LCST, hydrogen bonding between amide and water groups is preferred over water-water hydrogen bonding. As the temperature increases, water-water and amide-amide hydrogen bonding is more favorable and this difference in energies drives the collapse transition suggesting an enthalpic effect.
This result is supported by IR results presented by Lin et al.\textsuperscript{97} A change in the amide I and II peak positions in IR spectra is seen as the polymer collapses through the LCST. Presumably, intermolecular bonding between the amide groups and water is replaced with intramolecular hydrogen bonding between the amide groups at the LCST. However, even in its collapsed state, experimental observations show that PNIPAm contains appreciable amounts of water, meaning attractive hydrogen bonding between water and amide groups are still somewhat active.\textsuperscript{98, 99}

The loss of amide-water hydrogen bonding may be a result of the LCST, induced by the energetic costs of maintaining the liquid – vapor interface around hydrophobic groups of PNIPAm, rather than the driving force for LCST. Otake et al.\textsuperscript{100, 101} and Fujishige et al.\textsuperscript{102, 14} explain the collapse and aggregation of PNIPAm on hydrophobic interactions – indicating entropic effects are more prominent.

Molecular simulations allow a more idealized discussion through the ‘activation’ or ‘deactivation’ of forces, which is not possible under experimental conditions. This approach provides useful insights into the LCST behavior of PNIPAm in water.

\textbf{4.3.1. Simulation studies}

A simplistic but comprehensive view of transition behavior in hydrophobic polymer – water systems with attractive interactions has been presented by Athawale et al.\textsuperscript{103} We will use this simulation to explain PNIPAm LCST transition in water.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4_4.png}
\caption{LJ and WCA force fields used to describe attractive forces and lack of attraction respectively.\textsuperscript{104}}
\end{figure}
The simulation considers two polymer chains with different degrees of polymerization (short chain \( x = 25 \) vs. long chain \( x = 50 \)). Polymers simulated with Lennard-Jones (LJ) potentials are designed to mimic attractive forces (namely Van der Waals forces) and short range excluded volume effects. Polymers simulated with a Weeks-Chandler-Anderson force field (WCA) mimic the excluded volume effects with no long-range attractions (Figure 4.4).

The Gibbs free energy of folding, \( \Delta G_u \), is the energy difference of the collapsed state (collapsed state) and the swollen state (swollen state). A negative value of \( \Delta G_u \) indicates the swollen state the polymer is preferred.

Figure 4.5 shows computed temperature dependence of the Gibbs free energy for unfolding \( \Delta G_u \). As is expected, with or without Van der waals forces, the solvation of hydrophobic polymers at most temperatures is unfavorable for larger chain length polymers (\( x = 50 \)).

Smaller hydrophobic polymers interestingly show a parabolic trend in \( \Delta G_u \), with swollen states being favorable both at low and high temperatures. This trend mimics the closed miscibility – loop, showing both a LCST and UCST phenomena. Polymers simulated using WCA potentials (no attractions), show unfolding only at high temperatures i.e. a UCST. Therefore, we can establish that attractive polymer-water forces are responsible for polymer mixing at lower temperatures. In a purely hydrophobic polymer, the only attractive forces are Van der Waals
forces, and are relatively weak. However in PNIPAm, attractive forces (local hydrogen bonding) will be more dominant and the unfolded state will be more easily favored.

Molecular dynamics simulations of PNIPAm and other water-soluble polymers support the above mentioned outcome (Figure 4.6).\textsuperscript{106, 107} The residence rate $P_{\text{res}}(t)$ is defined as the probability that the water molecule exists at one region for a time interval $t$. Longer residence rates are an indication of hydrogen bonding between water and the functional group under observation. Regions around the amide linkages have larger residence rates due to hydrogen bonding effects when compared to hydrophobic regions. Attractive hydrogen bonding, an enthalpic effect stabilizes the unfolded polymer state.

The dissolution of a polymer in a solvent will increase the entropy of the system, clearly a favorable outcome for polymer dissolution. However, for polymer – water systems, there is another consideration. The entropic cost of maintaining the unfolded state in water i.e. local structuring effects. This effect is demonstrated in simulations by Athawale et al(Figure 4.7).\textsuperscript{103} $\Delta G_{\text{hyd}}$ is the contribution of water to the free energy of hydration $\Delta A_U$ for unfolding for varying polymer sizes. The colors denote chain length. Filled and unfilled circles represent results obtained from LJ and WCA force field applied polymers.\textsuperscript{103}
energy of unfolding $\Delta G_u$. $\Delta G_{\text{hyd}}$ contribution to polymer unfolding is large, positive, independent of the force fields applied. Three conclusions can be drawn from the simulation study pertaining to the LCST of PNIPAm.

Firstly, $\Delta G_{\text{hyd}}$ is identical and positive for both LJ and WCA force field polymers. This equivalence is expected because $\Delta G_{\text{hyd}}$ captures only the influence of water. The structuring effect should be qualitatively similar on the hydrophobic moieties of PNIPAm i.e. the hydrophobic backbone and isopropyl groups.

Secondly, $\Delta G_{\text{hyd}}$ shows a clear dependence on the polymer size i.e. larger polymers have a greater tendency towards collapse, driven by the hydration contribution to free energy. This conclusion suggests the LCST of PNIPAm should depend on molecular weight.

Finally, the hydration free energy profile may be reasonably represented by the change in surface area $\Delta A_u$ and the formation of a liquid – vapor like interface (due to broken hydrogen bonds) with monotonically decreasing surface tension $\gamma(T)$ (empirically predicted by Eőtvős relationship).\textsuperscript{108} This conclusion indicates that modification of the surface tension of bulk water will affect the hydration free energy and thereby can affect the LCST of PNIPAm.

The conclusions drawn from the simulation studies can be qualitatively verified by experimental results.

### 4.4. Effect of molecular weight on the LCST of PNIPAm

Until recently, a difficulty with systematic LCST studies of PNIPAm was polydispersity of the samples used to identify the LCST.\textsuperscript{109} Previous reports show no dependence of LCST on the
molecular weights. Later improvements in the polymerization techniques for PNIPAm involve controlled radical polymerization (CRP) to obtain reduced polydispersity.

Atom transfer radical polymerization (ATRP) of PNIPAm and corresponding cloud point measurement show PNIPAm LCST decreases with increasing molecular weights (Figure 4.8). Very small PNIPAm chains are easily dissolved in water. Increasing the molecular weight depresses the LCST. Similar results are seen in simulation studies performed by Deshmukh et al, who see no transition at 37 °C for PNIPAm chains up to ~ 1kDa, however observe the LCST at ~ 3 kDa.

This molecular weight dependence can be explained on the basis of hydration contributions to PNIPAm folding. Increasing the PNIPAm molecular weight correlates to an increase in the hydration contribution to polymer collapse, an entropic effect. This hydration contribution refers to the ordering of water effects in water. The hydration contribution therefore drives LCST to lower temperatures as the molecular weight of PNIPAm increases.

It must be noted that the LCST is independent of molecular weight after ~ 15 kDa. This effect may be a combination of two entropic effects, i.e. the increase in entropy due to the increasing molecular weights.
molecular weights, as predicted by Flory – Huggins theory, and the decrease in entropy due to structuring of water.

4.5. Effect of co-solutes on the LCST of PNIPAm

The Hofmeister series ranks the ability of various ions to precipitate proteins. The rankings are explained on the ability of anions to ‘make’ or ‘break’ water structure in the first hydration shell around co-solutes. The order for anions on the series is:

$\text{CO}_3^{2-} > \text{SO}_4^{2-} > \text{S}_2\text{O}_3^{2-} > \text{H}_2\text{PO}_4^- > \text{F}^- > \text{Cl}^- > \text{Br}^- > \text{NO}_3^- > \text{I}^- > \text{ClO}_4^- > \text{SCN}^-$

with $\text{CO}_3^{2-}$ being the most potent at precipitating proteins via the disruption of hydrogen bonds in water.

Figure 4.9 shows depression in LCST behavior of PNIPAm due to the addition of salts containing these anions in water. Evidently LCST of PNIPAm follows the Hofmeister series. Barring thiocyanates and iodides that show a slight increase of LCST at low concentrations, all other anions depress the LCST of PNIPAm.

Figure 4.9 LCST depression in PNIPAm-water system in the presence of anions. 

Figure 4.10 Surface tension dependence on concentration of anions and monovalent cations. 

64
The Hofmeister series can be explained on the basis of hydration contribution of water to PNIPAm folding. Since hydration contribution to folding directly depends on the surface tension at the interface of PNIPAm and water, its contribution to polymer folding will be affected due to changes in the liquid – vapor interface.

Figure 4.10 shows the effect of different anions on the surface tension of water.\textsuperscript{110} Anions in the Hofmeister series increase the surface tension of water solutions linearly with concentration, in the same ranking as the series itself. The increase in surface tension favors the collapsed state, thereby driving PNIPAm precipitation and decrease in LCST.

### 4.6. Effect of co-polymers on the LCST of PNIPAm

PNIPAm-copolymer systems can also be explained on a similar basis. Introducing a purely hydrophobic copolymer will reduce attractive unfolding interactions while simultaneously increasing hydration contribution. Subsequently PNIPAm-\textit{co}-hydrophobic polymers should show a depression in the LCST.

The complementary holds true for PNIPAm-\textit{co}-hydrophilic polymers. A hydrophilic component will reduce hydrophobic interactions while also increasing attractive unfolding potentials. Subsequently PNIPAm-\textit{co}-hydrophilic polymers should show an increase in the LCST.

Copolymer data collected from various sources is presented in Table 4.1.\textsuperscript{16, 111, 112} An notable exception to this trend is polyhydroxyethyl methacrylate (PHEMA) copolymers. It is likely that some balancing effects are in play during LCST of this copolymer, which warrants further investigation.
In light of the above discussions we can conclude that most experimental observations of the LCST behavior of PNIPAm in water can be explained qualitatively through a consideration of hydrophobic interactions (favoring folding) and attractive amide-water bonding (favoring unfolding). This argument can be extended to any polymer-water system.

### 4.7. Conclusions

PNIPAm has amide linkages that participate in hydrogen bonding with water. These bonds increase the stability of unfolded configurations at lower temperatures. This effect is primarily enthalpic in nature. Local structuring effects around the hydrophobic moieties drive the collapse transition – an entropic effect, which can be explained through the formation of a liquid – vapor like interface. Interplay of the enthalpic and entropic factors drives the LCST phenomenon. However since the hydration

<table>
<thead>
<tr>
<th>PNIPAm Copolymers</th>
<th>PNIPAm Molar ratio</th>
<th>LCST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>32.8</td>
</tr>
<tr>
<td></td>
<td>.978</td>
<td>34.4</td>
</tr>
<tr>
<td></td>
<td>.955</td>
<td>36.3</td>
</tr>
<tr>
<td></td>
<td>.934</td>
<td>38.0</td>
</tr>
<tr>
<td></td>
<td>.926</td>
<td>38.2</td>
</tr>
<tr>
<td></td>
<td>.919</td>
<td>39.0</td>
</tr>
<tr>
<td></td>
<td>.856</td>
<td>43.7</td>
</tr>
<tr>
<td>N-ethylacrylamide</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>74</td>
</tr>
<tr>
<td>N,N-dimethylacrylamide</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>32.8</td>
</tr>
<tr>
<td></td>
<td>0.893</td>
<td>36.9</td>
</tr>
<tr>
<td></td>
<td>0.853</td>
<td>38.1</td>
</tr>
<tr>
<td></td>
<td>0.827</td>
<td>39.8</td>
</tr>
<tr>
<td></td>
<td>0.788</td>
<td>41.1</td>
</tr>
<tr>
<td></td>
<td>0.686</td>
<td>46.0</td>
</tr>
<tr>
<td>N-propylacrylamide</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>28.3</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>26.3</td>
</tr>
<tr>
<td></td>
<td>0.15</td>
<td>25.5</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>24.5</td>
</tr>
<tr>
<td>Hydroxyethyl methacrylate</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>32.8</td>
</tr>
<tr>
<td></td>
<td>0.916</td>
<td>30.3</td>
</tr>
<tr>
<td></td>
<td>0.830</td>
<td>26.6</td>
</tr>
<tr>
<td></td>
<td>0.742</td>
<td>21.4</td>
</tr>
<tr>
<td></td>
<td>0.651</td>
<td>17.0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>NA</td>
</tr>
</tbody>
</table>
component to LCST is large, entropic considerations due to structuring of water is the major
driving factor and can explain most experimental observations.

Some notable exceptions however can be observed. For example co-polymers of hydrophilic
PHEMA with PNIPAm have lower LCST temperatures (Table 4.1). Also a slight elevation in the
LCST of PNIPAm (at low concentrations of thiocyanates and iodides) cannot be explained on
the basis of hydrophobic interactions. The discussions have not dealt with geometry of PNIPAm,
which is crucial to its observed transition temperature. Small changes to the PNIPAm structure,
i.e. replacing the isopropyl group with n-propyl groups can significantly alter the LCST
temperatures. Evidently subtle variations in monomer structure can affect polymeric behavior in
water. Similarly the effect of chain tacticity on LCST behavior has been ignored in experimental
studies. There is a need for more comprehensive experimental and theoretical studies to explain
to understand the influence of PNIPAm structure. Nonetheless understanding the influence of
hydration interactions provides a qualitative understanding for many of the phenomena observed.
5. Synthesis and control of PNIPAm thin-film morphology

5.1. Introduction

Thermally responsive substrates synthesized using poly (N-isopropylacrylamide) (PNIPAm) actuate the release of entire cell-sheets with intact extracellular matrix (ECM) and cell-cell junctions. Current understanding however, of the influence of polymer morphology on cell adhesion and release is incomplete.\textsuperscript{113} We intend to identify the physical basis for cell-adhesion and release from PNIPAm thin-films. Therefore, it is imperative to devise synthesis strategies that present the best control over the morphology of PNIPAm thin-films.

The simplest approach to synthesize polymeric thin-films is spin-coating. Spin coating yields thin, uniform and easily reproducible films on flat substrates and does not require expensive equipment. Nash et al. utilized spin-coating for facile synthesis of PNIPAm thin-films which demonstrated excellent cell-adhesion and release characteristics.\textsuperscript{114} Morphological evaluation of the synthesized PNIPAm thin-films was inadequate to assess the impact of film morphology on adhesion and release. The thickness and roughness of a spin-coated film depends on the quality of the solvent, polymer molecular weight and concentration.\textsuperscript{115} Also, the molecular weight of a PNIPAm used for film preparation will impact its LCST.\textsuperscript{81, 82} The above listed factors can complicate spin-coating of PNIPAm thin-films.

Spin coated films are not covalently bound to the underlying substrate. Therefore, after reducing the temperature below the LCST, spin-coated PNIPAm will be released from the substrates. The mechanism for metabolism of PNIPAm or other acrylamide based polymers under \textit{in vivo} conditions is unknown and the release of PNIPAm can result in unwanted complications. Grafted
PNIPAm films can avoid such complications. Therefore, spin-coating as an approach to PNIPAm film synthesis was discarded. Furthermore, only synthesis protocols that form covalent bonds with the underlying substrate were favored.

Physical polymerization strategies like plasma polymerization and e-beam polymerization are excellent approaches that facilitate large scale thin-film synthesis. Okano et al.’s original works for instance, utilize physical polymerization to synthesize PNIPAm films.\textsuperscript{12, 18, 116, 117} These strategies however can suffer from batch-based and instrument-based variability.

Rather than relying on a physical polymerization approach, surface-initiated polymerization schemes were developed to synthesize linear, end-grafted PNIPAm from silicon and glass substrates. Since end-grafted PNIPAm are bound only at the substrate, the transition behavior and corresponding conformational changes in PNIPAm were expected to be pronounced as compared to cross-linked films.

There are two approaches to immobilize end-grafted linear chain polymers on surfaces.\textsuperscript{118} One is the “grafting to” approach (attachment of end functionalized polymers to an activated surface) and the other is “grafting from” approach (surface initiated polymerization (SIP)).\textsuperscript{118} Well-defined polymers with narrow molecular weights can be attached to surfaces via the “grafting to” approach. However, areal graft density and film thickness are limited by the polymer structure and molecular weight. SIP involves immobilization of the polymerization initiator at the interface, resulting in high graft densities, thick polymer films and good control over polydispersity and topography. Therefore, SIP strategies were evaluated during the synthesis of PNIPAm thin-films.
The first was an immobilized chain transfer agent based approach. A chain transfer agent was coupled to 3-aminopropyltriethoxysilane (APTES) functionalized substrates to immobilize a thiol functionality. Standard free-radical polymerization of NIPAm in the presence of chain-transfer agent immobilized substrates results in PNIPAm thin-film growth at the substrate. PNIPAm films synthesized using the chain-transfer agent based approach showed reversible protein adsorption and release above and below the LCST respectively. However, the film thickness was limited (2 ± 1 nm).

The second approach utilized controlled radical polymerization, specifically atom transfer radical polymerization (ATRP). ATRP allows for excellent control over the polymer molecular weights. Expanding the synthesis protocol developed by Jones et al., 119 PNIPAm films between 0 – 150 nm were synthesized. Films prepared using ATRP also showed reversible protein adsorption and release.

ATRP offers flexibility and control over the polymerization, however, deoxygenation of the reaction mixture is crucial. The synthesis of PNIPAm thin-films on large surfaces (for example glass slides / tissue culture dishes) while maintaining a deoxygenated environment is cumbersome. Any contamination with oxygen deactivates copper (I) complexes and stops the polymerization.

To simplify the ATRP reaction scheme and adapt it for larger surfaces, the stringent de-oxygenation requirement was relaxed using “activators generated by electron transfer” (AGET) ATRP approach. 120 AGET ATRP utilizes stable Cu (II) complexes reduced to their active state (Cu (I)) using a reducing agent in solution. The reaction scheme mitigates the necessity of
complete de-oxygenation of the reaction mixture and storage of Cu (I) salts. PNIPAm film thickness was controlled between 0 – 155 nm using the AGET ATRP approach.

Finally, a graft density control protocol was developed to achieve independent control of PNIPAm graft density. The combination of graft density control and thickness control allowed the synthesis of model PNIPAm thin-films suitable for identifying the relationship between film structure and cell-adhesion and release.
5.2. Experimental section

5.2.1. Substrate preparation

Fisherbrand (Fisher scientific) polystyrene dishes were washed with water and isopropanol prior to e-beam polymerization. N-doped single side-polished 0.5 mm thick silicon wafers (1 0 0) (College Wafers Inc., Boston, MA) and Fisherfinest glass slides (Fisher scientific) were pre-cleaned with ethanol, acetone and chloroform. The wafers and glass slides were rinsed with deionized water and exposed to a piranha solution (3:1 (v/v) sulfuric acid (Pharmaco-AAPER) : hydrogen peroxide (Fisher) overnight. This procedure eliminated any organic contaminants from the wafers. The samples were removed from solution and rinsed with copious amounts of deionized water, dried under nitrogen.

5.2.2. Physical (e-beam) polymerization

E-beam induced polymerization of PNIPAm over polystyrene dishes was attempted at the e-beam laboratory at University of Dayton research institute (UDRI) according to the procedure employed by Okano et al.\textsuperscript{12} A 40 % (w/w) solution of NIPAm in isopropanol was spread over tissue culture polystyrene dishes. Starting with 0.17 MGy, the radiation dose was increased to 0.25 MGy.

5.2.3. Interfacial functionalization

Pretreated silicon wafers and glass slides were placed in a gently stirred solution of (N (3-triethoxysilylpropyl hydroxy-butyramide)) (from Gelest, Inc) (2.5 % v/v) (for hydroxyl group functionalization) or 3-aminopropyltriethoxysilane (APTES) (Sigma-Aldrich) (1 % v/v) (for amine group functionalization) in toluene (TEDIA) for two hours. The silane coupled substrates
were rinsed with ethanol, acetone and toluene. Finally, the functionalized substrates were cured at 120°C for one hour. Silane coupled substrates were stored in a dessicated environment and used within a week of functionalization.

5.2.4. **Chain transfer agent immobilization**

Mercaptopropionic acid was reacted to amine functionalized substrates in order to immobilize thiol functionality at the silicon wafer interface. Briefly, amine functionalized silicon wafers were placed in 0.2 mmol mercaptopropionic acid (Sigma-Aldrich), 0.25 mmol $N,N'$-Dicyclohexylcarbodiimide (DCC) (Sigma-Aldrich) and 0.05 mmol 4-Dimethylaminopyridine (DMAP) (Sigma-Aldrich) in 10 ml methylene chloride at 0 °C and left overnight at room temperature using procedure adapted from Neises et al.121 The thiol functionalized substrates were washed with toluene and acetone prior to polymerization. PNIPAm polymerization over the thiolated substrates was carried out using a 25 % (w / w) NIPAm (Sigma-Aldrich) and 20 mg / ml azobisisobutyronitrile (AIBN) (Sigma-Aldrich) solution in dioxane (Fisher) maintained at 60 °C for 8 hours. After polymerization, the substrates were cleaned using soxhlet extraction with isopropanol (Fisher) as the solvent for 24 hours.

5.2.5. **ATRP initiator immobilization**

A tertiary bromide group is a commonly used ATRP initiator. In order to facilitate surface-initiated ATRP polymerization of PNIPAm, the tertiary bromide group was immobilized on the silicon wafer substrates, following interfacial functionalization as in section 5.2.3. 0.835 ml (0.12 M) of triethylamine (Sigma-Aldrich) was added to 50 ml toluene and cooled to 0 °C. After cooling, 0.62 ml (0.1 M) of acid bromide (2-bromoisobutyrlbromide) (Sigma-Aldrich) was added drop-wise to the triethylamine solution and stirred. The amine / hydroxyl terminated silane-coated substrates were added to solution and stored at 0 °C for twelve hours. This reaction
ensures immobilization of the ATRP initiator. After initiator immobilization, the substrates were rinsed with two ethanol washes; acetone and chloroform wash and dried under nitrogen.

5.2.6. **ATRP polymerization of PNIPAm**

The ATRP polymerization procedure for PNIPAm was adapted from Jones et al.\textsuperscript{119} NIPAm (10 \% (w / v)) (Sigma-Aldrich) recrystallized from n-hexane and \(N, N', N'', N'''-pentamethyldiethylenetriamine\) (PMDETA) (0.0668 M) (Sigma-Aldrich) were dissolved in 50 \% (v / v), deionized water and methanol and then degassed by at least three freeze – thaw cycles. Cu (I) Br (0.0222 M) was added to the reaction solution in a degassed reaction flask via cannula transfer, stirred and finally transferred to tertiary bromine functionalized substrates in degassed vials. The polymerization was carried out at room temperature. PNIPAm film thickness was controlled by varying monomer concentration and polymerization time. The functionalized substrates were washed with copious amounts of water, ethanol, acetone and chloroform.

5.2.7. **AGET-ATRP polymerization of PNIPAm**

Deionized water was deoxygenated by bubbling nitrogen for at least one hour. In a typical AGET ATRP reaction, NIPAM (10 \% (w / v)) recrystallized from n-hexane (Sigma-Aldrich), 1,1,4,7,10,10-hexamethyltriethylenetetramine (HMTETA) (0.06 M) (Sigma-Aldrich) / \(N, N', N'', N'''-pentamethyldiethylenetriamine\) (PMDETA) (0.06 M) (Sigma-Aldrich), and Cu (II) Cl /Cu (II) Br (0.021 M) /were dissolved in deionized water. 0.0016 M ascorbic acid was added to the reaction mixture, stirred for five minutes and transferred to ATRP functionalized substrates placed in nitrogen flushed reaction containers. By controlling the reaction time, PNIPAm grafts of desirable thickness were generated. The PNIPAm functionalized substrates were washed with water, ethanol, acetone and chloroform through at least two cycles to remove any unreacted monomer which is toxic to cells.
5.2.8. Graft density control

In order to control graft density of the ATRP initiators, a dummy initiator, propionyl bromide (Sigma-aldrich) was utilized in conjunction with 2-bromoisoobutyrylbromide during immobilization of the ATRP initiator. The experimental procedure was previously described in section 5.2.5. First 0.835 ml (0.12 M) of triethylamine (Sigma-Aldrich) was added to 50 ml toluene and cooled to 0 °C. After cooling, 0.1 M of acid bromide mixtures (2-bromoisoobutyrylbromide and propionyl bromide mixed in ratios depending on the extent of separation required between the immobilized chains) was added drop wise to the triethylamine solution and stirred. The silane functionalized substrates were added to reaction mixture and stored at 0 °C for twelve hours. After initiator immobilization, the substrates were rinsed with two ethanol, acetone and chloroform washes and dried under nitrogen.

5.2.9. X-ray reflectivity

X-ray reflectivity (XRR) is a surface sensitive, non-destructive characterization technique wherein reflected X-ray intensities are analyzed in order to determine physical characteristics of ultra-thin films (0 – 1 μm). Layer structure, film thickness, scattering length density (SLD) and interfacial roughness can be obtained from a typical reflectivity scan and analysis. A limitation for the technique however is the necessity for ultra-smooth substrates. Silicon wafers are ideally suited to reflectivity analysis due to its low roughness (~ 5Å). XRR analysis was also found to work with untreated microscope glass-slides with larger interfacial roughness (interfacial layer thickness – 44 ± 3 Å, roughness 10 ± 7 Å). Initial parameters chosen during analysis depended on the choice of substrate.

The PANalytical X’pert Pro Materials Research Diffractometer at the Advanced Materials and Characterization Center (University of Cincinnati) was used to obtain XRR results. The x-ray
source (Cu Kα=1.542 Å) was operated at 1.8 kW (45 mV, 40 mA) and XRR analysis was carried out using PANanalytical X’Pert reflectivity v. 1.1.

The number of layers chosen during fitting was based on the immobilization protocol. Based on the model obtained, the SLD profile (SLD dependence on perpendicular distance from the interface) was generated using Irena Macros 2.38 for Igor Pro 6.17. The SLD profile relates to material composition and density, thereby, knowing the material composition, density can be accurately determined.

5.2.10. Ellipsometry

Ellipsometry was also used to characterize PNIPAm film thickness owing to the ease of data collection and fitting as compared to x-ray reflectivity. PNIPAm grafted substrates were analyzed using multiple wavelengths (300 – 900 nm in 4 nm steps) using the PhE-102 spectroscopic ellipsometer (Angstrom Advanced). The measured parameters are expressed as delta (Δ) and psi (φ). These parameters describe the change in polarization that occurs due to reflection of light from the substrate and the film. A model was developed using silicon wafer as the substrate, a native oxide layer and a Cauchy layer to describe the PNIPAm film. PNIPAm film thickness was obtained based on fitting the model to Δ and φ values obtained at multiple wavelengths.
5.3. Results and discussion

5.3.1. Physical (e-beam) induced polymerization of PNIPAm

The e-beam induced polymerization approach utilizes a high energy electron beam directed towards a monomer solution. The electron beam ionizes the target monomer thereby forming anions, cations, free radicals and other chemical species. Bond scission and bond formation simultaneously occur due to this ionization, driving polymer breakdown and cross-linking. Since the NIPAm monomer is spread over polystyrene dishes, bond scission and cross-linking occurs within the NIPAm film and the underlying polystyrene substrate. The polymerization of NIPAm in conjunction with cross-linking events at the NIPAm – polystyrene substrate interface during e-beam polymerization immobilizes PNIPAm thin-films.

We observed visible discoloration of the polystyrene dishes after exposure to the electron beam. This discoloration alluded to the partial degradation of polystyrene substrates, which is
undesirable. The contact angles for e-beam treated polystyrene dishes changed from $96 \pm 2^\circ$ to $65 \pm 6^\circ$ (vs. water) indicating the interfacial structure is modified.

In addition to discoloration, gelation of the NIPAm film was also observed, indicating successful polymerization of NIPAm. However, the immobilization of PNIPAm at the polystyrene interface could not be confirmed. IR spectra showed the evolution of a peak at 1650 cm$^{-1}$ indicative of amide I (C = O) stretching (Figure 5.1). Corresponding amide II and isopropyl doublet indicative of PNIPAm however, could not be observed.

Although e-beam polymerization is convenient for thin-film formation, it is affected by batch-based variability, a disadvantage acknowledged by the inventors of cell-sheet engineering themselves.$^{123}$ Also due to the nature of ionizing radiation, cross-linking in PNIPAm is expected. Clustering of cross-linked regions in the PNIPAm film can occur during swelling and affect both swelling behavior and surface morphology.$^{124}$ Physical polymerization schemes may also be surface dependent.$^{113}$ Our collaborator - Dr. Yi-Gang Wang’s group at the cardiovascular center (CVC) at UC also attempted e-beam induced polymerization to synthesize PNIPAm films. However our efforts and our collaborators efforts did not yield thermo-responsive films that release cells from the underlying substrates.

Nonetheless, tissue culture dishes modified by PNIPAm using e-beam polymerization means are commercially available. These dishes have small $5 \times 5$ mm sections, separated by hydrophilic boundaries where cell-adhesion cannot occur. Pilot cell-adhesion experiments were performed on commercially available PNIPAm treated polystyrene dishes.
Following tissue culture of rat mesenchymal stem cells (MSCs) to the substrate above the LCST (37 °C), a cell-sheet is formed within each of these sections. After reducing the temperature below the LCST (20 °C), release of cells is also observed (Figure 5.2). The release is not spontaneous, starting from the edges of the cell-sheet at the hydrophilic boundaries, gradually progressing inward. Furthermore, the release is slow, with complete release of a 5 × 5 mm cell-sheet occurring in around 20 - 30 minutes. The small size of the released cell-sheets however places a limit on the applicability of these commercial dishes.

5.3.2. Free-radical polymerization of PNIPAm

Working with Dale Huber at Sandia National laboratories, a chain transfer-agent based scheme\textsuperscript{13} was used to synthesize PNIPAm grafts. A thiol functionality was introduced on the substrate (glass or Si wafer) using either 3-mercaptopropyltriethoxysilane or by the reaction of mercaptopropionic acid to an APTES thin-film via $N, N'$-dicyclohexyl-carbodiimide (DCC) (Figure 5.3).

Figure 5.2 Cell-culture on commercially available PNIPAm grafted tissue culture polystyrene dishes. The red arrow points to the initiation of cell-release.

Figure 5.3 Schematic for chain-transfer agent based PNIPAm thin-film synthesis.
Thiol groups are typically used as chain transfer agents. During free radical polymerization, the hydrogen is transferred forming a sulfur-atom radical as shown in Eq 5.1.

\[ R_{Mn} \cdot + SH \rightarrow R_{Mn}H + S \cdot \]  

Eq 5.1

Free-radical polymerization occurs from the surface attached free-radical, forming a PNIPAm thin film. IR spectra confirmed successful polymerization of PNIPAm over thiolated substrates (Figure 5.4). Radical – radical coupling events can also occur attaching polymer chains from solution directly to the substrate.

The reaction conditions however result in limited film growth, possibly due to early termination events (2 ± 1 nm confirmed using ellipsometry and XRR). Our results matched closely with results previously published by Yim et al.\textsuperscript{125}

Protein adhesion studies were carried out using red-dye-conjugated bovine serum albumin (BSA) (Figure 5.5 and Figure 5.6). BSA adsorption studies using fluorescence microscopy showed that while the protein adhered to PNIPAm-grafted substrates from a 37°C solution, a rinse in room temperature water (~20 °C) releases the protein.
PNIPAm films synthesized on glass dishes using the chain transfer agent based synthesis protocol however were ineffective in releasing mesenchymal stem cells (MSCs) (Figure 5.7). Good adhesion of cells was observed above the LCST (37 °C). However, after reducing the temperature (20 °C), the cells remained firmly attached to the substrate. Efforts to release the cells such as gently agitating the buffer solution or inducing a tear at the substrate were also ineffective in inducing cell-release. Since the PNIPAm films synthesized using this approach are very thin (~ 2 nm) and the film thickness is self-limiting, no further cell-adhesion tests were performed.

Another disadvantage to free-radical polymerization approach is the need for extensive washing of the substrates to remove unbound polymers. Typically at least a 24-h soxhlet wash of
isopropanol is needed to ensure free-standing PNIPAm is washed from the substrate. We intended to circumvent these shortcomings using controlled radical polymerization schemes.

### 5.3.3. ATRP of PNIPAm

ATRP is a controlled radical polymerization technique that utilizes electron transfer processes in transition metal complexes, usually copper, to initiate and propagate polymerization under equilibrium conditions (Figure 5.8).

\[
\text{R-X} + \text{Cu (I) Y/Ligand} \leftrightarrow \text{R} \cdot + \text{Cu (II) XY/Ligand}
\]

Figure 5.8 Schematic showing the mechanism of an ATRP reaction. X and Y in this representation are halogens. M is the monomer.

The livingness of an ATRP reaction is achieved by maintaining a low concentration of active, transient free radicals. This effect is called the persistent radical effect (PRE). After the first few monomeric additions and terminations, terminations are reduced substantially. By reducing the concentration of radicals, loss of control due to termination by recombination events that occur during free radical polymerization are limited.

A two-step procedure was devised to synthesize well-defined PNIPAm thin-films from glass and silicon wafer substrates. In the first step, a tertiary bromine group was introduced on the substrate. Following pre-treatment either APTES or hydroxyl terminated silane (N-(3-triethoxysilylpropyl)-4-hydroxybutyramide) was introduced at the interface to synthesize robust amine / hydroxyl terminated substrates. The functionalized substrates were reacted with 2-bromoisobutylbromide. This results in surface immobilization of a tertiary bromide group, an ATRP initiator.
PNIPAm growth kinetics using hydroxyl functionalized substrates are shown in Figure 5.10 and Figure 5.10. The growth kinetics are non-ideal, exhibiting rapid initial growth in 30 minutes followed by stagnation. In the ideal scenario, linear growth kinetics are expected when good control over the polymerization is achieved, assuming thickness correlates linearly with the molecular weight. However, since water is used as the solvent, acceleration of polymerization results in rapid film growth at the cost of polymerization control.127

A direct relationship was found between the monomer concentration and PNIPAm film thickness. Utilizing this aspect, varying monomer concentration and stopping the polymerization within 10 minutes, up to 155 nm thick PNIPAm films were prepared (Figure 5.10).

While ATRP offers flexibility and control over the polymerization, de-oxygenation of the reaction mixture is crucial. The synthesis of PNIPAm thin-films on large surfaces (for example glass slides) is cumbersome under deoxygenated conditions. Any contamination with oxygen deactivates copper (I) complexes and stops the polymerization. To simplify the ATRP reaction
scheme and adapt it for larger surfaces required for tissue culture studies, the stringent
deoxygenation requirement was relaxed using “activators generated by electron transfer”
(AGET) ATRP approach.\textsuperscript{120}

**5.3.4. AGET ATRP of PNIPAm**

AGET ATRP utilizes stable Cu (II) complexes reduced to their active state (Cu (I)) using a
reducing agent in solution. Ascorbic acid was used as the reducing agent and water as the solvent
during reaction to allow for accelerated PNIPAm synthesis.\textsuperscript{127, 128} The reaction scheme is shown
in Figure 5.11.

![Reaction scheme](image)

Figure 5.11 Schematic for synthesis of surface initiated PNIPAm thin-films over hydroxylated substrates.

Figure 5.12 shows the infrared spectra collected post-polymerization and cleaning of a PNIPAm
grafted silicon wafer. Characteristic N-H bond vibration is observed at 3297 cm\(^{-1}\) while amide I
and amide II vibrations are seen at 1658 and 1540 cm\(^{-1}\). In addition the isopropyl doublet is seen
at 1385 and 1365 cm\(^{-1}\) respectively confirming AGET ATRP polymerization of PNIPAm
immobilization from the silicon wafer substrate.
Two Cu (II) / ligand systems were evaluated during AGET ATRP synthesis of PNIPAm. The results of varying ascorbic acid concentration while using $N,N,N',N',N''$-pentamethyldiethylenetriamine (PMDETA), CuBr$_2$ are shown in Figure 5.13. Corresponding results using 1,1,4,7,10,10-Hexamethyltriethylenetetramine (HMTETA), CuCl$_2$ are shown in Figure 5.14.

The PMDETA - CuBr$_2$ metal – ligand combination results in rapid film growth followed by termination of growth in under 10 minutes, independent of ascorbic acid content. In comparison, the HMTETA – CuCl$_2$ system provided better control.

At higher ascorbic acid concentration (3.2 mM) and therefore greater initiator concentration, polymerization was rapid with thick ~140 nm film formation in under 30 minutes, followed by termination of growth. By reducing ascorbic acid content (1.6 mM), pseudo-linear PNIPAm film growth was observed in the first hour of polymerization, following which the polymer thickness levels out to a maximum of 79 nm (Figure 5.14). The loss of control in PNIPAm synthesis is likely due to possible termination events. However, for our purposes the AGET ATRP synthesis approach gave reasonable control with rapid growth kinetics.

The effect of the underlying amine/hydroxyl terminated silane on PNIPAm film thickness was also evaluated (data not shown). Under similar polymerization conditions, PNIPAm synthesized...
using the hydroxyl-terminated silane as the interfacial layer resulted in a five-fold thicker film as compared to amine-functionalized surfaces. Potentially, the hydroxyl-terminated silane present more accessible functional groups as compared to APTES and thereby results in PNIPAm films with higher graft densities. The differences in polymer growth may also occur due to differences in amide vs. ester radical stability. The significantly improved thickness-range prompted the choice of hydroxyl silane over APTES for all ATRP polymerization and cell-adhesion studies.

![Graph showing polymerization over time for different ascorbic acid concentrations](image1)

Figure 5.13 Effect of varying ascorbic acid content, while using PMDETA as the ligand. Poor control of polymerization is observed, with rapid termination in under 10 minutes of polymerization.

![Graph showing polymerization over time for different ascorbic acid concentrations](image2)

Figure 5.14 Effect of varying ascorbic acid content while using HMTETA as the ligand. At higher ascorbic acid content, rapid termination is observed. However better control over PNIPAm polymerization was obtained by controlling the ascorbic acid content. The ideal reaction conditions that allowed synthesis of PNIPAm films from 0 – 80 nm (ascorbic acid content 1.6 mM) was chosen to prepare substrates for tissue cultures.

5.3.5. **Molecular weight and graft density control**

The thickness of any end-grafted linear polymer film is dependent on its molecular weight and grafting density at the substrate. Since we have used surface initiated polymerization to synthesize PNIPAm thin-films, a high grafting density of PNIPAm is expected. End-grafted polymer chains fall in the “brush” regime, if the film height exceeds polymer end – end distance.
For polymer chains in the brush regime, the film-thickness (as determined by ellipsometry and XRR) relates directly to its molecular weight and graft density as shown in Eq 5.2.\textsuperscript{129}

\[
\sigma = \frac{dL_dN_A}{M_n}
\]

Eq 5.2

Here \(\sigma\) is the grafting density, \(d\) is the density of the PNIPAm graft, \(L_d\) is the thickness of the graft layer, \(N_A\) is Avogadro’s number and \(M_n\) is the molecular weight of the polymer chains.\textsuperscript{130}

From Eq 5.2, we can infer that at a constant graft density, the film thickness is directly proportional to its molecular weight.

In order to quantify the molecular weight of surface-grafted PNIPAm, the polymer was synthesized from silica nanoparticles. Small particle sizes present a large surface area, allowing for the cleavage and extraction of large amounts of the polymer. The molecular weight was obtained from PNIPAm films synthesized on 3 – 5 nm Cab-o-Sil. For \(~60\) nm film (measured from a flat surface), the molecular weight was determined to be 73.4 kDa, with a polydispersity of 1.34. The calculated graft density on the basis of this calculation (assuming PNIPAm film density is \(1\text{ g / cm}^3\)) is 0.49 chains / \(\text{nm}^2\). This estimated value is very close to graft densities reported for polymer brushes synthesized using the ATRP approach.
However there is some doubt on the validity of using very small nanoparticles to approximate flat surfaces for molecular weight determination.\textsuperscript{131, 132} Smaller particles have a large curvature. Therefore, as the polymer film grows, the net effect is a systemic decrease in the effective graft density and can substantially affect the molecular weight during synthesis (Figure 5.15). Dan et al. have indicated that in order to approximate a planar surface, the nanoparticle radius to brush thickness ratio must be greater than ten.\textsuperscript{133} A minimum radius of 150 nm is necessary to approximate 15 nm polymer growth from flat substrates.

Figure 5.15 Loss in effective graft density due to surface initiated PNIPAm growth from small nanoparticles. (The black dots represent ATRP initiators. As the polymerization proceeds the spacing between individual ATRP initiators increases and the corresponding grafting density decreases.

Figure 5.16 SEM image showing 321 nm silica nanoparticles synthesized using the Ströber process.

Figure 5.17 SEM image showing the spherical shape and smooth interfaces of the synthesized silica nanoparticles.
In order to determine accurate molecular weight and graft density, PNIPAm was also synthesized from larger size scale particles. Silica nanoparticles were synthesized using the Ströber process using conditions reported by Bogush et al. and Razink et al.\textsuperscript{134,135}

Particles synthesized by this technique were spherical and showed a dense smooth interface (Figure 5.16 and Figure 5.17). The particle diameter obtained was 321 nm (determined using particle size analysis from SEM data).

Thermogravimetric analysis of the silica nanoparticles after ATRP initiator functionalization and subsequent polymerization is shown in Figure 5.18. Roughly 18% of the nanoparticle weight is due to the PNIPAm thin-film synthesis. On the basis of the known particle size and weight loss, the thickness of a dry PNIPAm film can be calculated. This relates to a PNIPAm film thickness of 18 nm.

The molecular weight of PNIPAm was determined using gel permeation chromatography after cleaving the polymer from the nanoparticles. The molecular weight determined using this approach was 10.36 kDa, with a polydispersity of 1.37. Using Eq 5.2, this relates to an unusually high PNIPAm graft density of 1.04 chains / nm$^2$. Typically ATRP results in a chain density of 0.5 – 0.7 chains / nm$^2$.\textsuperscript{4,136,137} The value obtained is significantly higher than typically reported. A more systematic study of the influence of PNIPAm growth kinetics on the graft density may be used in the future to validate the graft densities.
5.3.6. Graft density control

A unique system was developed to allow for precise control of the graft density. Mixture of silanes can result in a non-linear density variations or phase separation as seen in Figure 5.19),\textsuperscript{4} Instead we used a graft density variation scheme involving reacting the hydroxyl silane monolayer with a mixture of active ATRP initiator (bromo isobutyrlbromide) and inactive (propionyl bromide) initiator. Since the reactivity of both acid bromides is almost identical with the hydroxyl terminated silane, the graft density is expected to vary linearly with initiator concentration.

Using contact angles, graft density control of the ATRP initiator was assessed. Cassie's law (Eq 5.3) is used to determine the effective contact angle for a liquid on a multicomponent surface:\textsuperscript{138}

\[
\cos \theta_e = f_1 \cos \theta_1 + f_2 \cos \theta_2
\]

Eq 5.3
Where $\theta_c$ is the effective contact angle $\theta_1$, $\theta_2$ are contact angles of pure components and $f_1, f_2$ are the fraction of surface area covered by components 1 and 2 respectively.

A linear trend in $\cos \theta_c$ variation is expected for perfect mixing in a two-component system.\(^4\) Contact angle measurements of a water droplet confirm linear control of grafting density based on the dummy acid bromide approach (Figure 5.20).

Linear control of the graft density implies that for constant polymerization conditions, the graft density is directly proportional to the film thickness (Eq 5.2). Following 15-minute PNIPAm polymerization, linear thickness variation is observed as the graft density is varied (Figure 5.21). Within experimental error, reducing the graft density by half results in a thickness decrease by half, consistent with Eq 5.2. This result also validates the brush regime assumption for PNIPAm thin-film synthesis.

In order to facilitate PNIPAm thin-film morphological identification, a nomenclature was introduced. The maximum grafting density (100 % initiator fraction) was identified as ‘1GD’
whereas the molecular weight of the film after 15 min of polymerization (using 1.6 mM conditions) was identified as ‘1MW’. Using the term ‘1GD1MW’ describes the molecular weight of PNIPAm after 15 min of polymerization from a 100 % initiator fraction functionalized surface and resulting in the synthesis of a 18 ± 4 nm film. Similarly, ‘0.2GD3MW’ indicates PNIPAm polymerization from a 20 % ATRP initiator fraction immobilized on the substrate, resulting in a molecular weight three times that observed from a 1GD1MW polymerized surface. The molecular weights for each individual sample were estimated from the film thickness, using Eq 5.2. This system of PNIPAm film identification allows for facile identification of thin-film morphology and is used repeatedly in chapter 6 while identifying cell adhesion and release characteristics.

5.3.7. Influence of substrate on PNIPAm thin-film structure

PNIPAm thin-films were synthesized over both silicon wafers and microscope glass slides using the AGET ATRP approach. Silicon wafers are smooth and give high intensities of reflected x-rays and neutrons during XRR and neutron reflectivity analysis. Microscope glass slides, while not as smooth, allowed the use of optical light microscopy facilities available at Dr. Yi-Gang Wang’s laboratory at the University of Cincinnati.

The interface of silicon has a native oxide layer similar in structure to glass. However soda-lime glass typically is composed of a number of other metal oxides, and may change the interfacial structure, thereby affecting silane deposition and PNIPAm structure. XRR was used to confirm the effect of the substrate on PNIPAm layer structure.

From previous experience with silicon wafers,68 the silicon substrate roughness was allowed to vary between 3 – 7 Å during fitting. We set the native oxide layer density at 2.2 g / cm³. To
account for substrate variability, the native oxide layer thickness was allowed to vary slightly (13 ± 3 Å).

Although reported to show low interfacial roughness (~5 Å),\textsuperscript{139} Fisherfinest premium glass interfacial structure determined by XRR showed single layer structure (less dense glass with lower SLD) and a roughened interface. XRR data with best fits and corresponding SLD profile are shown in Figure 5.22 and Figure 5.23 respectively.

Assuming microscope glass composition is soda-lime, the density of the substrate layer (SLD ~ 20.8 ×10\textsuperscript{-5} Å\textsuperscript{-2}) determined by XRR is 2.47 g / cm\textsuperscript{3} and interfacial roughness was determined to be 17 ± 3 Å. The interfacial less dense glass layer thickness, density and roughness were determined to be 44 ± 3 Å, 2.11 ± 0.1 g / cm\textsuperscript{3} and 10 ± 7 Å respectively based on fitting from multiple samples.

![Figure 5.22 XRR profile (dots) and corresponding best-fit (solid line) for a representative pre-cleaned Fisherfinest premium glass slide.](image1)

![Figure 5.23 SLD profile for a representative Fisherfinest premium glass slide from fitting shown in Figure 4. The dotted lines signify the layered structure utilized during fitting.](image2)
In order to confirm the structure of PNIPAm synthesized over both silicon and glass, the XRR
determined SLD profiles were compared. Figure 5.24 and Figure 5.25 show XRR data and
 Corresponding SLD profiles for a (~150 Å) PNIPAm film synthesized on different substrates.
PNIPAm layer structural information obtained from the SLD profile is presented in Table 5.1.

Table 5.1. PNIPAm film thickness over silicon and glass under similar reaction conditions.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Thickness (Å)</th>
<th>Density (g / cm³)</th>
<th>Roughness (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silicon</td>
<td>168</td>
<td>0.98</td>
<td>5</td>
</tr>
<tr>
<td>Glass</td>
<td>132</td>
<td>0.94</td>
<td>7</td>
</tr>
</tbody>
</table>

Minor differences were observed in PNIPAm layer density and roughness. However these fall
within experimental errors. Reflectivity analysis confirms PNIPAm thin-film structure is
identical irrespective of the substrate used.
5.4. Conclusions

Multiple thin-film synthesis techniques were evaluated to synthesize model PNIPAm thin-films. Both chemical and physical means of polymerization were explored. Of the techniques developed, surface initiated polymerization using the AGET ATRP approach showed the most promise. PNIPAm thin-films with varying thickness (0 – 155 nm) and reasonable control over the polymerization rate could be synthesized in a facile manner using AGET ATRP. Also, AGET ATRP allowed for high chain densities (~1.04 chains / nm$^2$) Therefore, all systematic cell-adhesion and release experiments were carried out on substrates synthesized using the AGET ATRP protocol.
6. Understanding cell adhesion and release from PNIPAm grafted substrates

6.1. Introduction

Thermally responsive substrates synthesized using poly (N-isopropylacrylamide) (PNIPAm) actuate the release of entire cell-sheets with intact (extracellular matrix) ECM’s and cell-cell junctions.\textsuperscript{12} Cell-release below the LCST has been proposed to occur in two steps.\textsuperscript{18} Initially, passive detachment occurs due to the conformational changes and penetration of water in PNIPAm films below the LCST. This step is followed by active-release, wherein metabolic changes within the cell induce detachment. Evidence supports the importance of metabolic responses to cell-release.\textsuperscript{140, 141} Little is known however regarding the influence of polymer morphology on cell adhesion and release.\textsuperscript{113}

A number of articles report a relation between the amount of grafted PNIPAm and release properties.\textsuperscript{12, 19, 20, 116, 123} In general, three regimes of tissue responses have been reported. A small amount of PNIPAm supports tissue growth, but no release is observed. At significantly larger amounts of PNIPAm, no tissue growth occurs. Tissue growth and release occurs between the two extremes, typically for PNIPAm coverage the range of 1.4 – 2.0 $\mu$g/cm\textsuperscript{2}. The amount of PNIPAm, however, is insufficient as a variable to characterize thin-film morphology. For instance cross-linked and end-grafted PNIPAm may present the same amount of polymer. However release characteristics are expected to be significantly different. The swelling behavior of cross-linked polymers depends on the extent of cross-linking\textsuperscript{142} whereas swelling of end-grafted linear polymers depends on graft density and molecular weight.\textsuperscript{143}
Cell-adhesion and release behavior has also been reported to depend on PNIPAm film thickness. However, there is inconsistency regarding optimum thin-film thickness for cell-adhesion and release. Mizutani et al. showed that maximum cell-adhesion and corresponding release occurs from very thin (~ 2 nm) PNIPAm films.\textsuperscript{123} This result is partially supported by Linhui et al., although a range of optimal cell-adhesion and release is observed (~20 – 45 nm).\textsuperscript{144} Increasing PNIPAm thickness discourages cell-adhesion, even completely preventing any cell-adhesion.\textsuperscript{117} Xu et al. however contest these results, suggesting increasing polymer thickness improves cell adhesion.\textsuperscript{145} This ability of thicker PNIPAm films to inhibit cell-adhesion has been attributed to increased hydration at the water – polymer interface. Here we characterize film morphology using X-ray and neutron reflectivity (XRR and NR) to establish the physical basis for cell adhesion and release.

In order to systematically study the influence of PNIPAm thin-film morphology on cell-adhesion and release, the activators generated by electron transfer atom transfer radical polymerization (AGET ATRP) approach was utilized. As discussed in section 5.2.7, the AGET ATRP approach facilitates independent graft density and molecular weight control in PNIPAm thin-films. Cell-adhesion and release characteristics of two cell lines - human bone marrow stromal cells (hBMSCs) and rat endothelial cells were correlated to PNIPAm film morphologies synthesized using the AGET ATRP approach.

Neutron reflectivity (NR) was used to determine the effect of varying graft density and molecular weight on cell-adhesion and release behavior. On the basis of NR results, PNIPAm films with high graft densities (1 GD, 1.04 chains / nm\textsuperscript{2}) were expected to inhibit cell-adhesion due to partial swelling of the film at 37\textdegree C. Tissue cultures are incubated at this temperature and partial
swelling prevents cell-adhesion and proliferation. Lower graft densities on the other hand were expected to encourage cell-adhesion.

Our cell-adhesion results agree well with NR observations. At higher graft densities (1GD, ~1.04 chains / nm²), low-molecular-weight, thin (~10 kDa, 18 ± 4 nm) PNIPAm films are more suitable for cell-adhesion as compared to high-molecular-weight, thick films (~29kDa, 50 ± 4 nm) films. This result is attributed to partial hydration in PNIPAm films above the LCST and confirmed using NR. Specifically, when PNIPAm thin-films show less than 32 vol % water penetration, cell adhesion is observed. At or above 39 vol % water penetration, cell adhesion is inhibited.

This research is the first comprehensive work exploring structure – property relationships for multiple cell-lines, PNIPAm grafting densities and molecular weights. From the tissue cultures and NR analysis, some generalizations can be made regarding the influence of PNIPAm molecular weight and graft density on cell-adhesion and release. Cell-adhesion to PNIPAm grafted substrates is inversely proportional to its molecular weight, with lower molecular weight films encouraging cell adhesion, and higher molecular weight films inhibiting cell-adhesion and growth. Cell-release from PNIPAm grafted substrates on the other hand, is directly proportional to the graft density. Cell-release is inhibited at lower graft densities, whereas increasing PNIPAm graft density accelerates cell-release. The generalizations obtained from this work can be used to design tailored responsive substrates in order to optimize specific responses.
6.2. Experimental section

6.2.1. Synthesis of PNIPAm thin-films using AGET ATRP

PNIPAm thin-films were synthesized on silicon wafers and glass slides using the protocols established in section 5.2.7. Following thin-film synthesis the substrates were washed and stored in a desiccator. Prior to cell culture experiments, the substrates were placed in tissue culture polystyrene dishes and flushed for a minimum six times using a 70 : 30 (v/v) Ethanol / water mixture and dried.

6.2.2. X-ray reflectivity

X-ray reflectivity (XRR) is a surface sensitive, non-destructive characterization technique wherein reflected X-ray intensities are analyzed in order to determine physical characteristics of ultra-thin films (0 – 1 μm). Layer structure, film thickness, scattering length density (SLD) and interfacial roughness can be obtained from a typical reflectivity scan and analysis. A limitation for the technique however is the necessity for smooth substrates. Silicon wafers are ideally suited to reflectivity analysis due to its low roughness (~ 5Å). XRR analysis was also found to work with untreated microscope glass-slides with larger interfacial roughness.

The PANalytical X’pert Pro Materials Research Diffractometer at the Advanced Materials and Characterization Center (University of Cincinnati) was used to obtain XRR results. The x-ray source (Cu Kα=1.542 Å) was operated at 1.8 kW (45 mV, 40 mA) and XRR analysis was carried out using PANalytical X’Pert reflectivity software v. 1.1.

The number of layers chosen during fitting was based on the immobilization protocol. Based on the model chosen, the SLD profile (SLD dependence on perpendicular distance from the interface) was generated using Irena Macros 2.38 for Igor Pro 6.17. The SLD profile obtained
reflects the material composition and density. Knowing the material composition, the film density profile normal to the substrate can be determined.

6.2.3. Neutron reflectivity

Neutron reflectivity (NR) on end-grafted PNIPAm thin-films was performed at Surface profile analysis reflectometer (SPEAR) at the Lujan Scattering Center, Los Alamos National Laboratory, Los Alamos, NM and the Liquids Reflectometer (LR) at the Spallation Neutron Source (SNS), Oak ridge national Laboratory (ORNL), Oak Ridge, TN. Both instruments use a time-of-flight methods.

Both dry and in situ measurements were obtained using the NR instruments. For the in situ measurements, a liquid cell was placed on a temperature-controlled stage. By varying the temperature of the stage, the temperature in the cell could be maintained precisely. The evolution in PNIPAm thin-film structure was determined under in situ conditions using deuterated water and deuterated phosphate buffered solutions (pH = 7.4 ± 0.1). A deuterated solvent provides excellent contrast to hydrogenated PNIPAm and allows for facile observation of the penetration of water in the film. Following acquisition of NR data, subsequent reduction was performed using Irena Macros 2.38 for Igor Pro 6.10. The reflectivity tool in Irena inverts the reflectivity data (reflectivity vs. wave vector, q) to provide the real space profile of the scattering length density (SLD), from which we determine the layer thickness, mass density and interfacial roughness for multiple layers.

6.2.4. Cell cultures

Rat endothelial cells (provided by Dr. Yi-Gang Wang’s laboratory, obtained from seed cultures using 0.25 % trypsin) were seeded on the center of sterilized PNIPAm substrates using DME/F
supplemented with 10% fetal bovine serum (FBS) at a concentration of roughly, $10^4$ cells / cm$^2$ and 150 µl volume. The glass slides were placed in tissue culture polystyrene dishes and incubated at 37 °C for four hours in a humidified atmosphere with 5% CO$_2$. Following seeding, 3 ml of pre-warmed DME/F supplemented with 10% FBS was added to the tissue culture dishes and the tissue culture dishes were incubated for a week at 37 °C.

For the cell-release studies, the serum medium was replaced with dulbecco PBS solution (maintained at room temperature). The total areal coverage of cells was tracked using optical microscopy at varying time intervals. A minimum of three measurements were obtained per time point from random regions on the PNIPAm functionalized substrates to obtain statistics for determination of standard deviation.

Human bone marrow stromal cells (hBMSC’s) were incubated on PNIPAm functionalized substrates in a similar manner. The cells however were seeded at $5 \times 10^3$ cells / cm$^2$.

### 6.2.5. Statistical analysis

The area of PNIPAm functionalized substrates covered with cells was computed using the image processing software – *ImageJ* 1.46r.\textsuperscript{146} Utilizing the convolution, edge finding, smoothing, image thresholds and analyze functions available in *ImageJ*, the total surface coverage of cells on the obtained images was determined. Since three images were obtained per time point during cell-release studies, the standard deviation in areal coverage with cells could also be determined.
6.3. Results and Discussion

6.3.1. Effect of PNIPAm thin-film morphology on LCST behavior

Precise control over PNIPAm thin-film morphology was obtained through graft density and molecular weight control, as discussed in section 5.2.7. However, the influence of graft density and molecular weight variation on the collapse transition of PNIPAm is unclear. Understanding changes in the collapse transition, structure – property relationships can be established between thin-film morphology and cell-adhesion and release characteristics.

Neutron reflectivity (NR) measures thin-film structure under deuterated solvents in a temperature-controlled liquid cell. Therefore, NR was used to assess the effect of changing graft density and molecular weights on the PNIPAm collapse transition.

Our original hypothesis was that maximizing conformational changes within the PNIPAm film i.e. a maximum increase in film thickness / uptake of water below the LCST would result in accelerated, potentially instantaneous cell-release (as compared to 30 – 120 minute release times). This expectation was influenced by Yim et al.’s work, which showed maximum conformational changes occurs at lower graft densities (~0.06 chains / nm²) and relatively high molecular weights (~150 kDa).

We analyzed neutron reflectivity from PNIPAm films synthesized at two conditions - a moderate graft density (‘0.5GD’, 0.5 chains / nm²), moderate molecular weight condition (~72 kDa) (Figure 6.1) and compared the same to low-density (‘0.2GD’, 0.2 chains/nm²) and high molecular weight PNIPAm films (~120 kDa) (Figure 6.2).

Some important morphological observations and their expected influences on cell-adhesion and release are presented below.
1. At elevated temperatures, PNIPAm thin-films are collapsed and present a sharp, hydrophobic interface with water, irrespective of grafting density. Hydrophobic interfaces encourage non-specific protein adhesion and can thereby promote cell adhesion. Reducing the temperature below the LCST causes two distinct changes in PNIPAm. First, the sharp interface becomes diffuse due to water penetration. Secondly, the PNIPAm film swells considerably, resulting in increased film thickness and reduced volume of the polymer. Water penetration

Figure 6.1 (top) Neutron reflectivity raw data (colored dots) and best fits (solid lines) for intermediate graft density PNIPAm thin-films swollen under D$_2$O at varying temperatures. (bottom) Volume fraction profiles of PNIPAm-thin films from the interface.

Figure 6.2 (top) Neutron reflectivity raw data (colored dots) and best fits (solid lines) for low graft density PNIPAm thin-films swollen under D$_2$O at varying temperatures. (bottom) Volume fraction profiles of PNIPAm-thin films from the interface.
also reduces the hydrophobicity of the PNIPAm graft, thus reducing the driving force cell-adhesion. The increase in thickness in conjunction with water swelling provides the necessary driving force for cell-release.

2. Changing the graft density had a substantial impact on PNIPAm swelling characteristics. Increases in graft density relate to denser packing of the PNIPAm chains. As the temperature is reduced below the LCST (33.5 and 30 °C), due to the closely packed structure, swelling is only seen near the water – polymer interface. Closer to the substrate the LCST transition does not occur at the observed temperatures (Figure 6.1). This behavior indicates vertical phase separation within PNIPAm film, normal to the substrate. Our NR results agree with Yim et al.\textsuperscript{136} who also observed vertical phase separation at a similar graft density. Low-density grafts show no such discontinuity in the SLD profiles. The phenomena of a vertical phase separation results in a dulled interfacial response to changes in temperature. Since the conformational changes within the PNIPAm film were expected to impact tissue release, high graft density and high molecular weight combinations were determined to be unsuitable for cell-adhesion and release.

3. Dense PNIPAm films (0.5 chains / nm\textsuperscript{2}) show an elevation in the LCST. At 37°C (the temperature at which cell cultures are incubated), the dense film shows ~200 Å increase in thickness while the sparse graft density PNIPAm films show no such swelling effect. Swelling at the LCST would result in increased hydration of the PNIPAm film. Since cells attach to the dehydrated PNIPAm above LCST, the increased hydration is likely to deter cell-adhesion.
6.3.2. Influence of PNIPAm molecular weight on cell-adhesion and release

On the basis of neutron reflectivity results, the molecular weight of PNIPAm thin-films especially at higher graft densities is expected to inhibit cell-adhesion. However, no reports exist that confirm this claim. Typically, the cell-adhesion and release characteristics of PNIPAm films have been related to PNIPAm film thickness. For instance, Mizutani et al. show maximum cell-adhesion and corresponding release occurs from very thin (~ 2 nm) PNIPAm films.\textsuperscript{123} This result is supported by Linhui et al., although a range of optimal cell-adhesion and release observed is different (~20 – 45 nm).\textsuperscript{144} Increasing PNIPAm thickness is seen to discourage cell-adhesion, even completely repelling any cell-adhesion above 70 nm. In contrast however, Xu et al., suggest that increasing polymer thickness can improve cell adhesion.\textsuperscript{145}

We prepared cell cultures on high-graft density PNIPAm thin-films (1 GD, 1.04 chains / nm\textsuperscript{2}), while varying the molecular weights (~3 – 44 kDa) to validate NR predictions. Assuming brush regime for the PNIPAm chains due to the high graft density we also determined the effect of varying PNIPAm film thickness (from Eq 5.2) on cell-adhesion and release characteristics.

Following PNIPAm thin-film synthesis and characterization, human bone marrow stromal cells (hBMSC) were cultured on PNIPAm substrates for a week at 37 °C (after seeding at 5000 cells / cm\textsuperscript{2}) and then incubated at 20 °C in buffer solution. This aspect of the tissue-culture studies were performed by Girish Kumar at NIST.

Figure 6.3 and Figure 6.4 show the XRR profile and corresponding SLD profiles for the PNIPAm thin-films synthesized on fisherfinest glass slides prior to tissue-culture studies. A three-layer model was used to fit the XRR data. The layers are – a glass interface layer, a ATRP initiator functionalized silane layer and finally the PNIPAm layer. Typically, the silane layer
thickness was held in the range 20 ± 5 Å, while the SLD was allowed to vary between (10 ± 2) × 10^6 Å^-2 based on previous experience with silane based thin-films.  

PNIPAm samples 1GD0.5MW (1GD1MW, 1GD3MW and 1GD5MW represent increasing film thickness as the molecular weight is increased (~ 3 – 44 kDa). While a continuously increasing thickness trend is observable in Figure 6.4, the SLD of the PNIPAm layer is constant. Therefore, PNIPAm film density is independent of thickness. The density of PNIPAm thin-films as determined from the XRR best-fits is 0.98 ± 0.04 g / cm³. PNIPAm film thickness values and experimental errors are reported in Table 6.1.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Thickness (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1GD0.5MW</td>
<td>5.2 ± 4</td>
</tr>
<tr>
<td>1GD1MW</td>
<td>13.2 ± 4</td>
</tr>
<tr>
<td>1GD3MW</td>
<td>49.9 ± 4</td>
</tr>
<tr>
<td>1GD5MW</td>
<td>73.5 ± 4</td>
</tr>
</tbody>
</table>
Significant differences in cell-adhesion behavior are seen after 1-day incubation at 37 °C (Figure 6.5). Samples 1GD0.5MW and 1GD1MW (thickness 5.2 nm and 13.2 nm) showed good cell-adhesion and proliferation, although confluent sheet formation was not observed. Samples 1GD3MW and 1GD5MW by contrast showed no cell-adhesion.

After a week of incubation at 37 °C, the thicker PNIPAm grafted substrates continued to inhibit cell-adhesion. However, thinner PNIPAm films showed confluent cell-sheet formation (Figure 6.6). Incubating the samples at 20 °C in a PBS buffer resulted in initiation of cell-sheet release in less than five minutes. Within 15 minutes, the confluent cell-sheet completely released from the
1GD1MW PNIPAm film while 1GD0.5MW film showed partial release. Complete release of the cell-sheet from sample 1GD0.5MW occurs in 25 minutes.

The observed cell-adhesion and release results validate predictions made on the basis of neutron reflectivity (NR). Our tissue-culture results agree with Mizutani et al. and Linhui et al. but are at odds with the observations of Xu et al. At low molecular weights ( < 15 nm), strong adhesion of cells to is observed above the LCST. However increasing the molecular weight results in inhibition of cell adhesion.

The extent of hydration within the PNIPAm film necessary to inhibit cell-adhesion was quantified using neutron reflectivity.
<table>
<thead>
<tr>
<th>Temperature</th>
<th>Incubation time</th>
<th>1GD0.5MW (52 Å)</th>
<th>1GD1MW (168 Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37 °C ,</td>
<td>1 week</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>20 °C</td>
<td>1 min</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td></td>
<td>5 min</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td></td>
<td>15 min</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
<tr>
<td></td>
<td>25 min</td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Figure 6.6 Cell-adhesion to and release from thin PNIPAm films (1GD0.5MW and 1GD1MW) after 1 week incubation at 37 °C and 25 minute incubation at 20 °C. The scale bar represents 500 µm.
6.3.3. Hydration of the PNIPAm film inhibits cell-adhesion

Neutron reflectivity was used to assess the effect of thickness on hydration in PNIPAm films above the LCST. Deuterated phosphate buffered silane (PBS) solution was used instead of pure D$_2$O in order to emulate experimental procedures used during cell-release. Considering the stark difference in cell-adhesion to thinner (≤ 20 nm) and thicker (≥ 40 nm) PNIPAm, two representative films were synthesized on silicon wafers.

Dry state reflectivity analysis (not shown) identified the film thickness as ~ 17 nm and 40 nm respectively. Figure 6.7 shows the neutron reflectivity (NR) data and best-fits for the PNIPAm films exposed to the PBS-D$_2$O buffer at 37 °C. Corresponding SLD profiles are shown in Figure 6.8.

Two significant results were obtained from NR analysis. First, a sharp interface is maintained between PNIPAm film and PBS-D$_2$O at 37°C. This temperature is above the LCST of PNIPAm, therefore phase-separation at the PNIPAm – water interface is expected. NR profiles confirm that a single layer density with low roughness is sufficient to describe the film structure. Secondly, substantial penetration of water was observed even though PNIPAm thin-films were maintained above the LCST. The penetration of water results in both SLD and thickness increases. The SLD’s of the swollen thinner and thicker PNIPAm films are $2.5 \times 10^{-6}$ Å$^{-2}$ and $2.9 \times 10^{-6}$ Å$^{-2}$ respectively. The thickness increases are 11.5 % and 7.5 % respectively (Table 6.2). These values correspond to 32 ± 4 vol % D$_2$O in the PNIPAm film that encourages cell-adhesion and 39 ± 2 vol % D$_2$O in film that inhibits cell adhesion.

<table>
<thead>
<tr>
<th></th>
<th>PNIPAm thin-film thickness (Å)</th>
<th>PNIPAm thick-film thickness (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry state</td>
<td>165</td>
<td>396</td>
</tr>
<tr>
<td>37 °C in PBS D$_2$O</td>
<td>184</td>
<td>426</td>
</tr>
<tr>
<td>% increase in thickness</td>
<td>11.5</td>
<td>7.5</td>
</tr>
</tbody>
</table>

Table 6.2. Increase in PNIPAm layer thickness after exposure to PBS-D$_2$O buffer solution.
This is an important observation. Typically the LCST transition in PNIPAm is described as a hydrophobic – hydrophilic transition. This model leads to a false expectation that water is completely expelled from PNIPAm above the LCST. In fact, even in its “hydrophobic state,” the water contact angles for PNIPAm films are in the range of 71 – 88°, indicating that PNIPAm grafted substrates films do not act as water barriers. A completely hydrophilic substrate would most likely repel cell-adhesion. However, it appears that cells can tolerate some water content in the PNIPAm film above the LCST. This water content limit lies in the range of 32 – 39 vol %.

The NR data presented, in conjunction to the cell-culture results, establish limits on the hydration of PNIPAm grafted substrates above the LCST. Cells will only adhere on PNIPAm thin-films that show water hydration of less than or equal to 32 ± 4 vol % water. A transition in the substrate behavior occurs between 33 vol % and 39 vol % water penetration resulting in the inhibition of cell-adhesion.

Figure 6.7 NR profiles (colored solid dots) and corresponding best-fits (solid lines) for representative PNIPAm films exposed to PBS-D$_2$O at 37 °C.

Figure 6.8 SLD profiles corresponding to the best-fits for representative PNIPAm films exposed to PBS-D$_2$O at 37 °C. The thicker PNIPAm film shows significantly greater D$_2$O penetration and may explain the lack of cell-adhesion previously observed.
Following successful cell-adhesion and release analysis for high graft density PNIPAm, a more comprehensive evaluation of PNIPAm thin-film morphology on cell-adhesion and release was performed by independently varying both molecular weight and graft density.

6.3.4. Effect of PNIPAm molecular weight and graft density on cell-adhesion and release

We evaluated the influence of both graft density and molecular weight of PNIPAm thin-films on cell-adhesion and release characteristics. These studies were performed using rat endothelial cells in Dr. Yi-Gang Wang’s laboratory. PNIPAm grafted glass slides were seeded with rat endothelial cells and incubated for a week at 37 °C. The utilization of a different cell-line, as compared to the human bone marrow stromal cells (HBMSC’s) used previously allowed for independent verification of the previously presented tissue culture results.

The PNIPAm thin-film morphology was represented using the nomenclature discussed in chapter 5.3.6. The term ‘1GD’ represents the maximum grafting density possible; ‘1MW’ represents PNIPAm molecular weight after 15 minutes of polymerization.

In order to quantify cell-release characteristics, the optical microscopy images were analyzed using ImageJ 1.46r, image processing software. Figure 6.9 and Figure 6.10 show endothelial cells attached on glass slides prior to and after image modification. The red regions seen in Figure 6.10 identify adhered cells and the extent of spreading of the cells. Rather than counting endothelial cells adhered on the substrate, the total areal coverage of endothelial cells was measured. Ideally a confluent cell-sheet is desirable after cell-adhesion on PNIPAm grafted substrates, corresponding to 100 % cell-coverage. For our system though, the maximum cell-adhesion observed was ~ 60 % of the total area. Comparison of cell-adhesion was made to this 60 % adhesion condition.
6.3.4.1. Influence of varying molecular weight on cell-adhesion and release (high graft density)

This tissue culture experiment was meant to validate cell-adhesion and release behavior previously observed using hBMSCs while using a different cell-line (rat endothelial cells). The grafting density was maintained at 1.04 chains / nm$^2$. However the molecular weight was systematically increased from (~1 kDa – 18 kDa). Following incubation, the tissue cultures were washed with PBS and maintained at room temperature (~20 °C). Cell-release characteristics were recorded at regular time intervals as shown in Figure 6.11.

Some important results from the tissue-culture experiment are presented below.

Figure 6.9 Rat endothelial cells adhered to a PNIPAm grafted substrate following 5 minutes incubation at 20 °C.

Figure 6.10 ImageJ processed Figure 6.9, showing clearly defined cell spreading and boundaries. These modified images were used for quantification of cell-coverage areas.

Figure 6.11 Cell-release kinetics (represented by the loss of areal coverage of cells) from PNIPAm thin-films maintained at a constant graft density while varying molecular weights. The dotted lines are meant to highlight the trends in cell-release behavior.
1. All PNIPAm grafted substrates showed both cell-adhesion and cell-release. The extent of both adhesion and release depended on the thin-film morphology. However compared to observations with hBMSC’s, the surface coverage was lower (100 % areal coverage for hBMSC’s as compared to ~ 50 % areal coverage for the endothelial cells).

2. At very low molecular weight (1GD0.1MW condition, ~ 1 kDa), good (maximum cell-adhesion observed for our system) cell adhesion is observed (54 ± 18 % area coverage). However the release of cells is stunted. 34 ± 9 % areal coverage of cells is observed after maintaining the substrate at 20 °C for 13 minutes. Extending the time at 20 °C to 72 minutes results in significantly greater cell-loss (19 ± 2 % areal coverage). However maintaining tissue cultures at room temperature for extended periods of time can affect tissue viability. High graft density, very low molecular weight conditions (estimated to be ~0.5 chains / nm², ~1.8 kDa) are unfeasible due to poor cell-release properties. Low molecular weight, high graft density conditions for PNIPAm thin-films encourage cell-growth. Unfortunately, cell-release from such a film is inhibited.

3. Increasing molecular weight significantly improves both cell-adhesion and release driving it to maximum cell adhesion in our system (~ 60 % areal coverage). (1GD0.33MW and 1GD1MW conditions). Good coverage of endothelial cells was observed above the LCST (> 50 % areal coverage) followed by accelerated release in under 20 minutes.

4. Thicker films (1GD2MW condition), inhibit endothelial cells in a manner consistent with that observed for hBMSC’s. Only 23 ± 5 % areal coverage with the endothelial cells was observed. Thus the hydration of a dense, higher molecular weight PNIPAm film inhibits cell-adhesion, irrespective of the cell-line used for tissue cultures.
6.3.4.2. Influence of molecular weight on cell-adhesion and release (low graft density)

Cell-release kinetics of PNIPAm grafted films with varying molecular weights was observed in a manner identical to section 6.4.1. The graft density in this case however was maintained at 0.2GD (0.2 chains / nm^2), five times lower than used in the previous tissue culture studies. Molecular weights were varied from 10 – 45 kDa. Following incubation at 37 °C for a week, cell-release characteristics were observed at 20 °C and presented in Figure 6.12.

The significant results of this study are reported below.

1. Good cell-adhesion (close to maximum adhesion observed in this system ~ 60 %) was observed for all substrates prior to incubation at 20 °C. In general, low grafting density favors cell-adhesion.

2. Poor release (~ 40 % cell coverage after 25 minutes as compared to 0 – 5 % cell coverage for complete release) was observed for the 0.2GD1MW PNIPAm films. At low graft densities, and low molecular weights, the adhered cells may be able to adhere to the underlying substrate, through the film, dampening the cell-release characteristics of the film.

3. Rapid cell-release (complete cell release in under 20 minutes as compared to 60 – 120 minute release times previously reported by Mizutani et al.)^{123} was observed for the intermediate condition - 0.2GD2MW. PNIPAm thin-films shows rapid cell-release with complete loss of...
cells (3 ± 1 % areal coverage remaining) after 18 minutes of room temperature incubation. This result is predicted from neutron reflectivity results presented in section 6.3.1.

4. Surprisingly at low graft densities and high molecular weights – 0.2GD5MW (∼ 45 kDa condition, cell-release is once again stagnated. This result is anomalous and not consistent with neutron reflectivity analysis. The lack of cell-release may need to be independently verified.

6.3.4.3. **Influence of graft density on cell-adhesion and release**  
The graft density of PNIPAm was systematically varied while maintaining a constant molecular weight (1MW, ∼10 kDa) condition while varying graft density conditions between 0.2 – 1 chains / nm². Following incubation at 37 °C for a week, cell-release characteristics were observed and are shown in Figure 6.13.

Some important results obtained from the cell-release studies are presented below.

1. Good and almost identical coverage of cells was observed irrespective of the graft density (close to maximum cell-adhesion observed).
2. Cell-release characteristics are similar for the higher grafting densities (0.5GD and 1GD). Cells are almost completely released from the substrates in 18 minutes (< 7 ± 3 % areal
coverage). Release of cells however is much slower as the grafting density is reduced (0.2 GD and 0.33 GD).

3. As the graft density is increased, cell-release kinetics accelerate. After 18 minutes of room temperature incubation, endothelial cells are almost completely released from the 0.5 GD (0.5 chains / nm²) and 1 GD (1.04 chains / nm²) conditions. In contrast, 22 ± 4 % of the 0.33 GD (0.33 chains / nm²) substrate area is covered by endothelial cells. 0.2 GD (0.2 chains / nm²) PNIPAm substrates show an even higher areal coverage (36 ± 5 %).

This result points to a low graft density limit on the applicability of PNIPAm grafted substrates. At low graft densities, cell-adhesion occurs, but cell-release is significantly stunted. This effect is most likely due to a muted transition in sparse films, especially at lower molecular weights.

The tissue adhesion and release study presented in this dissertation is the first comprehensive analysis of the effect of PNIPAm thin-film morphology on cell-adhesion and release. Previously, Takahashi et al. have presented a similar strategy to synthesize PNIPAm films using independent graft density and molecular weight control and cell-adhesion and release. However the range of graft densities considered is very small.

In addition to tissue culture, the morphology implications of PNIPAm thin-films were quantified using neutron reflectivity. The important conclusions from NR data and tissue cultures are reported below.
6.4. Conclusions

Well-defined PNIPAm thin-films were synthesized on silicon wafers and microscope slides using the AGET ATRP approach. The protocol allowed for rapid growth of PNIPAm films, with independent control over molecular weight and graft density.

Neutron reflectivity (NR) was used to evaluate the effect of varying graft density and molecular weight on PNIPAm transition behavior. On the basis of NR, the influence of graft density on PNIPAm LCST transition was observed. Evidence for vertical phase separation within the PNIPAm film was observed below the LCST, indicating high graft density, high molecular weight (> 0.5 chains / nm$^2$, >72 kDa) PNIPAm films are unsuitable for cell-release.

Cell-adhesion and release behavior of the PNIPAm films was evaluated using hBMSCs. Although some conflicting results are available in literature, our cell-adhesion results agree well with Mizutani et al. and Linhui et al.$^{123, 144}$ At higher graft densities (1GD, ~ 1.04 chains / nm$^2$), low molecular weight, thin (~10 kDa, < 18 ± 4 nm) PNIPAm films are more suitable for cell-adhesion as compared to high molecular weight, thick films (30 kDa, > 50 ± 4 nm) films. This result was attributed to partial hydration in PNIPAm films above the LCST.

Neutron reflectivity was used to quantify the limits on hydration in PNIPAm thin-films. Specifically when PNIPAm thin-films show less than 32 ± 4 vol % water penetration, cell-adhesion behavior is observed. At or above 39 ± 2 vol % water penetration, cell-adhesion is inhibited.

The influence of PNIPAm thin-film morphology on cell-adhesion and release was determined through independent graft density and molecular weight control. From tissue culture studies, some trends were observed.
1. The graft density of a PNIPAm film affects the kinetics of cell-release. Increasing the graft density at constant molecular weight accelerates cell-release. Low graft densities however inhibit cell-release. Presumably, at lower graft densities, the PNIPAm chains are spread out. Cell-adhesion therefore is influenced by the underlying substrate in addition to the PNIPAm film.

2. The molecular weight of a PNIPAm film affects the cell-adhesion behavior. Rapid release of cells was observed for the 1GD1MW (1.04 chains / nm$^2$, ~10 kDa) condition resulting in almost complete cell-release in 18 minutes. This release time is faster than previously reported by Mizutani et al. However for higher molecular weights, cell-adhesion is inhibited, due to previously reported partial hydration effects.

A thermo-responsive substrate optimized for tissue cultures must be capable of maximizing cell-adhesion above the LCST, while exhibiting rapid cell-release below the LCST. Our research provides the necessary quantitative data for the design of such a substrate. A high graft density of PNIPAm in conjunction with molecular weights limited to ~ 10 kDa is the “sweet spot” condition, which accelerates cell-release while simultaneously improving cell-adhesion above the LCST. The approach utilized in this work for PNIPAm film optimization can be extended to other responsive polymer systems as well in order to optimize their specific responses.
7. References


30. J. Kim, G.J. Holinga and G.A. Somorjai: Curing Induced Structural Reorganization and Enhanced Reactivity of Amino-Terminated Organic Thin Films on Solid Substrates:
Observations of Two Types of Chemically and Structurally Unique Amino Groups on the Surface Langmuir. 27(9), 5171 (2011).


59. D.M. Murphy, V.V. Ivanenkov and T.L. Kirley: Bacterial Expression and Characterization of a Novel, Soluble, Calcium-Binding, and Calcium-Activated Human Nucleotidase† Biochemistry. 42(8), 2412 (2003).


98. R. Pelton: Poly(N-isopropylacrylamide) (PNIPAM) is never hydrophobic J Colloid Interface Sci. 348(2), 673 (2010).


126. F. Hanns: The persistent radical effect in controlled radical polymerizations, City, 1999), pp. 1885.