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BIOPHYSICAL DYNAMICS OF SMOOTH MUSCLE CELL ON THERMO-RESPONSIVE AND BIO-FUNCTIONALIZED POLYMERS

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SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

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Abstract

Biophysical Dynamics of Smooth Muscle Cell on Thermo-responsive and Bio-functionalized Polymers

Chen Beiyi

Advisor: Vincent Chan

Biomaterial surface engineering plays a pivotal role in the design and development of various biomedical devices and engineered tissue equivalents. The modification of tissue culture substrate with thermo-responsive polymer (TRP) has led to the realization of the enzyme-free harvesting of anchorage-dependent cells through an acute increase in surface hydrophilicity of TRP across its lower critical solution temperature (LCST). To date, the intricate mechanisms of cell deadhesion/adhesion on TRP and other bio-functionalized polymer have not been fully investigated. The understanding of biophysical behavior of cells towards the thermo-physical stimulations and biological recognition on biomaterials will be critical to the design of highly tailored cell regeneration processes. In my thesis, highly integrative biophysical approaches are applied to develop quantitative correlation between the biophysics of cell adhesion/deadhesion, receptor-ligand interactions, cytoskeleton reorganization and biomaterial properties.

Thermo-responsive polymer (TRP) enables the enzyme-free harvesting of cells through an acute increase in surface hydrophilicity of TRP across its lower critical solution temperature (LCST), rendering feasible the generation of polymer-free cell sheets for regenerative medicine applications. Hydroxybutyl chitosan (HBC29) and poly(N-isopropylacrylamide) (PIPAAm) are the two main classes of TRP used in the current study. To date, the biophysical mechanisms of cell deadhesion/detachment on these two polymers as mentioned above still remain obscure. It was hypothesized that the existence of TRP (with/without biomaterials

grafting) would trigger the geometric transformation and cytoskeleton remodeling of smooth muscle cells (SMC). In the first part of my study, integrative biophysical techniques including confocal reflectance interference contrast microscopy (C-RICM) are applied to probe the thermal-induced deadhesion kinetics of on thermo-responsive HBC29 against different periods of SMC pre-culture time at 37 °C. Atomic force microscopy (AFM) demonstrates that both the surface topography and mechanical property of HBC29 film in water are acutely modulated across its LCST. Firstly, cells show negligible changes in adhesion contact area during low-temperature incubation on unmodified tissue culture polystyrene (TCPS). Secondly, the recession of adhesion contact and retraction of cell body for cells with different pre-culture times are triggered by HBC29 coating on TCPS. Interestingly, the initial rate of reduction in the normalized adhesion contact area of SMC is negatively correlated with the pre-culture time. Thirdly, the degree of cell deformation and average adhesion energy are reducing functions of time only for SMC with the lowest pre-culture time. In contrast, adhesion energy per cell is a reducing function of time irrespective of the change of pre-culture time. Lastly, the temporal dynamics of cytoskeleton organization and β -actin/smoothelin-B mRNA expression for SMC are strongly dependent on the pre-culture time. Overall, this study demonstrates that the thermal-induced deadhesion of SMC on TRP is characterized by the evolution of its contractile phenotypes.

In the second part of my thesis, the de-adhesion kinetics of SMC on PIPAAm surfaces is probed with our unique biophysical techniques. Water-immersion AFM first showed that the nano-topology of PIPAAm surfaces is dependent on the polymerization time and collagen coating. It is found that the initial rate of cell de-adhesion increases with the increase in polymerization time. Moreover, the degree of cell deformation (a/R) and average adhesion energy are reduced with the increase of grafted PIPAAm density after cell de-adhesion for 40 min. It has been shown that collagen coating regulates cell adhesion on biomaterial surface. Interestingly, lower collagen density on PIPAAm leads to higher adhesion energy

per cell during the initial 20 min compared to as-prepared PIPAAm while the initial rate of cell de-adhesion remains unchanged. In contrast, higher collagen density leads to 50% reduction in the initial rate of cell de-adhesion and higher adhesion energy per cell during entire 90 min. Furthermore, immunostaining of cytoskeleton protein actin provides supporting evidence that the de-adhesion kinetics is correlated with the cytoskeleton transformation during cell de-adhesion below LCST.

Cell adhesion has been known to be triggered by the specific interaction between adhesive receptor and extracellular matrix (ECM) proteins. However, the exact role of immobilized ECM proteins on the deadhesion/recovery of cells from thermo-responsive polymer in cell sheet engineering has not thoroughly established. In the third part of my thesis, the deadhesion kinetics of SMC from PIPAAm modified with various ECM proteins is determined with C-RICM. Thin layer of elastin (EL), laminin (LA), hyaluronic acid (HA) or collagen with a density of around $1\mu\text{g}/\text{cm}^2$ is either physically grafted or adsorbed on PIPAAm surfaces. Then X-ray photoelectron spectroscopy (XPS) and AFM validates the ECM protein immobilization on PIPAAm and probes the nano-topographic transformation of the protein modified PIPAAm surfaces, respectively. First, it is shown that EL induces the highest rates of reduction in both the degree of deformation and average adhesion energy for SMC during the initial state of deadhesion on PIPAAm surface at 18 °C. In contrast, HA-PIPAAm strongly resists SMC deadhesion as shown by the slowest adhesion contact recession and lowest initial reduction rates in a/R and average adhesion energy in comparison with CL-, LA-, EL- and plain-PIPAAm. LA is not ideal for effective cell recovery as the average adhesion energy of SMC on LA-PIPAAm is two times and six times higher than that on CL-PIPAAm and EL-PIPAAm, respectively, after 60 min deadhesion at 18 °C. Interestingly, the enhancement in deadhesion rate is shown to be correlated with the formation of stress fibers in SMC on EL-PIPAAm.

After above works on cell deadhesion kinetics on TRP, we also extends our research to extra application of natural products modified chitosan for fibroblasts

adhesion. Our result shows the successfully improvement on bio-compatibility of chitosan with whey and honey. Although fibroblasts defer to adhere on honey modified chitosan than whey modified chitosan, those cells all form strong contact after 2 hrs' incubation on both samples. Caseinomacropetides, which is constitution of whey and could bind with chitosan, is investigated to compared with whey. Results indicate caseinomacropetides is also effective to improve fibroblasts adhesion but the adhesion rate is slower on caseinomacropetides than those on whey modified chitosan. Honey samples at different pH environment are tested. Results find that gluconic acid, which provides hydrogen ion in honey solution does not support fibroblasts adhesion. Fibroblasts still can firmly attach to the honey modified chitosan even after the surface is neutralized. Fibroblasts prefer honey to initiate contact adhesion but they spread faster on neutralized honey after adhesion contact formation compared to that on unmodified honey.

TABLE OF CONTENT

ACKNOWLEDGEMENT	I
ABSTRACT	II
TABLE OF CONTENT	VI
LIST OF TABLES	XI
GLOSSARY	XII
CHAPTER 1 RESEARCH MOTIVATION	1
1. 1 THE APPLICATION OF THERMO-RESPONSIVE POLYMER IN BIOTECHNOLOGY	1
1.2 POLY(N-ISOPROPYLACRYLAMIDE) FOR TISSUE ENGINEERING	5
1.3 MECHANISM OF CELL DETACHMENT FROM THERMO-RESPONSIVE POLYMER	11
1.4 THE ROLE OF PROTEIN ON CELL DETACHMENT	12
1.5 EFFECT OF CYTOSKELETON ON CELL DETACHMENT FROM THERMO-RESPONSIVE POLYMER	14
1.6 OUTLINE OF THESIS	15
CHAPTER 3 ATOM TRANSFER RADICAL POLYMERIZATIONS (ATRP) REACTION	28
CHAPTER 4 MATERIALS AND METHOD	30
4.1 MATERIALS	30
4.2 HBC29 SURFACE PREPARATION	30
4.3 PIPAAm SURFACE PREPARATION	31
4.5 CONTACT ANGLE MEASUREMENT	32
4.6 X-RAY PHOTOELECTRON SPECTROSCOPY (XPS)	32
4.7 ATOMIC FORCE MICROSCOPY (AFM)	32
4.8 CELL CULTURE	33
4.9 CONFOCAL REFLECTION INTERFERENCE CONTRAST MICROSCOPY (C-RICM)	33
4.10 IMMUNOSTAINING AND FLUORESCENCE MICROSCOPY	34
4.11 REVERSE TRANSCRIPT-POLYMERASE CHAIN REACTION	35
4.12 DATA ANALYSIS	36
CHAPTER 5 AFM AND XPS CHARACTERIZATIONS OF THERMO-RESPONSIVE POLYMERS	37
5.1 NANO-TOPOGRAPHY OF HBC29 SURFACES ACROSS LCST	37
5.2 NANO-TOPOGRAPHY OF PIPAAm SURFACES ACROSS LCST	38
5.3 NANO-TOPOGRAPHY OF PROTEIN COATED PIPAAm ACROSS LCST	42
5.4 CHEMICAL COMPOSITION ANALYSIS OF PIPAAm AND PROTEIN MODIFIED PIPAAm SURFACE WITH XPS	46
CHAPTER 6 DYNAMICS OF SMOOTH MUSCLE CELL	

DEADHESION FROM THERMOSENSITIVE HYDROXYBUTYL CHITOSAN.....	50
6.1 SUMMARY	50
6.2 INTRODUCTION.....	51
6.3 RESULT AND DISCUSSION	53
6.4 CONCLUSION	68
CHAPTER 7 DYNAMICS OF SMOOTH MUSCLE CELL DEADHESION FROM THERMORESPONSIVE PIPAAm (POLY(N-ISOPROPYLACRYLAMIDE))	69
7.1 SUMMARY	69
7.2 INTRODUCTION.....	70
7.3 RESULTS AND CONCLUSION	72
7.3.1 <i>PIPAAm synthesis</i>	72
7.3.2 <i>Effect of Polymerization Time on SMC De-adhesion</i>	73
7.3.3 <i>SMC De-adhesion from Collagen-Coated PIPAAm Surfaces</i>	83
7.4 CONCLUSION	91
CHAPTER 8 THE INFLUENCE OF EXTRACELLULAR MATRIX PROTEIN COATED PIPAAm ON SMOOTH MUSCLE CELL DEADHESION DYNAMICS	93
8.1 SUMMARY	93
8.2 INTRODUCTION.....	94
8.3 RESULTS AND DISCUSSION	96
8.3.1 <i>Micro BCA Assay</i>	96
8.3.2 <i>C-RICM</i>	98
8.3.3 <i>Geometry transform and adhesive force</i>	101
8.3.4 <i>Immunofluorescence</i>	106
8.4 CONCLUSION	107
CHAPTER 9 ENGINEERING CHITOSAN/NATURAL HYDROGEL COMPOSITES FOR CELL PATTERNING	108
CHAPTER 10 FUTURE WORK.....	119

LIST OF FIGURES

Fig 2.1 (A) Principle of Reflection Interference Contrast Microscopy. (B) The schematic of RICM image of an adhering cell.....	23
Fig 2.2 Theoretical height profile of adhering soft shells.....	25
Fig. 3.1 Mechanism of metal complex-mediated ATRP.....	28
Fig. 5.1 AFM topographic images of HBC29 film.....	37
Fig. 5.2 AFM images of PIPAAm surfaces.....	38
Fig. 5.3.1 AFM images of collagen coated PIPAAm.....	43
Fig. 5.3.2 AFM images of ECM coated PIPAAm.....	44
Fig. 5.4.1 XPS of PIPAAm surfaces.....	47
Fig. 5.4.2 XPS of ECM coated PIPAAm.....	48
Fig. 6.3.1 Real-time phase contrast and C-RICM images of SMC on TCPS.....	53
Fig. 6.3.2 Real-time phase contrast and C-RICM images of SMC pre-cultured for 3 hrs on HBC 29.....	55
Fig. 6.3.3 Real-time phase contrast and C-RICM images of SMC pre-cultured for 9 hrs on HBC 29.....	56
Fig. 6.3.4 Real-time phase contrast and C-RICM images of SMC pre-cultured for 24 hrs on HBC 29.....	57
Fig. 6.3.5 Average a/a_0 and average a/R of SMC on HBC 29.....	59
Fig. 6.3.6 Average adhesion energy and adhesion energy per cell of SMC pre-cultured on HBC 29.....	62
Fig 6.3.7 Immuno-fluorescence image of SMC on HBC 29.....	63
Fig. 6.3.8 β -actin RT-PCR and smoothelin-B RT-PCR of SMC.....	66
Fig. 7.3.1 Illustration of ATRP reaction.....	72
Fig. 7.3.2.1 Real-time phase contrast and C-RICM images of SMC on PIPAAm-0.5hr.....	73
Fig. 7.3.2.2 Real-time phase contrast and C-RICM images of SMC on PIPAAm-1hr.....	75
Fig. 7.3.2.3 Real-time phase contrast and C-RICM images of SMC on	

PIPAAm-1.5hr.....	76
Fig. 7.3.2.4 Average a/a_o and average a/R of SMC on PIPAAm.....	77
Fig. 7.3.2.5 Average adhesion energy and adhesion Energy per cell of SMC pre-cultured on PIPAAm.....	80
Fig. 7.3.2.6 Immuno-fluorescence image of SMC on PIPAAm.....	82
Fig. 7.3.3.1 Real-time phase contrast and C-RICM images of SMC on collagen coated PIPAAm.....	84
Fig. 7.3.3.2 Average a/a_o and average a/R of SMC on PIPAAm and collagen coated PIPAAm.....	86
Fig. 7.3.3.3 Average adhesion energy and adhesion energy per cell of SMC on PIPAAm and collagen coated PIPAAm.....	87
Fig. 7.3.3.4 Immuno-fluorescence image of actin staining SMC on PIPAAm and collagen coated PIPAAm.....	89
Fig7.3.3.5 Immuno-fluorescence image of vinculin staining SMC on PIPAAm and collagen coated PIPAAm.....	90
Fig. 8.3.1 MicroBCA assay of ECM coated on PIPAAm.....	96
Fig. 8.3.2.1. Real-time phase contrast and C-RICM images of SMC on elastin coated PIPAAm.....	98
Fig. 8.3.2.2 Real-time phase contrast and C-RICM images of SMC on laminin coated PIPAAm.....	99
Fig. 8.3.2.3 Real-time phase contrast and C-RICM images of SMC on hyaluronic acid coated PIPAAm.....	100
Fig. 8.3.3.1 Average a/a_o and average a/R of SMC pre-incubated on ECM coated PIPAAm.....	101
Fig. 8.3.3.2 Average adhesion energy and adhesion energy per cell of SMC pre-incubated on ECM coated PIPAAm.....	102
Fig. 8.3.4 Immuno-fluorescence image of actin staining SMC on ECM coated PIPAAm.....	106
Fig. 9.3.1 Real-time phase contrast and C-RICM images of 3T3 fibroblasts on	

chitosan hydrogel.....110

Fig.9.3.2 Real-time phase contrast and C-RICM images of 3T3 fibroblasts on
chitosan/whey and chitosan/ caseinomacropetide hydorgel composite.....111

Fig.9.3.3 Average a/a_0 and average adhesion energy of 3T3 fibroblasts
pre-incubated on chitosan/whey and chitosan/ caseinomacropetide hydorgel
composite.....112

Fig.9.3.4 Real-time phase contrast and C-RICM images of 3T3 fibroblasts on
chitosan/honey and chitosan/neutralized honey hydrogel composite.....114

Fig. 9.3.5 Real-time phase contrast and C-RICM images of 3T3 fibroblasts on
chitosan hydrogel etched with gluconic acid.....115

Fig.9.3.6 Average a/a_0 and average adhesion energy of 3T3 fibroblasts
pre-incubated on chitosan/honey and chitosan/neutralized honey hydrogel
composite.....116

LIST OF TABLES

Table 5.2 RMS of PIPAAm series surfaces.....41

Table 6.1. Fitted parameters in the deadhesion kinetics of SMC on HBC 29.....59

Table 7.3.2. Fitted parameters in the deadhesion kinetics of SMC on PIPAAm and collagen coated PIPAAm surfaces.....78

Glossary

AFM: atomic force microscopy
ATCC: American Type Culture Collection
ATRP: atom transfer radical polymerization
C-RICM: confocal reflectance interference contrast microscopy
DI: deionized water
CL: collagen
CMPTS: 4(chloromethyl)phenyltrichlorosilane
DMEM: Dulbecco's modified eagle's medium
ECM: extracellular matrix
EL: elastin
FBR: foreign body reaction
FBS: fetal bovine serum
FITC-phalloidin: fluorescein isothiocyanate labeled phalloidin
HA: hyaluronic acid
HBC29: hydroxybutyl chitosan
HMTETA: 1,1,4,7,10,10-Hexamethyl-triethylenetetramino
LA: laminin
LCST: lower critical solution temperature
MDCK: Madin-Darby canine kidney cell
NAL: poly(N-isopropylacrylamide-co-acrylic acid)-b-poly(L-lactic acid)
NIPAAm: N-isopropylacrylamide
PBS: phosphate-buffered saline
PCR: polymerase chain reaction
PIPAAm: poly(N-isopropylacrylamide)
PLLA: poly-L-lactide
PRE: persistent radical effect
PVDF: Polyvinylidene Fluoride
RNA: Ribonucleic acid
RT: reversetranscribed

SMC: smooth muscle cell

ToF-SIMS: time-of-flight secondary ion mass spectrometry

TRP: thermo-responsive polymer

TCPS: tissue culture polystyrene

XPS: X-ray photoelectron spectroscopy

Chapter 1

Research Motivation

1.1 The Application of Thermo-responsive Polymer in Biotechnology

Thermo-responsive polymer (TRP) is an emerging class of biomaterial which has received much attention from the biomedical communities since last decade. The huge potential of TRP is stemmed from the acute physiochemical responses against the change of external temperature. One of the key thermophysical parameters of TRP is the lower critical solution temperature (LCST). For instance, TRP like poly(N-isopropylacrylamide), PIPAAm is fully dissolved in water to form a clear solution below LCST. When temperature goes above LCST, the solution of PIPAAm turns cloudy. The change as mentioned above is induced by the significant intermolecular aggregation of PIPAAm chains following the increase of hydrophobicity of PIPAAm above LCST. Based on this fascinating physical properties, TRP has been applied in several biomedical and pharmaceutical applications including gene transfection^{1, 2}, drug delivery^{3, 4}, and bio-separation^{5, 6}, and tissue engineering⁷.

Recently, Okano's group has used the thermo-responsive macromolecular complex composed of the copolymer of poly(N-isopropylacrylamide(PIPAAm), dimethylamino-ethyl-methacrylate (hDMAEMA) and butylmethacrylate (BMA) as synthetic carrier of pCMV-LacZ plasmid DNA for executing gene transfection in COS-1 cells¹. Their result indicates that the plasmid DNA can be completely stabilized through the complex formation with the thermo-sensitive co-polymer above the LCST and partially dissociated from complex below LCST. From the *in vitro* gene transfection assay, it is shown that the gene expression of COS-1 cells induced by the plasmid DNA is more effective for carrying 3 hr incubation at 20 °C (below LCST). The gene transfection efficiency is found to be temperature dependent and is increased by up to two times compared with cells without going through extra incubation at 20 °C. Also, homo-polymer of DMAEMA in the

absence of the thermo-sensitive PIPAAm block does not show the enhancement of gene transfection against the reduction of temperature. DNA delivery with novel TRP is precisely triggered by thermal stimuli as well as eliminating the pathogenic or immunogenic problems normally caused by traditional viral vectors. Moreover, the new system based on TRP opens new possibilities for optimizing the association and dissociation behavior of the DNA-TRP complex by changing the polymers/DNA ratio, polymer charge, molecular weight, etc.

In addition to the application in gene/drug delivery, the thermal sensitivity of TRP enables the exploitation of new technological applications in bioseparation⁵. In general, HiTrap ConA column and HiTrap WGL column, which are products from GE Healthcare Life Science for protein separation, are commonly used as the stationary phase in the purification of concanavalin A (ConA) and wheat germ lectin (WGL). But the technology of HiTrap affinity columns are not fault free since clogging or reduced flow rates inside the chromatography column has been reported. The application of TRP as the stationary phase for separation would be more cost effective and easy for operation. Proteins can be specifically reacted with the amino groups of PIPAAm through the peptide linkage. For instance, the analyte solution containing the oligosaccharides or glycopeptides is first perfused through the TRP column above the LCST. Along the way, the biomolecules are entrapped by the hydrophobic polymer layer. Then another solvent is applied to elute the oligosaccharides and glycopeptides below LCST when the TRP layer becomes highly hydrophilic. The result shows that the binding capacity of TRP system is the same as HiTrap ConA and is ten times higher than that of HiTrap WGL. TRP would be potentially useful for the separation of oligosaccharides and glycopeptides.

Recently, considerable progress has been made in the development of engineered tissue equivalents of various tissues including bone, cartilage, blood vessel, nerve, liver, and myocardium by integrating cells with various biomaterial scaffold systems. Usually, the cells are seeded and cultured on the scaffolds until they reach full confluency. Conventionally, the cells are detached by chemical

treatment such as disperse or by physical scraping. But both these methods of cell detachment lead to the disruption of intracellular junctions and alteration of native tissue organization which often leads to the failure of tissue regeneration or cell therapy. Another alternative is to cultivate tissue on biodegradable scaffold and to recover tissues after the scaffold will dissipate naturally. However, most biomaterials do not promote sufficient cell migration to establish adequate cell-extracellular matrix (ECM) and cell-cell interactions, both of which are critical to the physiological functions of tissues. Also, several biomaterial properties, such as surface chemistry, bioactivity, electrostatics, porosity, pore size, pore connectivity should be tightly controlled to realize cell regeneration. There is still presently plenty of room for improvement in the design of ideal biomaterial scaffold. Therefore, novel biomaterial scaffold with superior physiochemical properties is of greatest interests in the field of tissue engineering and regeneration as of today.

It is well known that anchorage-dependent cells prefer to grow on relatively hydrophobic surfaces. For instance, human aortic endothelial cells (HAECs) adhere and spread on hydrophobic PIPAAm surface but do not adhere on hydrophilic PDMAAm surface even in the presence of serum proteins⁸. It is because cell adhesion is prevented by the water molecules which are adsorbed on highly hydrophilic surfaces^{9, 10}. Interestingly, the switching between hydrophobic and hydrophilic properties of TRP against the change of external temperature opens new direction of biomaterial design for tissue engineering applications. Above LCST, cells remain adherent on the hydrophobic TRP while below LCST, cells detach from hydrophilic TRP. By culturing single cells on TRP above LCST and let them naturally reach confluence, then reducing the temperature to generate the confluent cell layer, a novel strategy for regeneration medicine and tissue engineering has been evolved.

Obviously, TRP has certain competitive advantages over existing biomaterials for tissue and cell recovery. Traditionally, cells harvesting on biomaterials require enzymatic digestion, including trypsin-EDTA, pronase, or collagenase for

cell detachment. These enzymatic reactions cause significant damages to cell surface and extracellular matrix proteins by hydrolyzing various membrane-associated proteins, proteolytically degrading the extracellular matrix (ECM) and disrupting the corresponding cell receptors, finally resulting in the impairment of several cellular functions^{11, 12}. Compared to several proteolytic enzymes, dispase seems to be less harmful but this neutral protease isolated from *Bacillus polymyxa* remained on the surfaces of detached cells. As a result, extensive washing is required to remove the remaining enzymes from the cells before applying the detached cell on wound healing. Although the detachment assay won't damage membrane proteins during washing, significant amount of deposited ECM is lost. Moreover, the dispase treatment leads to the up-regulation of Urokinase-type plasminogen activator and its receptor^{13, 14}. In contrast, the cell-ECM interaction mediated by integrin is not weakened even during lower temperature treatment at 4 °C¹⁵. Therefore using TRP to recover cell sheets by ramping down the temperature would satisfactorily maintain the ECM at large without breaking the linkage between cell membrane and its supported ECM layer. Specifically, the recovered ECM protects the detached basal cell membranes and function as a biological cue for subsequent cell transplant operations.

Most importantly, cells harvested by temperature reduction from TRP retain the specific differentiated functions of the originally seeded cells. Maintenance of specific phenotypes of cells harvested by trypsin digestion is rather difficult, but in the case of TRP detached cells, albumin secretion in hepatocytes and prostacyclin secretion in endothelial cells has been demonstrated to be conserved¹⁶. Low temperature treatment is exceptionally noninvasive, possibly due to the concurrent detachment of ECM proteins underlying the adherent cells from TRP. As a result, the functions of the cells are mostly preserved. At the same time, there is no need to remove the proteolytic enzymes once the cells have been detached. The competitive advantage as mentioned above is quite important for the realization of the successful implantation of detached cells into the host through the elimination of foreign body reaction (FBR), which is the main limitation on

longevity and performance of tissue engineering implant. Collectively, the TRP system would be effective for recovering adherent cells with minimum cell damage. Recovered cell sheets can then be transferred and adhered onto other surfaces with the use of transfer membranes¹⁷. It will also realize the formation and harvest of cell layer constructs mimicking organ function for realistic applications in tissue engineering. The field as mentioned above is known as "cell sheet engineering" which overcomes the limitations of tissue reconstruction using traditional biodegradable scaffolds or single cell suspension injection.

1.2 Poly(N-isopropylacrylamide) for Tissue Engineering

Application of TRP in tissue engineering has been pioneered by Okano and coworkers during the last decade. Generally, the group has successfully developed poly(N-isopropylacrylamide) PIPAAm, the most common TRP, in the separations of bioactive compounds^{18, 19}, drug delivery systems²⁰, and gene delivery¹. Among all the applications, the most attractive one lies in cell sheet engineering which has led to new breakthrough in regeneration medicine²¹⁻²⁴. The water contact angle of PIPAAm covalently coupled onto TCPS by electron beam irradiation is 48° at 37°C (above LCST), quite similar to the unmodified TCPS surface. Moreover, the behavior of cell growth on PIPAAm-modified TCPS at 37°C is very similar to that on non-modified TCPS because both surfaces are hydrophobic in nature. Contact angle of PIPAAm-modified TCPS (below LCST) is 30° at 10°C (below LCST) and the cells response to the acute increase of hydrophilicity on the PIPAAm surface by initiating detachment. For instance, nearly 100% of adherent bovine hepatocytes detached from PIPAAm-modified TCPS against temperature reduction across LCST compared to 10% cells detached from control TCPS dish under the same condition. Another significant fact is that up to 73% of cells detached from PIPAAm-modified TCPS surfaces have the ability to adhere and spread upon re-seeding on TCPS but only 14% of cells remains viable upon re-seeding after detachment with trypsin-EDTA²¹.

Urothelial cell sheet with intact intracellular contacts has been successfully

recovered from PIPAAm surface within 30 min after ramping down the temperature from 37 to 20°C (below LCST). In fact, almost all types of cell sheets which could be successfully detached from PIPAAm or its derivatives within 30 min during the thermal transition of PIPAAm across LCST. After cell detachment, non-cells components were left on the TRP surface. Despite the detached cell sheet is partially contracted, the histology of the harvested tissue has demonstrated the presence of 1 to 4 layers of normal urothelial cell. For example, the novel method seems to overcome the complication in the medical procedures of urinary diversion and reconstruction with the use of intestinal segment. It is because the reconstruction of urinary track with intestinal segment possibly causes electrolyte imbalance, stone formation, or mucus production²⁵. The regeneration of primary cardiac myocytes has also been achieved from PIPAAm, which exhibited improved tissue property with visible pulsatile amplitude which is not commonly found in other tissue generation system²⁶. On the other hand, this thermal-induced detachment of cells from TRP remains to be further exploited for various applications in tissue engineering and regeneration medicine.

In some cases, the cell sheet recovered from TRP would shrink to only one tenth of the original size before the thermal-induced detachment. The serious compaction of tissue leads to the disruption and aggregation of actin network within cell cytoplasm. This contraction of cell sheet has been insalubrious to the pulsation property of the myocytes cell sheet. Therefore chitin membrane is chosen as support material for transferring the cells from membrane to new TCPS surfaces²⁶. After the inclusion of support material, the actin organization, nuclei distribution and cell morphology are similar to those of the controlled cell sheet. If polyethylene meshes is used to substitute the chitin membrane, the pulsation efficiency will be increased by 20 times than chitin membrane. Further study is focused on the pilling up of several cardiac cell sheets and the maintenance of gap junction between adjacent cell layers, which is important for the formation of electrical connection²⁷.

Cell sheet engineering has also potential applications in skin regeneration. In

particular, keratinocytes are clinically used as artificial skin for wound healing. In detail, multilayered sheets of primary human epidermal keratinocytes harvested from PIPAAm have superior properties such as the maintenance of E-cadherin and laminin over tissues generated from physical scraping or dispase treatment. E-cadherin plays important roles in intracellular adhesion whereby it ensures cells within tissues are bound together to form a highly organized layer. The lack of E-cadherin possibly causes secondary infection in the detached tissues since bacteria can cross the basement membrane of the cell sheets and invade surrounding tissues. Moreover, E-cadherin down-regulation reduces the strength of cell-cell adhesion within a tissue, resulting in an increase in cellular motility. In parallel, laminin 5 (removed during conventional cell harvesting) binding to type IV collagen contributes to the self-assembly of the basal lamina with ECM proteins secreted by cells. Specifically, the recognition of laminin by growth cone integrins of apposing is important to the organization and function of the basal lamina which acts as a glue for cell attachment. The absence of laminin 5 significantly decreases the efficiency for cell attachment within tissues. From those points of view, thermal-induced detachment would be more advantageous than dispase treatment for non-invasive cell recovery because it neither disrupt E-cadherin organization nor reduces the expression of laminin 5 as done by dispase treatment.

To achieve optimized regeneration of keratinocytes, PVDF are covered on top of the cell sheet before thermal-induced detachment from PIPAAm to prevent cell sheet shrinkage, wrinkling, and folding which are driven by the contraction forces²⁸. Because the formerly deposited ECM proteins was also recovered together with keratinocyte sheets beneath the basal cell membranes, transferred keratinocyte sheets rapidly attached even to the unmodified TCPS dish surfaces. After 30 min of incubation at 37°C, tight adhesion of keratinocyte sheets onto the TCPS surfaces was established and PVDF is easy to peel off from the apical surface of cell sheet. Compared to chitin membranes used in the transfer of cell sheet upon thermal induced detachment, better results were obtained with the

PVDF membranes. The good contact between PVDF and cell surfaces and high flexibility in the peeling-off process seem to be the main reasons behind the superior performance of PVDF.

During cell deadhesion, the relationship between the cultivation time and the rate of cell detachment is a critical parameter for further development of cell sheet engineering. The relationship as mentioned above will directly affect the efficiency and effectiveness of the tissue regeneration method. Recently, a renal epithelial cell line, Madin-Darby canine kidney (MDCK) cell cultured on PIPAAm film was used as a model system for investigating the relationship between culture condition²⁴. The result indicates that the detachment rate is indirectly proportional to the incubation time of cell seeding prior to temperature reduction. For 3-week culture, it nearly takes 12 hrs for the cells to detach from PIPAAm at 20°C, but 4-week cultured cells only need 8 hrs to detach. Interestingly, 5-week cultured cells detached within one hour from PIPAAm. At the same time, E-cadherin down-regulation reduces the strength of cell adhesion within tissue and results in an increase in cellular motility. The immuno-blotting showed that the amount of ECM proteins such as fibronectin and vitronectin from the detached cell sheet is comparable with that of the control cell sheet. This is another evidence of the recovery of deposited ECM proteins together with cell sheet during thermal-induced detachment. More significantly, noninvasive cell sheet harvest and subsequent transplantation through TRP has not only been a successful technique in laboratory scale, it has also been extended to the ocular clinical operation. The regeneration of corneal epithelial cell was realized with the use of stem cells prepared from a single donor eye^{29, 30}, and the regenerated cell sheet has been extensively applied to the clinical trial involved 20 patients.

All cases as discussed above focus on the regeneration of monolayer cell sheet. However, the application of PIPAAm has not been limited to this field. It is also an effective biomaterial for developing co-culture system of specific cells. For example, lung cells mixture including epithelial and mesenchymal cells is cultured on PIPAAm before the transfer of the cell layer to larger TCPS with hydrophilic

PVDF³¹. Specifically, both type I and II epithelial cells express the characteristic morphologies which are similar to those observed at the beginning of cell culture up to 70 days. At the same time, the ratio of different cell populations is maintained throughout the entire period of cell culture. In long term, the use of TRP for cell co-culture would be more attractive than commonly used soft photolithography for its ease in controllability and interoperability to obtain the self-assembled co-culture cell layer. Usually, co-culture system achieved by soft lithography requires cultivating the first cell type under the absence of serum in the culture medium for homing the cells to the designated regions of the surface through the use of immobilized ECM proteins or peptides. This would avoid the cells from spreading onto the entire surface. Then the second type of cells would be cultivated with the normal procedure to fill up the empty area which was not seeded with the first cell type. But this method has a potential problem that several types of anchorage-dependent cells cannot be adhered to substratum without serum proteins. TRP technology is advantageous in this aspect as it avoids the necessary exclusion of serum proteins during the culture of first cell type. In TRP system, the spatial organization of cell adhesion lies on the control of grafted area of the TRP for selective cell attachment and detachment through the change of temperature. In addition, TRP system reduces the possible cytotoxicity due to the uses of significantly less organic solvent in comparison with that in photolithography.

Recently, co-culture of rat primary hepatocytes and bovine aortic endothelial cells has been demonstrated on PIPAAm³². First of all, PIPAAm was covalently grafted onto specific regions of TCPS dishes by using electron beam irradiation with a millimeter-scale metal mask of micro-patterns. Then hepatocytes were seeded homogeneously in the presence of serum proteins on the whole surfaces at 37°C. By reducing temperature to 20°C, hepatocytes are selectively detached from PIPAAm-grafted regions³³. Subsequently, endothelial cells are attached on the regions where hepatocytes have been previously detached. These two cell types were successfully co-cultured to confluence while heterotypic patterned cell

domains were maintained. It is also possible to directly cultivate rat primary hepatocytes onto the same micro-patterned PIPAAm surface as mentioned above at 20°C. Below LCST of PIPAAm, the cells adhered to the area lacking grafted PIPAAm in the presence of serum proteins. After the hepatocytes well adhere and spread, human fetal lung fibroblasts TIG-1 are subsequently added into the dish at 37°C. The fibroblasts only adhere to the selected area with exposed PIPAAm-grafted above LCST. Well-ordered patterns of the heterotypic cell sheet are generated by this process which seems to reduce the required number of first type of cells since no cell is detached and discarded during the whole co-culture process.

Conventionally, the cell co-culture depends on the use of specific ligands or ECM proteins for triggering adhesion of specific cell types. Thus there is no choice but the less adhesive cells should be cultured first on specific ligand coated area, such as fibronectin or collagen. For novel TRP system, it can be applicable to any desirable combination of cells and the sequence of cultivation would not be a problem because cell adhesion is not mediated by ECM proteins but the pre-designed selective TRP region. Moreover, the micro-patterned TRP surface can be utilized to co-culture three or more cell types, with the uses of distinctive grafted TRPs. For example, various TRPs exhibiting a range of LCSTs can be readily synthesized and can be spatially fabricated on different regions of the material surface with the simultaneous use of coordinated masks and co-polymerization³⁴. Different side-chains are introduced to PIPAAm monomer and this is an efficient way to control the mechanical and thermophysical properties of the TRP. Random copolymerization of NIPAAm with relatively hydrophilic monomers such as acrylamide and acrylic acid results in the increase in LCST, while the coupling azidophenyl groups can decrease the LCST by 17 °C³⁵. Poly(N-isopropylacrylamide-co-acrylic acid)-b-poly(L-lactic acid) (NAL), a linear block copolymer, was successfully used to culture and harvest cells sheet of Murine osteoblastic MC3T3-E1³⁶. Acrylic acid is chosen because it is highly hydrophilic and can increase the swelling and hydration of PIPAAm below the

LCST. PLLA is used because of its biodegradability, biocompatibility, hydrophobicity and good mechanical properties, since it has been widely used for fabricating tissue engineering scaffold. NAL is immobilized on planar glass or fused silica with spin-coating instead of electric beam irradiation. Another thermo-responsive copolymer, poly (N - isopropylacrylamide - co - acrylic acid) is used to culture Mouse fibroblast STO cells^{37, 38}. Usually, NIPAAm coupled with hydrophobic monomer such as methylmethacrylate is known to lower the LCST, while with hydrophilic monomer will increase the LCST. Also, copolymer with some other monomers with good mechanical properties can widen the application of TRP.

Of course, PIPAAm as a grafting material on TCPS has certain limitations against its potential applications. It has non-ideal biomechanical properties for the use as tissue-engineering scaffold. In addition, these materials are simultaneously polymerized and grafted to substrates by means of radiation. The process as mentioned above makes it difficult to graft the material evenly to surfaces with complex three-dimensional architectures.

1.3 Mechanism of Cell Detachment from Thermo-responsive Polymer

Cell adhesion onto material surface can be generally described by a two-step mechanism. Firstly, there is a passive adhesion which is driven by complex combinations including hydrophobic, coulombic and van der Waals forces between the cell membrane and the material surface. Secondly, active adhesion which is directly triggered by ligand-receptor interaction and the subsequent metabolic process occurs. Like any case of cell adhesion, cell detachment from PIPAAm-modified dishes is triggered by both passive and active processes. The passive process is related to the change of surface properties of PIPAAm from slightly hydrophobic to highly hydrophilic during the reduction of temperature below the LCST. Cells preferably adhere to slightly hydrophobic surface which is mediated by the adsorption of ECM proteins onto the surfaces. Therefore the detachment of cells from PIPAAm is likely driven by desorption of ECM protein

from the surface below LCST. Otherwise, these ECM proteins and/or cell receptors are readily degraded during cell detachment by dispase treatment.

The active mechanism of cell detachment towards temperature reduction is also demonstrated to be related to ATP-dependent cell metabolism. Active energy-dependent processes are mediated by intracellular signal transduction, including tyrosine phosphorylation and cytoskeleton reorganization, which lead to morphological change of the cell from spreading to round up after the change in the surface property^{22, 39}. Moreover, traction and contraction forces exerted by the cytoskeleton for cell adhering on a substrate are observed in viable cells. The cytoplasmic termini of the cytoskeleton are connected with the intracellular domains of ECM receptors, such as integrins. These cellular forces have been shown to detach coated ECM such as fibronectin from the culture dish surfaces²³. To validate the metabolic effect on cell detachment, sodium azide, a known inhibitor of cytochrome C oxidase in mitochondria which reduces ATP generation, is used on cardiac myocyte sheet and hepatocyte sheet detachment^{22, 39}. The result indicates that the chemical can retard the cell detachment, and also repress the shrinkage of cardiac myocyte sheet. The result clearly indicates that ATP-dependent metabolic process is one of the major mechanisms responsible for cell sheet detachment from PIPAAm-grafted surfaces. If the cells are incubated for additional 5 min at 25 °C after 30 min of 20 °C treatment, the cell detachment will be speed up. It is because the higher temperature accelerates the ATP generation^{22, 26}. All the result as mentioned above support the “active detachment” process. The mechanism of cell sheets detachment is also correlated to cell types. Different cell lines exhibit different temperature sensitivities. For hepatocyte, the optimum temperature for cell detachment is 10 °C and is significantly lower than that of endothelial cells which is 20 °C²². This is another evidence of active detachment theory.

1.4 The Role of Protein on Cell Detachment

As mentioned above, the variation in surface hydrophobicity of TRP is the

critical issue for the cell detachment which occurs with the simultaneous release of ECM proteins surrounding the cell body. For example, fibronectin will be detached together with adherent hepatocytes from fibronectin coated PIPAAm surfaces below LCST²³. In another case, bovine aortic endothelial cells were cultured on PIPAAm layer in the presence of serum and the cells maintained cell-cell junctions composing of monolayer cell sheets¹². In the absence of adherent cells, the reduction of temperature below LCST is not sufficient to lift up the protein layer from PIPAAm surface²³. On the other hand, fibronectin matrix deposited and accumulated on the PIPAAm grafted surfaces during the culture of cell sheets has been recovered from temperature-responsive surfaces by low-temperature treatment. The protein would be destroyed by trypsin digestion. Moreover, the recovery of fibronectin by low-temperature treatment was as high as that by physical scraping with a rubber blade. It is because the externally coated fibronectin is significantly thicker than the internally secreted layer. It is also possible that hydration of PIPAAm layer should be accompanied by cellular activities of the adherent cells for releasing of fibronectin layer²³. The intrinsic mechanism of cell sheet release under the change in substrate-protein-cell relationship is still unclear.

Although most ECM proteins are harvested with the cell sheet itself during low-temperature treatment, there are still some residues left on the substrate after cells will be lifted off. Molecular ion fragments from amino acids are demonstrated to be remained on the TRP surface based on measurements with X-ray photoelectron spectroscopy (XPS) and time-of-flight secondary ion mass spectrometry (ToF-SIMS). The remaining alanine, glycine, and proline left on PIPAAm samples are amino acid components which are abundant in collagen⁴⁰. The result strongly indicates that the lift off of ECM proteins is not completed. Despite the low temperature lift-off, laminin and fibronectin could be clearly seen around the cell body, while collagen I and IV are likely absent as shown by the immunostaining. However, the remaining collagen would not influence the cell deadhesion even if the collagen is coated on PIPAAm. The viable hepatocytes

are easily recovered by lowering the temperature regardless of the presence of collagen⁴¹. In fact, various proteins such as immunoglobulin, myoglobin, and lactalbumin can be adsorbed onto PIPAAm surface above LCST, and triggered to desorb from PIPAAm after the temperature was decreased below the LCST⁴².

The properties of biomaterials are not always optimized for cell adhesion. The introduction of specific functional groups in the design of PIPAAm copolymer system is a conventional way to improve biomaterial performance. Alternatively, the coating of ECM proteins on polymer surface is another choice for enhancement of cell adhesion. It is well known that protein adsorption on biomaterials remarkably enhanced cell spreading and adhesion. ECM proteins such as fibronectin and collagen are widely used to improve the bio-specificity of the polymer surface towards the targeted cells. These two types of ECM protein are also valuable for the design of TRP in tailored applications for tissue engineering. For instance, the conglomeration of fibronectin with albumin on a pattern-grafted thermo-responsive, azidophenyl-derivatized copolymer can effectively facilitate the selective cell detachment under some culture conditions such as serum-free culture or pre-adsorption of albumin. The pattern-grafting technique as mentioned above will be useful for making qualitative comparison between surfaces with distinct properties on one simple surface under the same culture condition³⁷.

To date, the role of ECM proteins on the dynamics of cell deadhesion from TRP remains to be purely qualitative as no further research has been carried to reveal the intrinsic mechanisms behind. In my thesis, the quantitative characterization on the influence of ECM proteins on cell deadhesion kinetics would be focused.

1.5 Effect of Cytoskeleton on Cell Detachment from Thermo-responsive Polymer

Actin is a protein which serves as a major structural component of cytoskeleton. Actin filaments provide mechanical support for the cell, determine

the cell shape, enable cell movements (through lamellipodia, filopodia, or pseudopodia); and participate in certain cell junctions, in cytoplasmic streaming and in contraction of the cell during cytokinesis. Moreover, actin plays an essential role, along with myosin, in muscle contraction^{43, 44}. Normally, F-actin resists mechanical stress and concentrates at the periphery of adherent cells. During the detachment of cells from PIPAAm, single cells still maintain peripheral rings of actin⁴⁵. Peripheral bands composed of actin filaments are known to exert contractile forces and to shrink the whole cell sheets after detachment. Such force driven by the cytoskeleton might be used in the detachment process as well as in sparsely plated cells since actin is not damaged during low temperature treatment⁴⁵.

Vinculin is another key cytoskeleton protein which is involved in focal adhesion formation. Vinculin regulates the cells motility by simply altering its conformation from active to inactive form. When vinculin is at its inactive state, vinculin's conformation is characterized by the interaction between its head and tail domains. Most importantly, vinculin is involved in the linkage between the intracellular domain of integrin (adhesion receptor) and actin of cytoskeleton^{46, 47}. More specifically, vinculin is associated with focal adhesion and adherence junctions, which are molecular complexes that nucleate actin filaments and crosslink between the external medium, plasma membrane, and actin cytoskeleton. Vinculin's ability to interact with integrins and cytoskeleton at the focal adhesion appears to be a critical factor for controlling of mechanochemical transduction, cell spreading and lamellipodia formation. Thus the organization of vinculin for cells during cell detachment from TRP likely affects the kinetics of cell deadhesion and detachment. Actin and vinculin would be the representative protein markers which for characterizing cell deadhesion process.

1.6 Outline of Thesis

The regeneration of tissues/cells for clinical application is the ultimate objective of cell sheet engineering with the use of TRP. In my thesis, I aim to

investigate the mechanism of cell deadhesion with the determination of biophysical responses of cells from TRP and protein modified TRP surfaces.

The first part of my thesis concentrated on the study of cell deadhesion dynamics of smooth muscle cells (SMC) from hydroxybutyl chitosan (HBC). Artificial blood vessels are tubes conventionally made of synthetic materials for restoring blood circulation. Synthetic grafts have been associated with excellent long-term efficacy in treating the disorders of large-diameter arteries where the blood flow is high and the resistance is low. However, the artificial graft for the replacement of small-diameter (<6 mm) arteries such as the coronary vessels is not so satisfactory. An ideal artificial blood vessel should be composed of viable tissue which contracts in response to hemodynamic forces and chemical stimuli, and secretes normal blood vessel products. Artificial blood vessels should also allow complete wound healing without any immunologic reaction and remodeling according to the needs of the environment. As the construction in artificial blood vessels are such an important topic in tissue engineering, we choose SMC as our model cell type herein⁷. Specifically, A7r5 SMC exhibiting the characteristic phenotypes of SMC, namely the morphologic plasticity in converting contractile cells to highly motile proliferative cells is an ideal platform for engineering specific biological responses of blood vessels. Therefore the cell line has served as a useful model for investigating the migration and colonization of SMC on vascular stent⁴⁸.

The choice of biomaterial as tissue engineering scaffold directly dictates the potential success for *in vivo* applications. For instance, chitin/chitosan is the second most abundant natural material on earth and has been proven to be biologically inductive, biodegradable, non-antigenic, and relatively biocompatible. Moreover, it is easy to control its physical properties of chitosan by crosslinking, grafting other chemical species into chitosan backbone or blending chitosan with other specific biomaterials such as proteins. Thus chitosan has been shown to support the adhesion and proliferation of various types of cell, such as MDCK epithelial cells⁴⁹, vascular endothelial and smooth muscle cells⁵⁰, chondrocytes⁵¹

etc. Chitosan has demonstrated the potentials in applications for wound healing⁵², artificial kidney membrane⁵³ and vascular grafting^{54, 55}. For example, SMC exhibits clear satellite phenotypes on chitosan/chondroitin sulfate blending surface⁵⁰.

Chitosan has a primary amino group at C₂ and hydroxyl group at C₆ positions, which allow it to undergo a host of chemical reactions in mild conditions. HBC, a chitosan derivative is synthesized by conjugation of hydroxybutyl groups to the hydroxyl and amino reactive sites of chitosan. Recently, Leong and co-workerd has shown the use of HBC29 for the culture of stem cells⁵⁶. Within the series of HBC polymer with different molecular weights, LCST can be controlled from 17°C to 29°C. The LCST of HBC29 is 29°C. Above the LCST of HBC29, the polymer film is hydrophobic and SMC adhere and spread on it. Vice Verse, when the temperature is reduced below its LCST, HBC29 would dissolve in medium and the attached cells would de-adhere.

After choosing SMC and HBC29 as the basic model system for our deadhesion dynamic study, we aim to reveal the influence of pre-culture time, the time SMC are cultivated on HBC29 hydrogel film before deadhesion, on cell deadhesion kinetics using our unique biophysical techniques, which would be described in the following part. Specifically, confocal reflectance interference contrast microscopy (C-RICM) in combination of phase contact microscopy would be used to determine key biophysical parameters of cell deadhesion including initial deadhesion rate, cell geometry, averaged adhesion energy and adhesion energy per cell of SMC. Immunostaining is used to reveal the cytoskeleton structure of SMC during cell adhesion and real time PCR is used to detect mRNA expressions of SMC markers β -actin and smoothelin-B.

The second part of my thesis is focused on studying the cell deadhesion dynamics of smooth muscle cells (SMC) from poly(N-isopropylacrylamide) (PIPAAm) surfaces with different thickness and the influence of different amount of collagen grafting on cell deadhesion. Unlike HBC which dissolves from the supporting substrate after ramping down of temperature, the main mechanism behind the physiochemical transformation of PIPAAm lies on the

temperature-dependent binding ability of its isopropyl groups to the water molecules. Increasing temperature above LCST would result in the increase of intramolecular and intermolecular hydrophobic interactions between the isopropyl groups. Thus these reversible chain swell-collapse transitions can be rooted from the balance between hydrophobic and hydrophilic groups. Hence the LCST of a PIPAAm copolymer can be modified over a broad range of temperature by either blending or copolymerization of PIPAAm with other biopolymers, making them very attractive for medical and biotechnological applications.

From our previous experience, the film thickness of TRP directly affects the cell deadhesion behavior⁵⁷. The optimized density of PIPAAm for supporting cell adhesion and triggering effective cell deadhesion is 1.5 ug/cm² to 2.0 ug/cm². When the density of PIPAAm falls below 1.5ug/cm², there is insufficient number of polymer chain which can extend out to make the surface hydrophilic for cell deadhesion. When the density of PIPAAm chain exceeds the limitation, cells neither adhere nor detach from PIPAAm surface. It is because there is too many polymer chains on the surface and part of them are far away from the aggregated chains locating towards the substrate surface. The chains remain to be hydrated underwater and make the surface not hydrophobic enough for cell adhesion. In this part of our work, we synthesized PIPAAm with various chain length onto glass coverslips with an improved two-step polymerization reaction instead of using electric beam irradiation to couple the PIPAAm chains to silicon coverslips. Our approach enables the control of polymer film thickness at nanoscale by modifying the polymerization time. This method makes it possible to tailor the amount of grafted PIPAAm by controlling the reaction time. It would be shown whether there is any correlation between the strength of cell-substrate interaction and the physiochemical properties of PIPAAm, and whether SMC functions could be stabilized during the course of cell regeneration and detachment.

On the other hand, the biocompatibility of PIPAAm is less ideal than conventional TCPS for inducing strong cell adhesion which is critical for effective tissue regeneration⁵⁸. It is shown that the biocompatibility of PIPAAm is improved

by the incorporation of the hydrophobic co-polymer into the PIPAAm chain^{36, 38}. Alternatively, the biocompatibility of PIPAAm can be enhanced by coating the polymer surface with extracellular matrix (ECM) proteins⁵⁹. For example, collagen is the most abundant ECM protein found in mammalian tissues and forms fibrous fiber with great tensile strength⁶⁰. Therefore collagen network provides structural integrity in adult muscularized arteries and plays key roles in the physiological regulations of SMC, e.g., proliferation inside blood vessel. It is also shown that cell adhesion and migration on biomaterial surfaces are dependent on the density of adsorbed collagen⁶⁰.

From the best of our knowledge, there is currently a lack of quantitative correlation between the physiochemical properties of PIPAAm films or collagen grafting and the resulting biophysical responses of cells upon temperature reduction. In this study, the dynamics of SMC deadhesion on a series of PIPAAm surfaces with different polymerization time and collagen coating would be probed with our unique biophysical techniques. In the meantime, the cytoskeleton structure of SMC during the course of thermal-induced deadhesion is detected by confocal fluorescence microscopy through immuno-staining of actin and vinculin.

The third part is focused on cell deadhesion dynamics of smooth muscle cells (SMC) from poly(N-isopropylacrylamide) (PIPAAm) surfaces modified with different ECM proteins such as elastin, laminin and hyaluronic acid to reveal the function of these proteins or biomolecular in dynamics term.

Works have demonstrate that cells may respond to minute differences in their surroundings by extruding differing amounts or types of collagen^{61, 62}. In the second part, we also discusses the influence of different concentration of collagen on cell deadhesion. However in tissue engineering of blood vessels, individual collagen constructs have a limited applicability due to lack of structural integrity⁶³ and single collagen would not reveal much about cell deadhesion. Here in this part, we would further apply other specific vascular related ECMs such as elastin and laminin, which undertake the basic biofunction in the substrate of SMC layers in the human body, and also non-sulfated glycosaminoglycan hyaluronic acid (HA),

which is known to affect the growth and migratory activity of SMC. This simplex research would be to some extent help to clarify the roles of these biomolecules in cell adhesion and worked as guidance for further combined action for the ECM outlay emulation.

Elastin is located in the medial layer of natural blood vessels jointly with laminin, collagen and some other ECM proteins to support the smooth muscle cells⁶⁴. These matrix proteins support the highly differentiated muscle cells, which contract in response to chemical and mechanical stimuli and take part in the control of blood pressure and flow^{65, 66}. Including all elastin is the dominant ECM protein deposited in this arterial wall and can contribute up to 50% of its dry weight⁶⁷, the content of which extracted from calf aortic medial layer is nearly four times higher than the derived collagen from the same tissue. It is believed to play an important role in the attachment and migration of SMC^{68, 69}, help SMC to maintain their contractile phenotype⁶⁵ and confer compliance to a blood vessel⁷⁰. Furthermore, more elastin secreted from SMC spread on fibrin gels than on collagen gels, and this has been proved to be the reason of the good elastic properties of SMC^{71, 72}. These properties make it possible to modulate the role of elastin on the incorporation, esterification and accumulation of lipid by SMC. Unfortunately, most of the studied artificial scaffolds are intend to induce the collagen biosynthesis of SMC^{73, 74}, while the biosynthesis of elastin during SMC cultivation has not received enough attention as it should be. For example, SMC tissues generated from type I collagen gels exhibited high collagen secretion, but the secretion of elastin could be neglected. As a result, the cell adhesion efficiency and spreading is not notably improved^{75, 76}. This awakes us to find out if the coating of elastin would bring us some special points during SMC adhesion process, which would be quite prospectus to advance the TRP substrate for the design of biofunctional blood vessel replacements.

Both elastin and laminin are the matrix component in the blood vessel wall. While elastin is the component of basement membrane, laminin is located in the basal lamina. Laminin is a family of oligomeric glycoproteins composed of

subunits encoded by different genes which help to maintain the SMC contractile phenotype and promotes the attachment and migration of vascular SMC. It provides binding sites for cell surface receptors, collagen, and other ECM molecules^{77, 78}, which help to initiate and control vascular responses in growth, differentiation, and wound repair^{79, 80}. It has been demonstrated to be lifted off from PIPAAm substrate after cell deadhesion at low temperature⁴⁰. Here in this paper, we would also study the influence of laminin on cell deadhesion as a concomitant and comparison of elastin.

Hyaluronic acid (HA) is non-sulfated glycosaminoglycan which as same as ECM proteins can regulate cell growth and migratory activity of SMC. The binding with HA by small oligosaccharides interferes the proliferation and migration of SMC and the cell morphology tend to be altered to a more flattened and spreading appearance during adhesion process⁸¹. Additionally, unlike the tension-bearing elements of the ECM proteins such as collagen fibrils, the nonfibrillar HA components are presumably much more malleable. These highly malleable substrates exerts less resistance to the cellular traction forces on the surrounding matrix which facilitate the cell to round up⁸² when the external driving force trigger the cell deadhesion and therefore have direct bearing on cell shape and mechanically coupled signaling⁸³. Thus HA is supposed to be beneficial to cell migration^{84, 85}. Here we want to test if HA would also promote the cell deadhesion during temperature triggering, as a comparison with the ECM protein EL and LA.

We have supposed the manually provided ECM proteins would help cells to achieve good acclimatization during cell adhesion and development. On the other hand, not only the low-temperature lifted-off cells yields the most reproducible ECM layer around its cell body, but also the substrate PIPAAm surface is covered with several ECM component⁶⁰. For instance, while laminin is believed to lift off with the cell, collagen is still partly left on the PIPAAm substrate. This indicates the partly loss the ECM from the cell body. We suppose the prior ECM coating would help the cells to maintain most of its ECM and subsequently their bio-functions.

Chapter 2

Adhesion Energy Model

The physiochemical properties of the polymer are known to influence the cell adhesion affinity and proliferation above LCST. Moreover, the time taken for complete cell detachment is dependent on the polymer characteristics. It has been shown that the time of cell sheet detachment from various thermo-responsive polymers ranges from 3 mins to 24 hrs after the temperature is ramped below LCST⁸⁶. Despite the intense exploitations in cell sheet engineering, there is currently a lack of engineering principle in the design of thermo-responsive polymers for optimizing cellular functions after cell detachment. Several scientific questions must be addressed before cell sheet engineering will be brought into clinical applications as follows: 1) How do the physiochemical properties of thermo-responsive polymer affect the thermal-induced cell adhesion/detachment dynamics from TRP surfaces: 2) Does the detachment kinetics of adherent cells from the polymer surface influence the biological functions of the detached cells? 3) What is the intricate interplay between ligand modification, cell detachment kinetics and cellular functions on the thermo-responsive polymer? All these fundamental questions drive the need of biophysical study on cell deadhesion kinetics from TRP.

Our group's approach to biomaterials surface engineering is in the development of a unique analytical system that is based on biophysical principles to investigate the effect of substrates has on cell adhesion kinetics. For example, cell adhesion mechanism on biomaterial surfaces are influenced by polymer grafting, ligand immobilization, biopolymer modification or alteration to the cytoskeleton⁸⁷⁻⁸⁹. However, the measurement of fundamental forces that mediate cell adhesion on solid substrates poses significant challenges to the existing analytical techniques. In order to characterize the biophysical responses of cell upon adhesion, there is a need to monitor the formation of cell adhesion contact

made with the biomaterial surface. Moreover the contour of membrane-surface separation in the cohesive zone of adhering cells is correlated to the adhesion strength and membrane bending modulus. However, several optical techniques such as the side-view phase-contrast microscopy are incapable of probing the adhesion contact area (membrane-substrate distance $< 50\text{nm}$) and separation in the range of 0.01 to 5nm between adhering cells or vesicles and solid substrate while the technique of reflection interference contrast microscopy (C-RICM) can be used to detect the distance between cell membrane and substrate within 100nm ⁹⁰.

Fig 2.1 (A) Principle of Reflection Interference Contrast Microscopy⁹¹. (B) The schematic of RICM image of an adhering cell⁹².

Since last late eighties, C-RICM has been used to determine bending modulus, membrane tension, and adhesion energy to make comparison between normal *Dictyostelium discoideum* cells and mutant ones⁹³. C-RICM images are formed by the interference of light reflected from the surface of the glass substratum (I_1) with light reflected from the cell surface (I_2), as shown in Fig. 2.1A. The maximum distance $h(x)$ between substratum and cell surface still leading to visible interference fringes decreases with increasing angle α of illumination. For the

setup used, the maximum distance is $\sim 1\mu\text{m}$. The schematic (Fig. 2.1B) of image of an adhering cell reveals that the adhesion area of a cell appears dark, because of a phase jump of light reflected at the buffer-cell interface. The contact line L circumscribes the adhesion area, where the cell forms close contact with the substratum. Outside of the area, the increasing distance between cell and substratum gives rise to a series of interference fringes. This interferometric technique can be used to analysis the local deformation in terms of commonly used boundary conditions.

Briefly, in elastic model of the cell contour near the substrate, the shell was soft and closed, the adhesion relates to adhesion energy W , surface tension γ , and bending elasticity κ , but not shear elasticity for the weak adhesion. For fluid lipid vesicles only bending elasticity is important, and a rigorous theory relating the shape of the adhering vesicle to the membrane tension, the osmotic pressure, and the adhesion energy is available⁹⁴. To describe the adhesion, two balances are established in boundary conditions. The first is the balance of tensions that can be expressed in terms of Young's Law:

$$W = \gamma(1 - \cos \vartheta_c) \quad (2-1)$$

ϑ_c is the contact angle and γ is the surface tension. Another condition is the balance between bending modulus κ and adhesion energy W :

$$W = \frac{1}{2} \kappa / R_c^2 \quad (2-2)$$

Because $R_c^{-1} = \frac{\partial^2 h(x)}{\partial x^2}$ is difficult to measure directly, this can also be represented

with the minimizing free energy ΔG , which is:

$$\Delta G = \int_0^\infty \left\{ \frac{\kappa}{2} \left(\frac{\partial^2 h(x)}{\partial x^2} \right) + \frac{\kappa}{2} \left(\frac{\partial h}{\partial x} \right)^2 \right\} dx - \int_{-\infty}^0 W(x) dx \quad (2-3)$$

When $\Delta G = 0$,

$$\gamma \frac{\partial^2 h}{\partial x^2} - \kappa \frac{\partial^4 h}{\partial x^4} = 0 \quad (2-4)$$

If define characteristic length scale as:

$$\lambda = \sqrt{\kappa / \gamma} \quad (2-5)$$

and use the boundary conditions: $h(x)=0$, within the contact area and

$h(x) = \vartheta_c x$, in proximity of the contact area, we can get the solution of $h(x)$:

$$h(x) = \vartheta_c (x - \lambda) + \vartheta_c \lambda e^{-x/\lambda} \quad x \geq 0$$

$$h(x) = 0 \quad x \leq 0 \quad (2-6)$$

Fig 2.2. Theoretical height profile of adhering soft shells near the contact area. The predicted height profile $h(x)$ perpendicular to the contact line is governed by two boundary conditions, the balance of tensions and the equilibrium of bending moments. The characteristic length λ separates two regimes. For distances $x < \lambda$ the cell contour is dominated by the bending energy and is therefore curved. For $x > \lambda$ contour is dominated by the lateral tension of the composite membrane, and approaches a straight line. Extrapolation of this line to the substratum defines both λ and the contact angle ϑ_c ⁹².

When only close proximity to strong pinning centers was considered, the contact angle ϑ_c can be directly measured by a least-squares fit to the tension-dominated linear part of the reconstructed cell contour and the characteristic length λ can be calculated by the distance between the contact line

and the intersection of the linear fit with the x axis, because the contact line is usually well defined by a sharp increase in the intensity of the RICM image at the edge of the adhesion area. With known θ_c , λ and formula (2-6), we can get the value of R_c^{-1} . Thus, with formula (2-1), (2-2) and (2-5), all the values of W , κ and γ are obtained. The theoretical height profile of adhering soft shells near the contact area is shown in Fig. 2.2.

While the application of thermo-responsive polymers seems so attractive, most of the previous research has focused on modification or producing biomaterial surfaces that either promote or deter cell adhesion to the substrate surface. But kinetic mechanism is mentioned not so much, which is also very important to study the cells and cell-sheet detachment.

New model for adherent cell has been needed for interpreting data from our unique biophysical technique. The theory from K.T.Wan etc⁹⁵ is used a lot to study the adhesion biophysical mechanism on liposome⁹⁶⁻⁹⁸, HepG2 cells^{93, 99, 100}, primary hepatocytes^{101, 102}, fibroblasts¹⁰³ and etc. The degree of cells deformation is defined as the ratio of the contact zone radius, which can be obtained from confocal reflectance interference adhesion contact zone, which is obtained from C-RICM, and the mid-plane radius R of an adherent cell, which is obtained from phase contrast microscopy. The adherent cell has been modeled as a truncated capsule with a thin wall, with the radius of adhesion contact zone, a :

$$\sin \mu = (a/R) = \alpha \quad (2-7)$$

The adhesion energy when the cell membrane is under a uniform equi-biaxial stress, $\sigma = C\varepsilon$, which is shown as (2-8):

$$W = (1 - \cos \mu)C\varepsilon + C\varepsilon^2 \quad (2-8)$$

where C is equivalent to eq.(2-9):

$$C = Eh/(1 - \nu) \quad (2-9)$$

in a linear system under small strain with E the elastic modulus and the Poisson's ratio, respectively, and h the film thickness.

ε is found by Eq. (2-10):

$$\varepsilon = \frac{1}{2} \left[\frac{2 + 2(1 - \alpha^2)^{1/2}}{4/R^2 - \alpha^2} - 1 \right] \quad (2-10)$$

So, if the elastic modulus of the cell type is known, we can calculate the trend of adhesion energy in the single cells detachment process.

Chapter 3

Atom Transfer Radical Polymerizations (ATRP) Reaction

Atom transfer radical polymerization (ATRP) is a “living” or “controlled” radical polymerization method¹⁰⁴. It allows the preparation of well-defined (nearly monodispersed) polymer brushes on various types of substrates^{105, 106} under easy experimental setup, using readily accessible and inexpensive catalysts and simple commercially available or easily prepared initiators. ATRP does not require stringent experimental conditions and allows for the polymerization and block copolymerization of a wide range of functional monomers in a controlled fashion, yielding polymers with narrowly dispersed molecular weights. Moreover, ATRP is tolerant of monomers with polar functionality. Thus, it allows the direct polymerization of functional monomers without involving the tedious protection and deprotection procedures.

The normal schematic of the ATRP equilibrium which emphasizes the repetitive nature of activation and deactivation is shown in Fig. 3.1:

Fig. 3.1 Mechanism of metal complex-mediated ATRP. Mt^m : transitional metal; L_n : complexing ligand; R: halogen chain; X: Cl or Br.

Most ATRP reactions require the addition, or in situ formation, of four essential components for an ATRP reaction: (1) initiator, a macromolecule which includes at least one transferable atom or group, frequently a halogen R-X (normally X = Cl or Br); (2) a transition metal (normally Cu(II)/Cu(I)); (3) a ligand that forms a complex with the transition metal to modify solubility and catalyst activity (normally pyridine or bipyridine); (4) one or more radically

(co)polymerizable monomers. Mechanistically, ATRP is based on an inner sphere electron transfer process, which involves a reversible halogen homolytic transfer between the propagating dormant chain end and a transition metal complex in the lower oxidation state (Mt^m/L_n) resulting in the formation of propagating radicals (R^*) and the metal complex in the higher oxidation state with a coordinated halide ligand (e.g. $X-Mt^{m+1}/L_n$)¹⁰⁷. The reaction progresses radical termination is diminished as a result of the persistent radical effect¹⁰⁸, chain length¹⁰⁹ and the equilibrium is strongly shifted towards the dormant species ($k_{act} \ll k_{deact}$)¹¹⁰.

At the initial stage of ATRP, a sufficient concentration of the deactivating Cu(II) complex is necessary to quickly establish an equilibrium between the dormant and active chains. If this equilibrium is not controlled properly, the process resembles that of the conventional redox-initiated radical polymerization¹¹¹. The higher oxidation state transition metal can be formed in situ by reaction with dissolved oxygen, or it can be added directly to a reaction prior to initiation which can help to increase the efficiency of initiation by reducing the fraction of low molecular weight termination reactions initially required to generate the persistent radical effect (PRE)¹¹². Addition of the PRE is of particular utility when conducting a "grafting from" reaction with a multifunctional initiator or grafting from a surface. It is also strongly recommended when ATRP is carried out in protic, particularly homogeneous aqueous media. In our work, we add Cu(II) complex ($CuCl_2$) to control the surface initiated ATRP and the feed ratio between monomer, transitional metal and ligand, $[NIPAAm]:[CuCl]:[CuCl_2]:[HMTETA]$ is 150:1:0.2:2.5 in 15 ml of distilled water at room temperature in a Pyrex tube containing the silalated substrate.

After synthesis of PIPAAm, there would be a Cl group left on the backbone of PIPAAm chain. These active or "living" chloride end groups provide the chance for substitution reactions with the nucleophilic $-NH_2$ groups of collagen.

Chapter 4

Materials and Method

4.1 Materials

High glucose Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), streptomycin, penicillin, 10X Tripson-EDTA (0.5%), 10X PBS (pH=7.4) were purchased from Gibco (Singapore). HBC29 is a gift from Professor Kam Leong from Johns Hopkins Singapore Biomeical Centre. Acetic acid was purchased from Sigma Pte. Ltd. (Singapore). Triton X-100, formaldehyde, FITC-phalloidin and anti-Vinculin-FITC conjugated antibody were purchased from Sigma Pte. Ltd. (Singapore). CuCl_2 , CuCl , N-isopropylacrylamide (NIPAAm), 1,1,4,7,10,10-Hexamethyl-triethylenetetramino (HMTETA), were purchased from Sigma Chemical Pte. Ltd. 4(chloromethyl)phenyltrichlorosilane (CMPTS) (97%) was obtained from Alfa Aesar (Singapore). Extracellular matrix protein type I collagen was bought from Benton Dickinson (Singapore). Elastin, laminin and hyaluronic acid and were purchased from Sigma Pte. Ltd. (Singapore). Highly purified 18.2M Ω water was obtained from water purification system (Sartorius, Germany). Quantipro BCA Assay kit was purchased from Pierce (Singapore).

4.2 HBC29 surface preparation

The thermo-responsive HBC29 surface is prepared with 1% HBC29 solution. In brief, HBC29 is dissolved into 1% acetic acid in distilled water and stirred overnight at room temperature. After centrifugation at 11400 rpm for 30 min, the supernatant (transparent solution) is collected. 500ul of above solution is transferred onto tissue culture polystyrene (TCPS) dishes and dried under room temperature for two days. The coating was then washed three times with 1X PBS at 37 °C. The amount of coated HBC29 is about 130 $\mu\text{g}/\text{cm}^2$.

4.3 PIPAAm surface preparation

Glass coverslips were cleaned with piranha solution (30% H_2O_2 with 70% H_2SO_4) for one hour, rinsed thoroughly with 18.2M Ω water and then dried under vacuum at room temperature. Then the glass coverslips were first immersed in 30ml chloroform, and followed by 0.5ml triethylamine and 2ml CMPTS. After reaction for 24 hrs, the coverslips were thoroughly washed with acetone and kept in acetone for another thirty mins to remove the un-reacted silane. The silylated coverslips were dried in air in the following step and they were added to 15ml of 0.2g/ml NIPAAm solution. 20mg of CuCl and 4mg of CuCl_2 were then added and the tube was degassed with argon for 20 min. Finally, 50 μ l of HMTETA was added into the mixture and the tube was sealed for the interfacial polymerization of NIPAAm. Throughout this report, PIPAAm surfaces obtained from different polymerization time of IPAAm are represented by the time following the polymer notation, e.g., sample with 0.5 hr of polymerization time is represented by PIPAAm-0.5hr.

4.4 Preparation of protein coated surface

The original collagen solution (2.9 mg/ml in 0.12N HCl) was diluted for 10, 25 and 100 times. 200 μ l of solution at each concentration was poured onto the PIPAAm surface and incubated at 4 °C for 24hrs. The surfaces were then neutralized with 1N NaOH for 20min and thoroughly washed with DI water to remove the excess ions. Finally, the surfaces were sterilized with 70% ethanol and stored in refrigerator before further experiments. Throughout this report, collagen was coated on PIPAAm-1.5 hr, and surfaces obtained using 0.29 mg/ml, 0.12mg/ml and 0.03 mg/ml of collagen in the coating process are represented by 0.3x, 0.12x and 0.03x, respectively, before the polymer notation.

200 μ l of elastin, laminin, protein hyaluronic acid, and collagen solution (0.02mg/ml) were poured onto the PIPAAm surface and incubated at 4 °C for 24 hrs to dry up. Then the surfaces were totally washed with DI water and dried in air. Another elastin adsorbed sample was PIPAAm surfaces immersed in 500 μ l of

elastin (0.02mg/ml) solution overnight and afterward the residue solution thrown away. Final surface was also totally washed with DI water and dried in air. These coating process are represented by 0.02x, or 'adsp'.

4.5 Contact angle measurement

Static contact angle measurements were conducted for HBC29 surface using a Krüss G10 instrument (Germany) and 18.2M Ω water as the probe liquid. For consistency, contact angle measurements were taken at exactly 60s after placement of a water droplet on the surface, and reported values represent the average and 95% confidence intervals obtained using at least three 20 μ l droplets on at least three surfaces.

4.6 X-ray photoelectron spectroscopy (XPS)

The chemical composition of the modified PIPAAm surfaces was determined by X-ray photoelectron spectroscopy (XPS). XPS measurements were performed on a Kratos AXIS HSi spectrometer using a monochromatized Al K X-ray source (1486.6 eV photons) at a constant dwell time of 100 ms and a pass energy of 80 eV for the wide-scan and 40 eV for the core-level spectra. The samples were mounted on the standard sample stubs by means of double-sided adhesive tapes. The core-level signals were obtained at a photoelectron takeoff angle (measured with respect to the sample surface) of 90°.

4.7 Atomic force microscopy (AFM)

A MFP-3D atomic force microscope (Asylum Research, CA, USA) was applied in all measurements, which were carried with contact mode in deionized water. The cantilever with a spring constant of 0.32N/m and a scanning rate of 1Hz were used in all experiments. A temperature-controlling fluid cell was applied to control the temperature of the liquid and immersed polymer film. Each AFM topographic image is a representative data of at least three samples and is highly reproducible under the same procedures of sample preparation. The spring

constant of each AFM cantilever used in this study was calculated with the built-in thermal noise method in the MFP-3D software. With the detected cantilever sensitivity and spring constant, the preset tip deflection (set point) value corresponding to the force of cantilever exerted on the surface can then be calculated.

4.8 Cell Culture

A7r5 smooth muscle cell (American Type Culture Collection (ATCC) CRL 1444) originally derived from embryonic rat aorta and exhibiting an adult smooth muscle phenotype were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS), 5mg/ml penicillin and 5mg/ml streptomycin (Gibco, Singapore). Cell cultures were maintained at 37 °C under a humidified atmosphere of 5% CO₂ in air. The medium was changed every 2 days, and the cells were passaged at least once a week. Cells were detached from the culture flask by the addition of a 1:10, 0.5% 5.3 mM trypsin-EDTA solution in phosphate-buffered saline (PBS).

Before experiment, the HBC29-coated TCPS are maintained above LCST (at 37 °C) in order to keep the surface hydrophobic. A7r5 cells at density of 5200 cell/cm² were then plated onto HBC29-coated TCPS at 37 °C and returned to the incubator to allow cell attachment. After 3 hrs, 9 hrs or 24 hrs of seeding on the thermoresponsive surface, the samples are immediately transferred to an online CO₂ incubator at 18 °C under a humidified atmosphere of 5% CO₂ in air for biophysical measurements.

4.9 Confocal Reflection Interference Contrast Microscopy (C-RICM)

The system is based on a laser scanning confocal microscope (Pascal 5, Carl Zeiss, Germany) and is integrated with an online CO₂ incubator (Carl Zeiss). The illumination source is an Argon ion laser with a maximum power of 1 mW and excitation wavelength of 488 nm. 20 X objective (NA: 1.25) was used in this study. The strong contact zone of the adhering cells appears as dark region on the image.

Simultaneously, a transmitted light analyzer (Carl Zeiss, Germany) attached to the confocal microscope is used for performing phase contrast microscopy.

In order to monitor the deadhesion/detachment dynamics of SMC from HBC29 below LCST, a series of C-RICM images on selected region is taken against time of incubation at 18 °C. The image analysis is based on image analysis software, ZSM 5 (Carl Zeiss, Germany). The contact area (πa^2) of each cell is measured by drawing a region of interest with a PC mouse based on the criteria of the dark region (representing strong adhesion contact). At the same time, the projected area of the adherent cells (πR^2) is determined from the phase contrast image. Therefore the radius of contact area a and R of each cell is determined by C-RICM and phase contrast microscopy, respectively. At each time point, the average value of a and R is measured from one hundred or more cells on at least four identical samples. Another biophysical parameter, normalized adhesion contact area is defined as the ratio of adhesion contact area at any time during cell deadhesion (πa^2) and adhesion contact area immediately after the temperature reduction from 37 to 18 °C (πa_0^2). Degree of deformation for each cell is simply the ratio a/R .

4.10 Immunostaining and Fluorescence Microscopy

The cells before deadhesion and at the half-time during cell deadhesion (when a/a_0 is reduced to 0.5) from HBC 29 surface are stained with FITC-phalloidin for labeling the actin filaments of cells. In brief, esophageal epithelia washed with cold PBS were fixed with 3 % formaldehyde in PBS at room temperature for 10 mins. The cells were permeabilized with 0.2 % triton X-100 in PBS for 10 mins at room temperature following by a wash with 1X PBS and blocking with 10% FBS/0.1% TritonX100 for another 10mins. For the localization and structure of actin filaments, the fixed and permeabilized epithelial cells were incubated with fluorescein isothiocyanate labeled phalloidin (FITC-phalloidin) or FITC-anti-vinculin in 5% BSA for one hour. After dried overnight, the cells could

be observed with fluorescence microscopy.

Fluorescence images of SMC immuno-stained with FITC-phalloidin or FITC-anti-vinculin were measured by confocal microscopy (Pascal 5, Carl Zeiss, Germany) fitted with a 20 X oil immersion objective, an Argon-ion laser as the excitation source (488 nm), at a resolution of 1024 x 1024 pixels with the slow scan speed. Excitation was set at 488nm and emissions between 505 and 530 nm were recorded.

4.11 Reverse transcript-polymerase chain reaction

A7r5 SMC were pre-cultured on HBC29 surface for 3 or 24 hrs, respectively. After pre-culture, the cells were then detached with 0.05% trypsin-EDTA after incubation at 37 °C for 0, 30, or 60 min. Each batch of cells was collected and the total mRNA was extracted by RNeasy Mini Kit (Qiagen, Singapore) according to the recommended protocol. One microgram of the total mRNA was used for amplification using onestep Titanium RT-PCR Kit (Clontech). The mRNA was reverse transcribed (RT) to cDNA at 50 °C for 1 hr, and then the cDNA was denatured at 94 °C for 5 min. Fifteen polymerase chain reaction (PCR) cycles consisted of 30s denaturation at 94 °C, 30s annealing at 65 °C, and 1 min extension at 68 °C for b-actin and 22 PCR cycles with the same reaction conditions for smoothelin-B. Primers specific for b-actin were 5'-GTG GGC CGC TCT AGG CAC CAA-3' (sense) and 5'-CTC TTT GAT GTC ACG CAC GAT TTC-3' (antisense). And primers specific for smoothelin-B were 5'-GCT GGC ATC CGC CGA GTG-3' (sense) and 5'- GCA CCT TAC CAG GGT CCA ATGT-3' (antisense). Upon completion of reaction, 5 mL of the DNA products were electrophoresed on 1.5% agarose gel for 20 min at 100 V. DNA was stained with ethidium bromide (Sigma, Singapore), visualized under UV light and imaged by ChemDot (BioRad, USA).

4.12 Data analysis

The details of the contact mechanics model of adherent cells have been reviewed in the part of introduction. In the absence of external force, the cell spontaneously adjusts its distance of approach towards the substrate until equilibrium is achieved. It has been shown earlier that the adhesion energy, W , is as follows:

$$W = (1 - \cos \mu) C \varepsilon + C \varepsilon^2 \quad (2-8)$$

$\cos \mu$ is related to the degree of deformation, $\sin \mu$. Briefly, the equilibrium geometry of a cell adhering on a non-deformable substrate is modeled as a truncated sphere with a mid-plane radius R . Thus we assume $\sin \mu = \alpha = (a/R)$, which is an experimentally measurable parameter. Here a and R are measured by C-RICM and phase contrast microscopy, respectively.

ε is the average biaxial strain, which could be directly calculated from R and α as follows:

$$\varepsilon = \frac{1}{2} \left[\frac{2 + 2(1 - \alpha^2)^{1/2}}{4/R^2 - \alpha^2} - 1 \right] \quad (2-10)$$

C is the stress equivalent and is equal to $Eh/(1-\nu)$ in a linear system under small strain where E , h and ν are the elastic modulus, membrane thickness and Poisson's ratio, respectively.

Based on the mid-plane diameter R (from phase contrast microscope) and the radius of contact zone, a (from C-RICM), W can be found by Eqs. (2-8) and (2-10). From Matsumoto's research, elastic modulus E of rat thoracic aortic smooth muscle cells is $9.3 \pm 2.8 \text{ KPa}^{113}$.

Chapter 5

AFM and XPS Characterizations of Thermo-responsive Polymers

5.1 Nano-topography of HBC29 Surfaces Across LCST

Surface topography of biomaterials at the nanoscale is known to play significant roles in cell adhesion^{114, 115}. It would dominate in cell orientation, rates of movement, and activations of the cells. The formation of focal adhesions stimulation has been proved to be related to the roughness of the substrate surfaces and cellular adhesion would be directed by substrate topography^{116, 117}. The interfacial properties of TRP film under physiological conditions is believed to directly tune the cellular responses of adherent cell during thermal transition across LCST.

Fig. 5.1 AFM topographic images of HBC29 film immersed in pure water at 37 °C and 18 °C.

Fig. 5.1 shows the AFM topographic images of HBC29 film immersed in pure

water at 37 °C and 18 °C. Above LCST, the polymer surface appears to be rough and is filled with small agglomerates with different size. The result is likely caused by the increase of hydrophobicity of the polymer chain and the subsequent dewetting of the polymer film. From the representative force curve at 37 °C at representative region under water (the spot on topographic image), the presence of a pull-off event (escape transition) at 108 ± 12 nm ($n = 10$) from the polymer surface during the retraction of AFM tip indicates that there is a manifestation of adhesion force between the AFM tip and polymer film (Bottom left curve on Fig. 5.1). Below LCST (18 °C), the AFM image indicates that the polymer surface is significantly smoother than that at 37 °C. Similar trend of force curve has been previously reported in poly-(butadiene)-b-poly(ethylene oxide) film supported on glass under aqueous medium¹¹⁸. The transformation of surface topology from 37 to 18 °C is supported by the significant reduction of root-mean-square roughness from 2.52 nm to 0.08 nm. At the same time, the representative force curve at 18 °C (the spot on topographic image) under water shows that the jump-in during the AFM tip retraction is abolished. The result strongly indicates that there is negligible adhesion between the AFM tip and the substrate below LCST and the indifference between the approach and retraction regimes on the force curve as mentioned above is similar to that of a bare glass substrate¹¹⁸. The result is likely caused by the increase of hydrophilicity of the polymer film or by the desorption of the polymer from the substrate into the aqueous medium.

5.2 Nano-topography of PIPAAm Surfaces Across LCST

It has been recently shown that the surface topography of PIPAAm-gelatin hydrogel is not affected by the use of pure water or culture medium during AFM imaging at 37 °C¹¹⁹. Therefore AFM is applied herein to characterize the surface morphologic map of the PIPAAm under DI water for simulating the micro-environment surrounding the PIPAAm film during cell deadhesion across LCST.

Fig. 5.2 Series of contact mode AFM images of PIPAAm5min at 18 °C in water (a). PIPAAm samples at 22 °C (b, d, f) and 37 °C (c, e, g) in water environment. They are PIPAAm0.5hr (b, c), PIPAAm1hr (d, e) and PIPAAm1.5hr (f, g).

Fig. 5.2 shows a series of AFM images of PIPAAm surfaces PIPAAm5min (a), PIPAAm0.5hr (b, c), PIPAAm1hr (d, e) and PIPAAm1.5hr (f, g), which are PIPAAm polymerized for 5 min, 30 min, 1hr and 1.5 hr at either 18 °C (a, b, d, f) or 37 °C (c, e, f) under water. Throughout this report, PIPAAm surfaces with different polymerization time of NIPAAm are represented by the time after the polymer notation. At 18 °C, PIPAAm doesn't fully cover the entire of the glass coverslip on PIPAAm-5min sample (Fig. 5.2a). The reaction time of five mins is obviously not enough to obtain a homogeneous thermo-responsive surface for our research. In contrast, PIPAAm-0.5hr surface looks rather uniform below LCST. The result supports that a complete PIPAAm film was formed on the glass surface because lesser time of NIPAAm polymerization leads to incomplete coverage of PIPAAm chain as shown in PIPAAm-5min. Similar nano-topology has been found in other polymer thin film immobilized on glass substrate¹²⁰. When the temperature moves above LCST to 37 °C, the evolution of small agglomerates with average diameter of around 85 nm occurs on the same region of PIPAAm-0.5hr surface (Fig. 5.2c). The result is likely caused by the intermolecular association of grafted PIPAAm chains following the acute increase of hydrophobicity above LCST^{121, 122}.

	RMS	
	22 °C	37 °C
PIPAAm30min	0.9	1.1
PIPAAm1hr	8.4	3.1
PIPAAm1.5hr	1.7	1.1
PIPAAm1.5hr+0.04x collagen	1.2	1.6

Table 5.2 RMS of PIPAAm series surfaces.

Moreover, the averaged root-mean-squares (RMS) roughness of PIPAAm-0.5hr surface is increased from 0.9 nm to 1.1 nm across LCST (Table 5.2). The relatively low RMS roughness across LCST is likely caused by either the lower density or molecular weight of PIPAAm chain under the minimum polymerization time among all samples herein. Usually for thermo-responsive

polymers, the intermolecular/intramolecular interactions of PIPAAm chains induced by hydrogen bonding and hydrophobic interactions become dominant when they are in water above LCST. When the temperature decreases to below LCST, the interaction between water molecules and PIPAAm chains dominates¹²³. Thus, the polymer is present in solution as hydrated chains at 18 °C, while the polymer coils collapse and aggregate at 37 °C^{121, 122}.

Towards an increase of polymerization time, larger domains with averaged diameter of 250 nm are found on PIPAAm-1hr at 18 °C (Fig. 5.2d). At the same time, smaller sponge-like domains of averaged diameter of 50 nm evolve throughout the whole surface. Both types of domain are absent on PIPAAm-30min below 29 °C (LCST of PIPAAm). Interestingly, the larger domains as mentioned above significantly shrink in size at 37 °C (Fig. 5.2e). The result is likely linked to the increased hydrophobic interaction between adjacent PIPAAm chains. The drastic change of nano-topography is further supported by the significant reduction of the height of the surface features during the thermal transition across LCST (Fig. 5.2d, 5.2e). The result is likely caused by the collapse of PIPAAm chains on the glass substrate above LCST. Simultaneously, RMS roughness is also decreased from 8.4nm to 3.1nm when temperature is increased from 18 °C to 37 °C. The more significant transformation of nano-topographic features on PIPAAm-1hr in comparison with that on PIPAAm-30min is likely originated from the higher density and length of grafted polymer chains. The further increase of polymerization time leads to the formation of smaller domain with averaged diameter of 50 nm on PIPAAm-1.5hr at 18 °C in comparison with that on PIPAAm-1hr. Also, the height of surface feature of PIPAAm-1.5hr at 37 °C is reduced compared to that within the same area of interest at 18 °C as shown by the change of 1-D line profile (Fig. 5.2f, 5.2g). Simultaneously, RMS roughness of PIPAAm-1.5hr is reduced from 1.7 nm to 1.1 nm against the increase of temperature from 18 °C to 37 °C. The trend of RMS roughness as mentioned above is similar to that on PIPAAm-1hr. Below LCST, the more inhomogeneous surface is caused by the complete swelling and extension of the hydrophilic PIPAAm chain

into the water phase on the substrate. Alternatively, the local topological fluctuation in the swollen polymer networks below LCST can be caused by the random cross-linking density of PIPAAm chains during polymerization¹²⁴. There is also a reduction of RMS roughness from PIPAAm1hr to PIPAAm-1.5hr because the longer PIPAAm chains in PIPAAm-1.5hr tend to cause the flattening of the polymer filled surface at the highest polymerization time used herein¹²⁵.

5.3 Nano-topography of Protein Coated PIPAAm Across LCST

The coating of ECM protein on PIPAAm surface likely modifies its surface nano-topography. First of all, collagen is coated on PIPAAm-1.5 hr with the use of 0.29 mg/ml, 0.12 mg/ml and 0.03 mg/ml collagen solution which is represented by 0.1x, 0.04x, and 0.01x, respectively, before the polymer notation. Fig. 5.3.1 shows the AFM topographic image of 0.04x-PIPAAm surface in (a) air at 18 °C, (b) water at 18 °C and (c) water at 37 °C. The result shows that dense collagen fibers are formed by the absorption of collagen from solution to PIPAAm surface (Fig. 5.3.1a). Similar structure of collagen fiber has been found on other substrate, e.g., self-assembled monolayer on gold¹²⁶.

Fig. 5.3.1 Series of contact mode AFM images of 0.04x collagen coated PIPAAm samples in air at 18°C (a) and in water at 18°C (b) and 37°C (c).

The collagen fibers remain on the polymer surface under water but they are less distinct from the PIPAAm base layer compared to that under air. Moreover, RMS roughness for the collagen coated surface is reduced from 3.3 nm to 1.2 nm when the medium of imaging is changed from air to water at 18 °C. The swelling and portraying of PIPAAm chains under water likely leads to the remodeling of the PIPAAm/collagen interface and caused the fattening of the surface as shown by the reduction of RMS roughness. From 18 to 37 °C, there is apparent increase in the number of small agglomerate with averaged diameter of 40 nm on 0.04x-PIPAAm. Also, RMS roughness of the same region of interest under water is increased by 32% to 1.6 nm. The trend in nano-topographic transformation is opposite and more subtle compared to that of bare PIPAAm surfaces because the collagen fibers shield the underlying PIPAAm layer from the AFM tip.

Fig. 5.3.2 A series of AFM images of protein coated PIPAAm samples at 18 °C (a, c, e,g) and 37 °C (b, d, f, h) in a water environment. They are (a, b) PIPAAm, (c, d) EL (0.02x) PIPAAm, (e, f) LA (0.02x) PIPAAm and (g, h) HA(0.02x) PIPAAm.

The morphologies of other ECM protein or glycosaminoglycan coated surfaces were also evaluated by AFM. Atomic force microscopy (AFM) probes the nano-topology of protein coated PIPAAm surfaces in fully hydrated state. Fig. 5.3.2 shows 2 x 2 μm AFM topographic images of a representative area on (a, b) PIPAAm, (c, d) EL-PIPAAm, (e, f) LA-PIPAAm and (g, h) HA-PIPAAm during the phase transition of PIPAAm from (a, c, e, g) 37 °C or (b, d, f, h) to 18 °C under water. Uncovered PIPAAm was evenly deposited on substrate at 37 °C (Fig. 5.3.2a). After temperature decreased to 18 °C, the polymer chains are extended out with the hydrophilic domains and many aggregates were formed with diameter of around 150 nm (Fig. 5.3.2b). The temperature below LCST is set at 18 °C herein based on the published results of other groups on cell recovery from PIPAAm¹⁻³. Different proteins may evolve into different nano-scale features upon immobilization on polymer surface^{127, 128}. In our previous work, CL has been shown to form fibular network on PIPAAm surface under high concentration regime of CL solution during coating¹²⁷. Compared to the relatively smooth PIPAAm surfaces, a dense layer of conical particulates are detected on EL-PIPAAm which indicates the successfully condensation of protein onto the PIPAAm surface. Elastin remains its conical shaped particulates with a dimension of around 200 nm under both 37 °C (Fig. 5.3.2c) and 18 °C (Fig. 5.3.2d). PIPAAm deformation during temperature reduction does not influence the surface morphology as well as the surface roughness of elastin layer. RMS of EL-PIPAAm keeps nearly constant with slightly change from 4.3 nm to 4.1 nm. Compared to the EL particulates, LA forms larger cloud-like spheres with an average diameter of 300-400 nm on PIPAAm surface at 18 °C. When the temperature goes above LCST, the spheres as mentioned above further coagulate into larger structures with average size of 800-1000 nm. At the same time, the RMS roughness is significantly reduced from 38.3 nm to 18.4 nm across LCST.

Unlike EL-PIPAAm and LA-PIPAAm, only a small number of HA particulates discernible on the HA-PIPAAm surface at 18 °C (Fig. 5.3.2f). Above LCST, the same region on the PIPAAm surface is entirely covered by HA particulates with an

average diameter of 200 nm at 37 °C (Fig. 5.3.2e). This difference between the nano-topographical transformation of ECM protein modified PIPAAm and HA-PIPAAm across LCST is likely to have resulted from the difference in their molecular structures. For instance, LA and EL have highly organized globular structures while HA is a linear polysaccharide chain. The more flexible chains of HA immobilized on PIPAAm at 37 °C (Fig. 5.3.2g) likely lead to distinct responses compared to the globular structure of proteins during the thermal transition as observed herein¹²⁹. Anyway, this aggregation could promote the formation of the island-like structure but the RMS roughness of HA-PIPAAm is still stabilize at around 4.1nm - 4.3nm.

5.4 Chemical Composition Analysis of PIPAAm and Protein Modified PIPAAm Surface with XPS

X-ray photoelectron spectroscopy (XPS) probes the chemical composition within a limited depth from the sample surface. XPS complements AFM measurement which mainly reveals the distinct nano-topography of adsorbed biomacromolecules on PIPAAm surfaces.

Fig. 5.4.1. XPS C 1s and N 1s core-level spectra of (a, a₀) PIPAAm-0.5hr, (b, b₀) PIPAAm-1.5hr, (c, c₀) 0.04x-PIPAAm and (d, d₀) 0.1x-PIPAAm.

Fig. 5.4.1 shows the C1s and N1s core-level spectra of (a, a₀) PIPAAm-0.5hr, (b, b₀) PIPAAm-1.5hr, (c, c₀) 0.04x-PIPAAm and (d, d₀) 0.1x-PIPAAm. The N1s peak represents nitrogen atoms found in the backbone of PIPAAm and those in the peptide bond and side chains of amino acids in CL. Its core-level spectra of PIPAAm-1.5hr, 0.04x-PIPAAm and 0.1x-PIPAAm are all located at around 399.0 eV because the difference in the chemical groups adjacent to nitrogen atoms on these samples does not directly influence the electronic state of the nitrogen. The C1s core-level spectra of PIPAAm-0.5 hr and PIPAAm-1.5hr can be curve-fitted into three peak components with binding energies (BEs) of about 284.6, 285.7 and 287.4 eV, which is attributable to the C-H, C-N and O=C-N species of PIPAAm, respectively. The increase of polymerization time (PIPAAm-0.5hr vs. PIPAAm-1.5hr) has led to a higher surface density of grafted PIPAAm, as shown by the slight increase of the intensities of C-N, and O=C-N peaks. The C1s core-level spectra of 0.04x-PIPAAm and 0.4x-PIPAAm can be curve-fitted into five peak components with BEs at about 284.6, 285.7, 286.2, 287.5 and 288.4 eV, which is attributable to the C-H, C-N, C-O, O=C-N and O=C-O species, respectively. The C-N and O=C-N peak components are associated with the peptide bonds in collagen, as well as the components of PIPAAm. The C-O and O=C-O peak components are solely associated with collagen. With the increase in collagen density on PIPAAm surface (0.04x-PIPAAm vs. 0.1x-PIPAAm), the intensities of the C-N, C-O, O=C-N and O=C-O components increase substantially. The results as mentioned above are consistent with the successful immobilization of PIPAAm layer and the collagen on the glass substrate.

Fig. 5.4.2. XPS C 1s and N 1s core-level spectra of (a, a₀) EL (0.02x) PIPAAm, (b, b₀) EL (absorption) PIPAAm, (c, c₀) HA (0.02x) PIPAAm and (d, d₀) LA (0.02x) PIPAAm.

XPS complements AFM measurement which mainly reveals the distinct nano-topography of adsorbed biomacromolecules on PIPAAm surfaces. The presence of PIPAAm layer and proteins coating were first confirmed by XPS analysis. Fig. 5.4.2 shows the C 1s and N 1s core-level spectra of ECM molecule coated PIPAAm surfaces: (a, a₀) EL-PIAAm, (c, c₀) HA-PIAAm, (d, d₀) LA-PIAAm, and (b, b₀) EL(ad)-PIPAAm surfaces. The N1s core-level spectra of EL-PIPAAm, EL(ad)-PIPAAm and LA-PIPAAm are all located around 399.0 eV. The N1s peak represents nitrogen atoms found in the peptide bond and side chains of amino acids in these ECM proteins while nitrogen atoms are found in the N-acetyl glucosamine structure of HA. The N1s peak is at the same binding energy in all four XPS spectra because the difference in the chemical groups adjacent to nitrogen atoms on the four ECM molecules does not influence the electronic state of the nitrogen.

The C 1s core-level spectra of EL-PIPAAm, EL(ad)-PIPAAm and LA-PIPAAm are best fitted with five peaks with binding energies of 284.55, 285.34, 286.15, 287.6, and 288.2 eV, which are attributed to C-H, C-N, C-O, O=C-N and O=C-O groups, respectively. C-H, C-N, and O=C-N groups as

mentioned above originated from the PIPAAm surface, while C-N, C-O, O=C-N (amine species) and O=C-O (alcohol species) are associated with the peptide bonds in ECM proteins. Compared to the bare PIPAAm surfaces with C-H group as the most significant peak¹²⁷, the contribution of protein groups in the XPS spectra of the ECM molecule modified PIPAAm surfaces is most prominent. This confirms that proteins have been successfully coated onto PIPAAm either through physical coating or chemical adsorption.

By comparing the protein representative peaks of EL-PIPAAm and EL(ad)-PIPAAm, higher signal due to the C-O and O=C-O species was detected on EL-PIPAAm. This result as mentioned above is likely caused by the different preparation methods. Casting of elastin solution on top of PIPAAm surface triggers the condensation of all the elastin molecules into a hydrogel-like layer, while the chemical adsorption of elastin onto PIPAAm surface is driven by the combinations of intermolecular forces between PIPAAm chain and elastin, e.g., hydrophobic forces, electrostatics, etc^{130, 131}. It would be in further interest to investigate on the surface chemistry of these elastin films when they are prepared under different methods. Anyway, PIPAAm and proteins are both mainly composed of carbon, nitrogen, and oxygen, XPS cannot be used as a reliable indicator of film thickness.

Chapter 6

Dynamics of Smooth Muscle Cell Deadhesion from Thermosensitive Hydroxybutyl Chitosan

6.1 Summary

The modification of tissue culture dish with thermo-responsive polymer (TRP) has enabled the enzyme-free harvesting of cells through an acute increase in surface hydrophilicity of TRP across its lower critical solution temperature (LCST). To date, the intricate mechanisms of cell deadhesion and detachment from TRP surface have not been investigated. Biophysical insights into the mechanochemical transduction of cells undergoing the temperature-triggered responses on TRP will facilitate the engineering of tailored processes for cell regeneration. In this part of my work, highly integrative biophysical techniques are applied to probe the thermal-induced kinetics of smooth muscle cell (SMC) deadhesion on thermo-responsive hydroxybutyl chitosan (HBC29) against a range of pre-culture time at 37 °C. From the last chapter, atomic force microscopy demonstrates that both the surface topology and mechanical property of HBC29 film are acutely transformed across LCST. As a control, it is shown that cells have negligible changes in adhesion contact area during low-temperature incubation on unmodified tissue culture polystyrene (TCPS). Secondly, the presence of HBC29 coating on TCPS has triggered the recession of adhesion contact and retraction of cell body of SMC with different pre-culture times. Interestingly, the rate of reduction in the normalized adhesion contact area of SMC is negatively correlated with the pre-culture time. Thirdly, the degree of cell deformation and average adhesion energy are reducing functions of time only for SMC pre-cultured for 3 hrs. In contrast, adhesion energy per cell is a reducing functions of time in irrespective to the pre-culture time. Lastly, the transformation of cytoskeleton organization of SMC during the thermal-induced deadhesion is strongly dependent

on the pre-culture time. Overall, our study has demonstrated that the deadhesion kinetics of thermal-induced on TRP is quantifiable with our biophysical approaches and will provide new design principles for cell sheet engineering.

6.2 Introduction

As mentioned previously, thermo-responsive polymer (TRP) is an emerging class of biomaterial which has received much attention from the biomedical communities. The huge potential of TRP is stemmed from its dramatic physical responses against the change of external temperature²². One of the key physical parameters characterizing the thermal sensitivity of TRP is the lower critical solution temperature (LCST). Specifically, the significant increase in hydrophobicity of TRP above LCST induces the intermolecular aggregation of TRP. The acute switching between hydrophobic and hydrophilic properties of TRP against the change of temperature opens new opportunities in tissue engineering. It is because cells adhere and detach on the hydrophobic TRP (above LCST) and hydrophilic TRP (below LCST), respectively¹³². By culturing cells on TRP above LCST until the reach of confluent layer, the resulted cell sheet is released into the culture medium below LCST. The use of TRP eliminates the uses of proteolytic enzymes like dispase or physical scraping for recovering cells from tissue culture dish. Both methods of cell/tissue recovery as mentioned above lead to the disruption of intracellular junctions and alteration of native tissue organization. During the past decade, Okano and coworkers has pioneered the use of poly(N-isopropylacrylamide) PIPAAm which is chemically grafted onto tissue culture polystyrene (TCPS) for the regeneration and recovery of keratinocytes, Madin-Darby canine kidney (MDCK) cell, hepatocyte, etc²⁴.

The physiochemical properties of TRP and cell type are known to influence the time taken for complete detachment of cell sheet. It has been shown that the time of cell sheet detachment from various forms of PIPAAm ranges from 3 mins to 24 hrs after the temperature is ramped below LCST¹³³. Despite the rapid development of cell sheet engineering with TRP, there is clearly a lack of

quantitative correlation between the strength of cell-substrate interaction, biophysical responses of cells, physiochemical properties of TRP, and ultimately cell functions during the course of cell regeneration and detachment. In particular, the kinetics of cell deadhesion and detachment on TRP during the thermal-induced process on TRP is not known. Our group has actively engaged in the development of integrated biophysical techniques for elucidating the adhesion contact dynamics of several cell types on biomaterials during the initial cell seeding¹⁰². We hypothesize that our unique biophysical approaches would provide new insights into the cell deadhesion/detachment kinetics towards temperature drop on TRP surface.

Hydroxybutyl chitosan (HBC), a promising TRP which is synthesized by conjugation of hydroxybutyl groups to chitosan is used herein. Recently, HBC29 with LCST of 29°C has been developed for the therapeutic applications related to injectable delivery of human mesenchymal stem cells⁵⁶. In this study, the deadhesion kinetics of SMC from HBC29 supported on TCPS is probed with confocal reflectance interference contrast microscopy (C-RICM) in combination of phase contact microscopy. Smooth muscle cell is chosen as our model system herein because the design and development of artificial blood vessel containing native SMC is critical area of tissue engineering¹³⁴. Specifically, A7r5 SMC cell exhibiting the characteristic phenotypes of SMC, namely the morphologic plasticity in converting from contractile cells to highly motile proliferative cells is an ideal platform for engineering specific biological responses. We aim to investigate the biophysical mechanism of cell deadhesion on HBC29 against the change of cell culture time at 37 °C beforehand.

Along the way, several kinetic parameters including normalized contact area and degree of cell deformation against time of low-temperature incubation for SMC with different pre-culture time is determined. Also, the average adhesion energy and adhesion energy per cell of SMC during the course of deadhesion from HBC29 surface is determined. Moreover, the cytoskeleton structure of SMC during the course of thermal-induced deadhesion is probed with fluorescence microscopy

through immuno-staining of actin. Our results as described herein will provide a solid foundation for developing new design principles in cell sheet engineering for cells/tissues regeneration with the desirable phenotypes.

6.3 Result and Discussion

Fig. 6.3.1. The real-time phase contrast images and C-RICM images of a typical SMC pre-cultured for (A) 3 hrs and (B) 24 hrs at 37 °C and 5% CO₂ on untreated TCPS surfaces after 2 mins and 15 hrs of incubation at 18 °C and 5% CO₂. The scale bar represents 10 μm.

Before we can correctly attest the effect of the thermal transition of TRP (coated on TCPS) on the biophysical dynamics of adherent cells, we need to investigate the behavior of seeded cells on unmodified TCPS during a temperature drop across LCST. Fig. 6.3.1A shows the real-time phase contrast images and C-RICM images of a typical SMC (pre-cultured for 3 hrs at 37 °C and 5% CO₂) on untreated TCPS surfaces after 2 mins and 15 hrs of incubation at 18 °C and 5% CO₂. From the phase contrast image, it is demonstrated that the cell adopts the typical elongated morphology after seeding on TCPS for three hrs at the physiological temperature. From C-RICM image, it is shown that strong adhesion contact (dark region) is formed between the cell and TCPS surface. Also,

the projected area and adhesion contact area is $479.8 \mu\text{m}^2$ and $356.0 \mu\text{m}^2$, respectively, after 2 mins of 18°C incubation. Based on conventional geometry of adherent cell, the adhesion contact area will never exceed the projected cell area. Interestingly, the cell still maintains its elongated morphology despite its subjection to 15 hrs incubation at 18°C . Cell spreading continues as shown by the increase of projected area from $479.8 \mu\text{m}^2$ to $499.6 \mu\text{m}^2$ from 2 mins to 15 hrs after low-temperature incubation. In parallel, the adhesion contact zone slightly grows from $356 \mu\text{m}^2$ to $360.0 \mu\text{m}^2$ during the same period. The result demonstrates that a temperature drop alone is not sufficient to trigger SMC deadhesion/detachment from unmodified TCPS surface after strong adhesion contact has been established during 3 hrs of pre-culture at 37°C .

It is interesting to investigate the effect of pre-culture time at physiological temperature on the biophysical responses of SMC during low-temperature incubation. Fig. 6.3.1B shows the real-time phase contrast images and C-RICM images of a typical SMC (pre-cultured for 24 hrs at 37°C and 5% CO_2) on untreated TCPS surfaces after 2 mins and 15 hrs of incubation at 18°C and 5% CO_2 . The SMC initially adopts a more elongated morphology after 24 hour pre-culture in comparison to that of SMC after 3 hr pre-culture. After 15 hrs, the phase contrast image indicates that the middle part of cell body (arrowhead) significantly contracts through the formation of longer lamellipodium. During the same period, C-RICM shows that the adhesion contact area recesses within the same region of SMC contraction. Specifically, the projected area and adhesion contact area is reduced from 985.3 to $715.2 \mu\text{m}^2$ (by 27 %) and 721.0 to $637.8 \mu\text{m}^2$ (11.5 %), respectively, from 2 mins to 15 hrs at 18°C . In comparison with the SMC at lower pre-culture time of 3 hrs, the result indicates that the prolonged culture at 37°C leads to the higher % reductions in projected area and adhesion contact area during low-temperature incubation. Interestingly, the cell remains strongly adhered on TCPS as shown by the moderate reduction in adhesion contact area. Our result is supported by a recent study which demonstrated that the viability, expansion and phenotype of peripheral blood lymphocytes are unaltered

after low-temperature incubation¹³⁵. The cell contraction is likely caused by the depletion of the nutrients in the culture medium after 24 hrs of 37 °C pre-culture. In the absence of TRP, there is no significant thermal-induced deadhesion of SMC on untreated TCPS for prolonged period which is significantly longer than that for our cell deadhesion assay on TRP.

Fig. 6.3.2 A series of (a) phase contrast images and (b) C-RICM images for a typical SMC on HBC 29 surface which has been pre-cultured at 37°C for 3 hrs at different times during 18°C incubation. The scale-bar represents 10 μm .

The physical properties of HBC29 film as mentioned above likely alter the thermal-induced response of adherent cell. Fig. 6.3.2 shows a series of (a) phase contrast images and (b) C-RICM images of a typical SMC pre-cultured at 37 °C for 3 hrs on HBC29 surface during different times of 18 °C incubation. After 5 mins of low-temperature incubation, the cell demonstrates an elongated morphology with a projected area of 295.2 μm^2 as shown by the phase contrast image. Simultaneously, C-RICM image demonstrates that the initial adhesion contact with an area of 179.9 μm^2 completely falls within the projected area. Our result validates that HBC29 is bio-compatible for SMC adhesion compared to unmodified TCPS. From 5 to 10 mins, it is shown that the adhesion contact acutely recedes (arrowhead on C-RICM image) in parallel with the retraction of the lamellipodium at the bottom half of the cell body (arrowhead on phase contrast image). During the period, the cell transforms from an elongated to round

geometry. At 10 mins, the projected area and adhesion contact area is $164.9 \mu\text{m}^2$ and $120.8 \mu\text{m}^2$, respectively. From 10 to 20 mins, the adhesion contact area is further reduced to $84.8 \mu\text{m}^2$ while the projected area remains constant. From 20 to 60 mins, the cell remains round in shape without any lamellipodium formation and has further reduction in adhesion contact area from 84.8 to $36.1 \mu\text{m}^2$. Overall, the adhesion contact area and projected area is reduced by 45 % and 82 %, respectively. The result also indicates that cell retraction doesn't synergize with the adhesion contact recession. Generally, our results are caused by the acute reduction in hydrophobicity of HBC29 during its thermal transition across its LCST (29°C). The lack of complete cell detachment during low temperature incubation is likely linked to the presence of serum proteins which replenish the desorbed ECM proteins on HBC 29 surface during low-temperature incubation.

Fig. 6.3.3. A series of (a) phase contrast images and (b) C-RICM images for a typical SMC on HBC 29 surface which has been pre-cultured at 37°C for 9 hrs at different times during 18°C incubation. The scale-bar represents $10 \mu\text{m}$.

The change of pre-culture time on TRP may affect the subsequent biophysical responses of SMC during low-temperature incubation. Fig. 6.3.3 shows a series of (a) phase contrast images and (b) C-RICM images of a typical SMC pre-cultured at 37°C for 9 hrs on HBC29 surface at different times during 18°C incubation. After 5 mins, the cell maintains an elongated morphology with a

projected area and adhesion contact area of $669.1 \mu\text{m}^2$ and $345.4 \mu\text{m}^2$, respectively. From 5 to 10 mins, the lamellipodium at the bottom cell body slightly retracts upward (projected area: $559.1 \mu\text{m}^2$) without the abrupt recession of adhesion contact (with area of $335.7 \mu\text{m}^2$) as found in cell pre-cultured for 3 hrs. From 10 to 20 mins, phase contrast image shows that the lamellipodium significantly reduce in size (projected area: $226.6 \mu\text{m}^2$) and fails to form strong adhesion contact (contact area: $124.7 \mu\text{m}^2$). At 20 mins, the relative length of the lamellipodium of the cell pre-cultured for 9 hrs (Aspect ratio: 3.33) is significantly longer than that of cell pre-cultured for 3 hrs (Aspect ratio: 1.42). From 20 mins onward, the cell continues to transform from elongated to elliptical shape until the lamellipodium disappears at 60 mins. Overall, the projected area and adhesion contact area is reduced by 75 % and 70 %, respectively. Generally, the increase of pre-culture time leads to a change of cell deadhesion mechanism on HBC29 surface, e.g., prolonged presence of lamellipodium.

Fig. 6.3.4 A series of (a) phase contrast images and (b) C-RICM images for a typical SMC on HBC29 surface which has been pre-cultured at 37°C for 24 hrs at different times during low-temperature (18°C) incubation under 5% CO_2 . The scale-bar represents $10 \mu\text{m}$.

Fig. 6.3.4 shows a series of (a) phase contrast images and (b) C-RICM images for a typical SMC on HBC29 surface which has been pre-cultured for 24 hrs (at 37°C) at different times of 18°C incubation. After 5 mins, phase contract image indicates the SMC is highly elongated and form highly extended lamellipodium.

Also, the projected area and adhesion contact area is $921.2 \mu\text{m}^2$ and $593.7 \mu\text{m}^2$, respectively. From 5 to 20 mins, the cell moderately retracts (projected area: $771.4 \mu\text{m}^2$) and reduces in strong adhesion contact (with area of $492.7 \mu\text{m}^2$) in contrast to the rapid retraction found in cell pre-cultured for 3 hrs at 37°C . Interestingly, the relative length of the lamellipodium at 20 mins is highest (Aspect ratio: 4.3) among cells under different pre-culture times. From 20 to 30 mins, the cell significantly retracts (projected area reduces from 771.4 to $379.1 \mu\text{m}^2$) and transform from an elongated to elliptical geometry. Also, the abrupt retraction is concurrent with the recession of adhesion contact (area reduces from 492.7 to $272.4 \mu\text{m}^2$). After 60 mins, thin lamellipodium which are not found in cell pre-cultured for 3 or 9 hrs remain on the cell body. Overall, the projected area and adhesion contact is reduced by 63 % and 62 %, respectively. Interestingly, the trend as mentioned above indicates that further increase of pre-culture time delays the retraction and deadhesion of cell on HBC 29 surface. Our results herein haven't demonstrated the migration and complete detachment of cells like BAECs on PIPAAm surface¹³⁶. It is because the physiochemical properties of HBC29 are different from those of PIPAAm. Moreover, SMC chosen in our study is different from BAECs. The exact difference in the mechanisms of thermal-induced responses between our HBC29 system and PIPAAm system needs to be clarified by future biophysical investigations.

C-RICM has been proven as an effective tool for probing the adhesion contact dynamics of mammalian cells on biomaterials during the initial stage of cell seeding¹⁰¹. In this study, we aim to exploit the change of cell-substrate interaction towards the thermal transition of TRP. Therefore a new parameter known as the normalized adhesion contact is defined herein by taking the ratio between the initial adhesion contact area (a_0) and the adhesion contact area at anytime during the low-temperature incubation (a).

(a)

(b)

Fig. 6.3.5. (a) Average a/a_0 and (b) the average a/R for SMC pre-incubated for 3 hours, 9 hours and 24 hours against time during low-temperature incubation at 18°C. The error bar is the standard error of at least 100 cells on five identical samples.

Culture Time (hr)	m	n
24	0.54	0.0288
9	0.535	0.0377
3	0.688	0.0401

Table 6.1. Fitted parameters in the deadhesion kinetics of smooth muscle cells on HBC 29.

Fig. 6.3.5a shows the average a/a_0 for SMC pre-incubated for 3 hrs, 9 hrs and 24 hrs against time of low-temperature incubation at 18 °C. The error bar is the standard error of at least 80 cells on five identical samples. In general, a/a_0 against time follows the trend of exponential decay until it reaches a steady-state level regardless of the pre-culture time. The reduction of a/a_0 is faster at the early stage of cell deadhesion due to the acute retraction of cell bodies and loss in adhesion contact area as mentioned above. By fitting the experimental data with the following exponential decay function, $y = y_o + me^{-nt}$, (fitted lines on Fig. 7.3.2.4a), where y represents the degree of deformation a/R and t represents the period of cell deadhesion. When $t=\infty$, y is equal to y_o , which represents the degree of deformation when cell deadhesion reach steady state. When $t=0$, y is equal to $(y_o + m)$, the initial degree of deformation. The kinetic coefficient m , and n are obtained (Table 6.1). The resulted fittings of the experimental data capture both the fast decay of a/a_0 at earlier time point and the reach of steady-state level at later time. It is determined that the steady-state value of a/a_0 which is equal to y_o for cell pre-cultured at 3 hrs, 9 hrs and 24 hrs is 0.31, 0.47 and 0.46, respectively. Moreover, n for cells pre-cultured at 3 hrs, 9 hrs and 24 hrs is 0.0401 min^{-1} , 0.0377 min^{-1} and 0.0288 min^{-1} , respectively. The result indicates that initial rate in the reduction of a/a_0 is highest for cells which are pre-cultured for 3 hrs at 37 °C. Moreover, the increase of pre-culture time leads to significant reduction of n by 6 % and 28 % in cells pre-cultured for 9 and 24 hrs, respectively, compared to cells pre-cultured for 3 hrs. The trend of n is caused by the increase of cell integration with HBC29 surface against the increase of pre-culture time at the physiological temperature.

Several biological functions including cell fusions, proliferation and differentiation are induced by cell deformation upon adhesive interactions on ECM or biomaterials. So far, the kinetics of adhesion contact recession for SMC on HBC 29 during the thermal-induced process is investigated. However, the change of A/A_0 is not directly correlated with the geometric transformation of the cell during contact deadhesion. To quantify the change of geometry of SMC, a

biophysical parameter known as the degree of cell deformation (a/R) plays a crucial role⁹⁵. Fig. 6.3.5b shows the average a/R for SMC pre-incubated for 3 hrs, 9 hrs and 24 hrs (at 37 °C) against time during low-temperature incubation at 18 °C. The error bar is the standard error of at least 80 cells on five identical samples. The result indicates that average a/R for cells pre-cultured on HBC 29 for 9 and 24 hrs remains constant at around 0.78 ± 0.2 and 0.84 ± 0.2 , respectively, during 150 mins of low-temperature incubation. The constant value of a/R during the cell deadhesion indicates that the cell morphology and adhesion contact simultaneously recede against time. In contrast, SMC pre-cultured for 3 hrs demonstrates a significant reduction of average a/R from 0.81 ± 0.2 to 0.64 ± 0.2 from 15 to 150 mins. This reduce of a/R indicates that cell adhesion contact a recedes faster than cell morphology R . Compared to the 9 hrs and 24hrs preculture samle, we could conclude that the reduction of pre-culture time to 3 hrs leads to the loss of synergy between adhesion contact and cell morphology. Specifically, the higher rate of adhesion contact recession in comparison with cell body retraction may induce the “pop up” of cell from the substrate. In the current study, a/R is directly calculated from the square root of the ratio of adhesion contact area and projected area, which doesn't affect the general analysis of cell geometry [Wan K. T., personal communication].

Average adhesion energy is directly correlated with the degree of deformation and elastic modulus of cell as shown in our contact mechanics model⁹⁵. Fig. 6.3.6a shows the average adhesion energy for SMC pre-cultured for 3 hrs, 9 hrs and 24 hrs against time during low-temperature incubation at 18 °C. The error bar is the standard deviation of at least 80 cells on five identical samples. The result shows that the average adhesion energy of cells pre-cultured for 9 and 24 hrs stays constant at around $1.1 \pm 0.1 \times 10^{-7}$ and $1.4 \pm 0.1 \times 10^{-7}$ J/m², respectively, from 5 to 120 mins after low-temperature incubation. The trend as mentioned above may still imply a change on the adhesion strength between cell and substrate because the energy unit is normalized with adhesion contact area which is a reducing function of time. In contrast, average adhesion energy of cells

pre-cultured for 3 hrs steadily reduces from $1.3 \pm 0.1 \times 10^{-7}$ to $3.0 \pm 1.1 \times 10^{-8} \text{ J/m}^2$ (by around 2.4 times) from 5 to 150 mins of low-temperature incubation. The result strongly supports that the pre-culture time directly moderates the dynamics of average adhesion energy during cell deadhesion on TRP below LCST.

(a)

(b)

Fig. 6.3.6. (a) The average adhesion energy and (b) the adhesion energy per cell for SMC pre-incubated for 3 hours, 9 hours and 24 hours against time during low-temperature incubation at 18 °C. The error bar is the standard error of at least 100 cells on five identical samples.

The adhesion energy per cell is calculated by average adhesion energy times adhesion contact area. It represents the overall adhesion energy that is necessary to be counteracted when the single cells are lifted up from the substrate. Fig. 6.3.6b shows the adhesion energy per cell for SMC pre-incubated for 3 hrs, 9 hrs and 24 hrs against time during low-temperature incubation at 18 °C. The error bar is the standard error of at least 80 cells on five identical samples. The result shows that the adhesion energy per cell of SMC pre-cultured for 9 and 24 hrs is reduced from $3.8 \pm 0.1 \times 10^{-17}$ to $1.8 \pm 0.2 \times 10^{-17}$ J (by around 2 times) and $5.3 \pm 0.2 \times 10^{-17}$ to $3.8 \pm 0.2 \times 10^{-17}$ J (by around 1.4 times), respectively, from 5 to 150 mins after 18 °C incubation. The trend as mentioned above is differed from that of the average adhesion energy because the adhesion energy per cell takes the change of adhesion contact area against time into account. Moreover, the extent in the reduction of adhesion energy per cell from $4.7 \pm 1 \times 10^{-17}$ to $5.3 \pm 0.7 \times 10^{-18}$ J (by around 9 times) is highest on cell pre-cultured for 3 hrs. Overall, the result strongly indicates that the adhesive interaction between individual cells and HBC 29 surface is reduced for all three types of cells and the extent of reduction in adhesion energy per cell is negatively correlated to the pre-culture time.

Fig 6.3.7. The fluorescence image of a typical SMC (actin staining) pre-cultured for (A) 3 hours, (B) 9 hours and (C) 24 hours at 37°C at 2 minutes, 30 minutes, and 60 minutes after low-temperature incubation at 18 °C. The scale-bar represents 10 μ m.

In response to environmental cues, an anchorage-dependent cell changes both its shape and its extent of attachment to substratum. These changes are often triggered by the rearrangements of the cytoskeleton. For instance, F-actin which is a major component of cytoskeleton induces finger-like protrusions of the plasma membrane known as filopodia during cell spreading and migration¹³⁷. If actin is assembled as a mesh-network, it supports sheet-like protrusions known as ruffles or lamellipodia. If actin forms bundles coupled to adhesion plaques, actin 'stress fibers' exert force against the substratum. It is important to correlate the kinetics of thermal-induced deadhesion with the cytoskeleton remodeling of SMC on TRP. Figure 6.3.7A shows the immuno-fluorescence images of a typical SMC pre-cultured for 3 hrs at 37 °C at 2 mins, 30 mins, and 60 mins after low-temperature incubation at 18 °C. The scale-bar represents 10 µm in length. After 2 mins, the result indicates that actin concentrates at the periphery of the elongated SMC. There is also sign of microfilament formation in the cytoplasm of the cell. The presence of actin in a parallel filamentous array suggests the development of a contractile structure, which is the characteristic phenotype of contractile SMC¹². The result supports that the cytoskeleton organization of SMC on HBC29 remains intact during the early stage of thermal-induced deadhesion. From 2 to 30 mins after low-temperature incubation, the actin further concentrates at cell periphery during the significant retraction of the cell. Also, the characteristic morphology of contractile SMC is lost. After 60 mins of low-temperature incubation, further recession of the actin ring at cell periphery is found to be coincided with the reduction of adhesion contact area.

The effect of pre-culture time may affect the cytoskeleton organization during cell deadhesion on TRP. Similar to cell pre-cultured for 3 hrs, actin concentrates at the cell periphery of SMC pre-cultured for 9 hrs during the first 2 mins of 18 °C incubation (Fig. 6.3.7B). At the same time, parallel actin microfilaments are found in the cytoplasm of the cell. From 5 to 30 mins, cell retraction has led to an increase of actin concentration at cell periphery. At the same time, an intense microfilament is formed in the middle of the cell body during the transformation

from elongated to elliptical shape. Interestingly, the typical phenotype of contractile SMC at 30 mins is more obvious than that of the cell pre-cultured for 3 hrs. After 60 mins of low-temperature incubation, the actin forms circular ring at the leading edge of lamellipodia during further retraction of cell. At the same time, more actin concentrates at the lamellipodium which is site for active mechanochemical transduction during cell adhesion in comparison with the cell pre-cultured for 3 hrs. The result indicates that the further increase of pre-culture time to 24 hrs leads to the expression of the typical SMC phenotypes after 2 mins of low-temperature incubation as shown by the active formation of lamellipodium and microfilaments composed of actin (Fig. 6.3.7C). Moreover, the cell still maintains the more convoluted shape (arrows) as outlined by the actin distribution after 30 mins in contrast to cells with less pre-culture time. Finally, the cell retraction from 30 to 60 mins leads to the formation of circular ring of actin at cell periphery. However, the convoluted extensions which are not found in cells with lower incubation time remains after 60 mins. Therefore, cytoskeleton transformation during low-temperature incubation is correlated with the biophysical dynamics of cells on HBC29. Taken together, the result reported herein on cytoskeleton organization is in accord with the result from the harvest of BAECs cell sheet. For BAECs cell sheet detachment, the peripheral bands composed of actin filaments are also preserved for shrinking the whole cell sheets¹².

Actin is a ubiquitous protein present in large quantity in all cells and tissues. For SMC, actin is distributed uniformly within the cell. It can be used to realize force development and tension maintenance for extended time intervals. In the actin family, β -actin is a cytoskeletal protein and induces the formation of microfilaments and stress fibers that influence the cytoskeleton architecture of the cell. In vascular SMC, β -actin rather than α -actin expression becomes dominant, particularly when SMC undergoes phenotypic modulation¹³⁸. During canine myocardium vascular remodeling, β -actin mRNA is upregulated in transmyocardial by direct-current (DC) shock treatment¹³⁹. Therefore β -actin gene

expression has served as a key marker for phenotypic modulation of SMC and vascular remodeling.

Fig. 6.3.8. (A) The β -actin RT-PCR products arise from amplification of mRNA extracted from detaching A7r5 Smooth Muscle Cells from HBC29. Lane 1 shows the negative control. Lane 2 shows the 0 hr incubation at 18 °C after 3 hr-precultured at 37 °C. Lane 3 shows the 0.5 hr incubation at 18 °C after 3 hr-precultured at 37 °C. Lane 4 shows the 1 hr incubation at 18 °C after 3 hr-precultured at 37 °C. Lane 5 shows the 0 hr incubation at 18 °C after 24 hr-precultured at 37 °C. Lane 6 shows the 0.5 hr incubation at 18 °C after 24 hr-precultured at 37 °C. Lane 7 shows the 1 hr incubation at 18 °C after 24 hr-precultured at 37 °C. (B) The smoothelin-B RT-PCR products arise from amplification of mRNA extracted from A7r5 Smooth Muscle Cells from HBC29 surface. Lane 1 shows the negative control. Lane 2 shows the 0 hr incubation at 18 °C after 3 hr-precultured at 37 °C. Lane 3 shows the 0.5 hr incubation at 18 °C after 3 hr-precultured at 37 °C. Lane 4 shows the 1 hr incubation at 18 °C after 3 hr-precultured at 37 °C. Lane 5 shows the 0 hr incubation at 18 °C after 24 hr-precultured at 37 °C. Lane 6 shows the 0.5 hr incubation at 18 °C after 24 hr-precultured at 37 °C. Lane 7 shows the 1 hr incubation at 18 °C after 24 hr-precultured at 37 °C.

Fig. 6.3.8A shows the temporal trend in β -actin mRNA expression after cell pre-cultured at 37 °C for 3 hrs (lane 2-4) and 24 hrs (lane 5-7). For 3 hr-precultured cells, after 30 mins of incubation at 18 °C, the expression of β -actin mRNA is significantly reduced compared to cells at 37 °C. Towards 60 mins of 18 °C incubation, the level of β -actin expression is unaltered compared to the cells with 30 mins of 18 °C incubation. This result is consistent with the phase contrast images in Fig. 6.3.2. After 30mins of incubation at 18 °C, most cells change to round morphology and unaltered until 60 mins. For 24 hr pre-cultured cells, the expression of β -actin mRNA is significantly higher than that of 3 hr-precultured cells before incubation at 18 °C. The result is in accordance with the higher degree of cell spreading on the TRP surface against the increase of pre-culture time after seeding. It is because cells synthesize more cytoskeletal β -actin when they are spreading and growing. After incubation of the 24 hr pre-cultured cells at 18 °C for

30 mins, the β -actin expression does not change significantly. The result is supported by the slower changes in cell morphology and adhesion contact area for 24 hr pre-cultured cells during first 30mins of incubation at 18 °C (Fig. 6.3.4). After 60 mins of incubation at 18 °C, the mRNA expression is significantly reduced to the level close to that of 1 hr pre-cultured cell. This result follows the trend of the cell morphology and adhesion kinetics data. For example, after 60mins of incubation at 18 °C, both the 3 hr-precultured and 24 hr-precultured cells shrink to a truncated spherical shape.

Smoothelin-B is another SMC specific protein which is not commonly found in all vascular tissues but only restricted to smooth muscle cells, mostly in contractile and vascular SMC. Cells with SMC-like characteristics, such as myofibroblasts and myoepithelial cells, as well as skeletal and cardiac muscle did not contain smoothelin¹⁴⁰. Moreover, smoothelin-B expression is more sensitive to change of phenotypes than smooth muscle α -actin and desmin. The distribution of smoothelin-B positive cells indicates a correlation between the smoothelin-B expression and contractility of smooth muscle tissue. The differentiation and dedifferentiation are important features in the development of the vascular tree and in proliferative vascular diseases^{66, 141-143} and the expression of smoothelin-B coincides with the degree of differentiation of SMC. Therefore smoothelin-B is used as specific molecular markers to describe the phenotypes of SMC¹⁴⁴.

Fig 6.3.8B shows the temporal trend in the expression of smoothelin-B mRNA after cell pre-cultured at 37 °C for 3 hrs or 24 hrs. Before the low temperature incubation, both kinds of cells show the positive expression of smoothelin-B which indicates the contractile phenotype of the SMC (Lane 2 and Lane 5). For both kinds of pre-cultured cells, the expression of smoothelin-B significantly increases during the detachment process. Significant increase in smoothelin-B expression occurs at 30 mins and 60 mins of incubation at 18 °C for 3 hr pre-cultured cells and 24 hr pre-cultured cells, respectively. The increase of lag time of smoothelin-B expression with the increase of pre-culture time coincides with the delay in the biophysical responses of SMC on TRP below its LCST.

After 60 mins of incubation at 18 °C, the 3 hr pre-cultured cells express more smoothelin-B than 24 hr pre-culture cells. The result strongly indicates that the low temperature treatment alter the cells contractility during cell deadhesion. In previous studies, for porcine coronary artery SMC, the smoothelin-B expression is reduced when cells are transferred from primary culture to the later passage¹⁴⁵. In our experiment, the result shows that the smoothelin-B expression of SMC at the same passage alters against the change of pre-culture time. The increase of smoothelin-B expression indicates that the cells are more differentiated during the cell detachment process.

6.4 Conclusion

In summary, we have thoroughly demonstrated the new possibility of engineering the cell deadhesion dynamics with cell culture parameters on a model TRP in order to control the cytoskeleton organization and specific phenotypes of SMC. It is shown that several key biophysical parameters including normalized adhesion contact area, degree of deformation and adhesion energy per cell are correlated with the pre-culture time of SMC before low-temperature incubation. Lastly, the cytoskeleton remodeling is correlated with the deadhesion dynamics of SMC.

Chapter 7

Dynamics of Smooth Muscle Cell Deadhesion from Thermoresponsive PIPAAm (poly(N-isopropylacrylamide))

7.1 Summary

Poly(N-isopropylacrylamide) (PIPAAm) has been demonstrated as an effective thermo-responsive polymer (TRP) for non-invasive cell regeneration/recovery. Despite the advance in surface functionalization techniques of PIPAAm, little is known on the intricate relationship between the biophysical response of cells and physiochemical properties of PIPAAm during cell recovery. In this study, the deadhesion kinetics of smooth muscle cells (SMC) on PIPAAm surfaces under different polymerization time or collagen coating is probed with biophysical microscopy. It is found that the initial rate of cell deadhesion is increased against the increase of polymerization time of PIPAAm. However, the initial rate and extent of reduction in the degree of cell deformation (a/R) and the adhesion energy per cell against the density of grafted PIPAAm shows a biphasic behavior. The results are further correlated with the distinct trends in the nano-topological transformation under different PIPAAm density across its lower critical solution temperature (LCST) as shown by water-immersion AFM. Moreover, collagen coating on PIPAAm surface which enhances the adhesion contact formation during cell seeding still supports cell deadhesion below LCST. Interestingly, lower collagen density on PIPAAm enhances the initial deadhesion rate and extent of reduction in averaged adhesion energy compared to plain PIPAAm while the adhesion energy per cell is unaffected by collagen. In contrast, higher collagen density nearly abolishes cell deadhesion as shown by unaltered a/R and adhesion energy during cell deadhesion. Furthermore, immunostaining of actin and vinculin supports that the contractile phenotypes of SMC are preserved on various PIPAAm surfaces below LCST.

7.2 Introduction

Although chitosan and its derivatives like (HBC29 used in previous chapter) are bio-compatible materials, they have not been widely used applied in tissue engineering as of today. Poly(N-isopropylacrylamide) (PIPAAm) is one of the earliest members of TRP family, and has emerged as a promising class of biomaterials for the regenerations of hepatocytes, endothelial cells, urothelial cells, epithelium cells, Madin-Darby canine kidney and fibroblasts^{21, 22, 146}. The application of PIPAAm for the non-invasive recovery of cells/tissues is brought about by its acute switching of physiochemical properties across its LCST. Moreover, LCST of PIPAAm can be engineered with the design of block copolymers containing PIPAAm and other polymer. To date, PIPAAm and its copolymers have been widely exploited for several applications in bio-separation, drug delivery cell and other thermal responsive devices¹⁴⁷⁻¹⁴⁹.

Chemical grafting of PIPAAm onto tissue culture polystyrene (TCPS) can be realized by electrical beam irradiation¹⁵⁰. Alternatively, PIPAAm films may be prepared by physical adsorption of polymer chain onto glass¹⁵¹ and by plasma enhanced polymerization of NIPAAm onto TCPS¹⁵². It has been shown that the acute increase of hydrophilicity on PIPAAm films against the reduction of temperature drives the deadhesion of confluent cell layer. Moreover, the time required for complete cell sheet detachment from various types of PIPAAm films is highly dependent on cell types¹³³. Besides, the thickness of PIPAAm film would also affect the cell detachment mechanisms⁵⁷. Therefore the elucidation of the kinetics of cell deadhesion from different PIPAAm films would likely provide important insights for designing tailored process for cell regenerations.

The biocompatibility of PIPAAm is less ideal than conventional TCPS for inducing strong cell adhesion which is critical for effective tissue regeneration⁵⁸. It is shown that the biocompatibility of PIPAAm is improved by the incorporation of the hydrophobic co-polymer into the PIPAAm chain^{36, 38}. Alternatively, the biocompatibility of PIPAAm can be enhanced by coating the polymer surface with extracellular matrix (ECM) proteins⁵⁹. For example, collagen is the most abundant

ECM protein found in mammalian tissues and forms fibrous fiber with ideal tensile strength^{153, 154}. Therefore collagen network provides structural integrity in adult muscularized arteries and plays key roles in the physiological regulations of smooth muscle cells (SMC), e.g., proliferation inside blood vessel. SMC is an interesting model system for TRP because the non-invasive recovery of engineered tissues containing native SMC is critical area of vascular tissue engineering¹³⁴. It is also shown that cell adhesion and migration on biomaterial surfaces are dependent on the density of adsorbed collagen⁶⁰.

Our group has actively engaged in the development of integrated biophysical techniques for elucidating the adhesion contact dynamics of several cell types on biomaterials during the initial cell seeding^{102, 103}. In the last chapter, I have discussed the deadhesion kinetics of single SMC from hydroxybutyl chitosan by using this unique approach. In this part of my work, I have developed an improved two-step reaction for coupling PIPAAm chains to silicon coverslips. The method as mentioned above enables the control of polymer film thickness at nanoscale through the change of polymerization time⁸⁶. From the best of our knowledge, there is currently a lack of quantitative correlation between the physiochemical properties of PIPAAm films and the resulting biophysical responses of cells upon temperature reduction. Based on the pivotal roles of collagen in SMC adhesion and proliferation, it is interesting to exploit the effect of collagen on the deadhesion kinetics of SMC from PIPAAm.

In this study, the dynamics of SMC deadhesion on a series of PIPAAm surfaces with different polymerization time and collagen coating are probed with our unique biophysical techniques. Specifically, confocal reflectance interference contrast microscopy (C-RICM) in combination of phase contact microscopy determined key biophysical parameters of cell deadhesion including initial deadhesion rate, cell geometry, averaged adhesion energy and adhesion energy per cell of SMC. In the meantime, the cytoskeleton structure of SMC during the course of thermal-induced deadhesion is detected by confocal fluorescence microscopy through immuno-staining of actin and vinculin. It is demonstrated that the

deadhesion dynamics and cytoskeletal transformation of SMC is influenced by both nano-topology of PIPAAm surfaces and collagen modification.

7.3 Results and Conclusion

7.3.1 PIPAAm synthesis

Fig. 7.3.1 Illustration of ATRP reaction, R: PIPAAm chain

We use a two step atom transfer radical polymerization (ATRP) reaction to synthesize the PIPAAm grafted glass surface. The simple scheme for the ATRP is shown in Fig. 7.3.1. For the preparation of polymer brushes on the glass surface, a uniform and dense monolayer of polymerization initiators which includes at least one transferable atom or group is indispensable was immobilized on the glass surface. Usually, halogen R-X (X - Cl or Br) is widely used initiator for ATRP reaction. In this part of our work, we used 4-(chloromethyl)phenyltrichlorosilane (CMPTS) to introduce halogen onto glass surface and Si-O cross-linked supporting layer before carrying ATRP. Cu(II) complex (CuCl_2) was chosen as the transition metal to control the concentration of the deactivating Cu(II) complex during the surface-initiated ATRP process on the Si-CMPTS surface.

1,1,4,7,10,10-Hexamethyl-triethylenetetramino (HMTETA) is chosen as a ligand to form a complex with the Cu(II) complex to modify solubility and catalytic activity. The [NIPAAm (monomer)] : [CuCl (catalyst)] : [CuCl₂ (deactivator)] : [HMTETA (ligand)] ratio was controlled at 150:1:0.2:2.5. By controlling the polymerization time, the PIPAAm grafted surfaces with different polymer density are obtained.

7.3.2 Effect of Polymerization Time on SMC De-adhesion

Fig. 7.3.2.1 A series of (a) phase contrast images and (b) C-RICM images for a typical SMC on PIPAAm-0.5hr surface which has been pre-cultured at 37°C for 3 hours at different times during 18°C incubation. The scale-bar represents 10 μ m.

C-RICM has been proven to be an effective tool for probing the real-time change in cell-biomaterial interactions¹⁰¹. Four types of PIPAAm surfaces with NIPAAm polymerization times of 5min, 0.5hr, 1 hr and 1.5hr were used herein. As indicated by AFM, 5 min of NIPAAm polymerization is not enough to completely cover the whole surface with PIPAAm chains (Fig 5.2.1(a)). Moreover, C-RICM result indicates that PIPAAm-5min is ineffective to trigger the cell de-adhesion even if temperature falls below LCST (data not shown here). Therefore, most cells firmly adhered on the silane layer on top of glass surface which solely supports cell adhesion. However, the typical elongation of SMC is

not detected on PIPAAm-5min.

Since cells failed to de-adhere from PIPAAm-5min, a series of PIPAAm with higher polymerization time is used for our biophysical study. Fig. 7.3.2.1 shows a set of (a) phase contrast images and (b) C-RICM images of a typical SMC (pre-cultured for 3 hrs at 37 °C and 5% CO₂) in real-time on PIPAAm-0.5hr from 0 to 120 min after incubation at 18 °C and 5% CO₂ atmosphere. At 37 °C, the cell adopts a diamond shape with the formation of lamellipodium (Fig. 7.3.2.1a) and establishes strong adhesion contact (dark region) after 3 hr culture on PIPAAm-0.5hr (Fig. 7.3.2.1b). The initial projected and adhesion contact area is 650 μm^2 and 525.3 μm^2 respectively, at 37 °C. Based on the elliptical or quasi-spherical geometry of most adherent cells, the adhesion contact area never exceeds the projected cell area. It takes 2 min for the temperature inside the on-line incubator to drop from 37 to 18 °C. During sample cooling, cell shape becomes elongated due to the recession of lamellipodium (0 min on Fig. 7.3.2.1a). During this period, the projected area and adhesion contact area is reduced to 545 μm^2 (by 16.2 %) and 443.5 μm^2 (by 15.6 %), respectively. From 0 to 20 min of 18 °C incubation, the projected area and contact area is significantly reduced from 545 μm^2 to 250.5 μm^2 (by 61.5 % in the initial area) and from 443.5 μm^2 to 198.1 μm^2 (62.3% in the initial area). The rate of recession of both areas is reduced after 30 min. After 60 min, the cell has shrunk to a round shape due to the active retraction of cell body. Overall, the projected and contact area is reduced to 205 μm^2 (by 68.5% in the initial area) and 143.2 μm^2 (by 72.7% in the initial area).

Interestingly, the contour of adhesion contact and that of cell projection synergistically recede during different stages of cell de-adhesion. The general mechanism of cell sheet detachment has been previously discussed by Okano *et al.* for PIPAAm-grafted TCPS¹⁰¹. First of all, the hydrophobic to hydrophilic transition of PIPAAm chains with the temperature drop below LCST has been known to induce passive detachment of cells. This is followed by active detachment triggered by cellular metabolism in order to complete cell detachment. Our result demonstrates that sufficient coverage of PIPAAm on the substrate has

successfully induced an acute change in the surface chemistry and has provided passive driving force to induce de-adhesion of single-cell within a relatively short time interval of low-temperature incubation. However, single cells fail to completely detach from the PIPAAm surface in the system used herein. The discrepancy is likely caused by the presence of intracellular contacts in confluent cell sheet as shown in a previous study²².

Fig. 7.3.2.2 A series of (a) phase contrast images and (b) C-RICM images for a typical SMC on PIPAAm-1hr surface which has been pre-cultured at 37°C for 3 hours at different times during 18°C incubation. The scale-bar represents 10 μm .

Since we have demonstrated that the polymerization time is related closely to the film surface topography, we could establish a relationship between the adhesion kinetics and the surface nano-topography with the polymerization time. Fig. 7.3.2.2 shows a series of (a) phase contrast images and (b) C-RICM images of a typical SMC (pre-cultured for 3 hrs at 37 °C and 5% CO₂ in air) in real-time on PIPAAm-1hr from 0 to 120 min after incubation at 18 °C and 5% CO₂. The result shows that the projected area (initial area: 777.5 μm^2) and adhesion contact area (initial area: 681.7 μm^2) is reduced by about 7.5% and 17.4%, respectively, immediately following sample cooling from 37 to 18 °C (0 min on Fig. 7.3.2.2a and 7.3.2.2b). The most dramatic change occurs from 0 to 10 min as the projected area and contact area are reduced from 719.2 μm^2 to 364.7 μm^2 (by 45.6

% in the initial area) and from $563.1\mu\text{m}^2$ to $279.8\mu\text{m}^2$ (by 41.5 % in the initial area), respectively. Moreover, the cell changes from a triangular to quasi-elliptical shape with the disappearance of the two small lamellipodia at the bottom part of the cell during this period (Fig. 7.3.2.2a). Finally, the cell adopts a round geometry after 20 min of incubation at 18 °C when the projected area and contact area becomes $220\mu\text{m}^2$ (reduced by 71.7 % from initial value) and $200\mu\text{m}^2$ (reduced by 70.7 % from initial value), respectively. The initial recession rates as mentioned above are significantly higher than that on PIPAAm-0.5hr.

Fig. 7.3.2.3 A series of (a) phase contrast images and (b) C-RICM images for a typical SMC on PIPAAm-1.5hr surface which has been pre-cultured at 37°C for 3 hours at different times during 18°C incubation. The scale-bar represents 10 μm .

(a)

(b)

Fig. 7.3.2.4. (a) Average a/a_0 and (b) average a/R for SMC pre-incubated on PIPAAm-0.5hr, PIPAAm-1hr and PIPAAm-1.5hr against time during low-temperature incubation at 18 °C. The error bar is the standard error of at least 60 cells on three identical samples.

Fig. 7.3.2.3 shows the corresponding series of images for SMC on PIPAAm-1.5hr from 0 to 120 mins after incubation at 18 °C and 5% CO₂. The initial projected area and adhesion contact area are reduced by 3.5% and 3.6%, respectively, during the first 2 min of cooling from 37 to 18 °C. From 0 to 10 min, the cell loses its elongated shape while the more dramatic change in cell shape

occurs when the projected area and adhesion contact area is reduced from 371.3 to 202 μm^2 (by 45.7% from initial value) and from 287.5 to 137 μm^2 (by 52.3% from initial value), respectively. The result shows that the initial recession rate of adhesion contact on PIPAAm-1.5hr is highest among the three surfaces used herein. For the all above three PIPAAm surface, no total cell detachment was found.

Normalized adhesion contact which is defined as the ratio between the adhesion contact area at anytime during the low-temperature incubation (a) and the initial adhesion contact area at 37 °C (a_0) was used to quantify the rate of adhesion contact recession during low-temperature incubation below the LCST. Fig 7.3.2.4a shows a plot of a/a_0 against time during SMC deadhesion on PIPAAm-0.5hr, PIPAAm-1hr and PIPAAm-1.5hr at 18 °C. The error bar is the standard error of at least 60 cells on three identical samples. As the adhesion contact area shrinks during deadhesion, a/a_0 for SMC on all three samples follows an exponential decay until it reaches a steady state at longer time. The extent of reduction in a/a_0 during the initial 10 min of deadhesion is lowest on PIPAAm-0.5hr. By fitting the experimental data with a typical exponential decay function, $y = y_o + me^{-nt}$, (fitted lines on Fig. 7.3.2.4a), where y represents the degree of deformation a/R and t represents the period of cell deadhesion. When $t=\infty$, y is equal to y_o , which represents the degree of deformation when cell deadhesion reach steady state. When $t=0$, y is equal to $(y_o + m)$, the initial degree of deformation. The kinetic coefficients m and n are obtained as shown in Table 7.3.2:

	y_o	m	n
PIPAAm-0.5hr	0.473	0.492	0.023
PIPAAm-1hr	0.619	0.307	0.057
PIPAAm-1.5hr	0.55	0.401	0.051
PIPAAm1.5hr+0.1x collagen	0.619	0.338	0.031
PIPAAm1.5hr+0.04x collagen	0.413	0.561	0.051
PIPAAm1.5hr+0.01x collagen	0.451	0.514	0.043

Table 7.3.2. Fitted parameters in the deadhesion kinetics of smooth muscle cells on PIPAAm and collagen coated PIPAAm surfaces.

A comparison of the n values, which quantify the initial decay rate of a/a_0 , shows that the lowest value is obtained for PIPAAm-0.5hr. This result thus

indicates that the rate of reduction in adhesion contact area of 0.023 min^{-1} is lowest for cells on PIPAAm-0.5hr. For PIPAAm-1hr and PIPAAm-1.5hr surface, n is 0.051 min^{-1} and 0.057 min^{-1} respectively. The result indicates that the initial decay rate of a/a_0 on the two surfaces is two times higher than that of PIPAAm-0.5hr but it is indifferent against the increase of polymerization time from 1 hr to 1.5 hr. Interestingly, cells on PIPAAm-0.5hr has the highest decay rate of a/a_0 beyond 45 min of 18°C incubation compared to that on PIPAAm-1hr and PIPAAm-1.5hr. This trend is also reflected in the lowest value of y_0 (0.473) on PIPAAm-0.5hr which represents the residual value of a/a_0 beyond 90 min of deadhesion.

While a/a_0 serves to probe the recession of adhesion contact area on various PIPAAm surfaces but it does not provide any insight into the actual geometry of adherent cells during the course of deadhesion. The degree of cell deformation (a/R) becomes critical⁹⁵ for quantifying the geometric transformation of the entire cell during deadhesion. Fig. 7.3.2.4b shows the average a/R for SMC against time during low-temperature incubation on PIPAAm-0.5hr, PIPAAm-1hr and PIPAAm-1.5hr at 18°C . The result indicates that the initial rate of reduction in a/R for PIPAAm-0.5hr, PIPAAm-1hr and PIPAAm-1.5hr surface is 0.005 min^{-1} , 0.0025 min^{-1} and 0.01 min^{-1} , respectively. Analogous to the trend of a/a_0 , the highest initial rate in reduction of a/R is found in PIPAAm-1.5hr. The result indicates that cell geometry on PIPAAm-1.5hr quickly changes from a highly deformed ellipsoid to a truncated sphere. At the same time, the adhesion contact area reduces faster than the projected area. In contrast to the trend of a/a_0 , the initial rate in reduction of a/R on PIPAAm-0.5hr is higher than that on PIPAAm-1hr. During the entire 18°C incubation, a/R is reduced from 0.89 to 0.75 (by 15.7 %) and 0.88 to 0.67 (by 23.8 %) on PIPAAm-1hr and PIPAAm-1.5hr, respectively. Interestingly, a/R of SMC continues to reduce significantly beyond 60 min only on PIPAAm-0.5hr. Similar to the trend of a/a_0 , the overall extent of reduction a/R from 0.85 to 0.56 is highest (by 34.1%) for SMC on PIPAAm-0.5hr.

(a)

(b)

Fig. 7.3.2.5. (a) Average adhesion energy and (b) adhesion Energy per cell for SMC pre-incubated on PIPAAm-0.5hr, PIPAAm-1hr and PIPAAm-1.5hr against time during low-temperature incubation at 18 °C. The error bar is the standard error of at least 60 cells on three identical samples.

The average adhesion energy is positively correlated with a/R and elastic modulus of cell as shown in a contact mechanics model of adherent cell⁹⁵. Fig. 7.3.2.5a shows the average adhesion energy of SMC on the series of PIPAAm surfaces during de-adhesion (at 18 °C) after pre-cultured for 3 hrs at 37 °C and 5% CO₂. Among the three samples, SMC on PIPAAm-0.5hr surface show the highest

reduction in average adhesion energy from $1.8 \pm 0.1 \times 10^{-7} \text{ J/m}^2$ to $1 \pm 0.1 \times 10^{-8} \text{ J/m}^2$ (by 18 times) from 0 to 90 min. Despite the higher initial rate of decay in average adhesion energy, the average adhesion energy on PIPAAm-1hr and PIPAAm-1.5hr is reduced from $1.8 \pm 0.1 \times 10^{-7} \text{ J/m}^2$ to $1.0 \pm 0.1 \times 10^{-7} \text{ J/m}^2$ and $1.8 \pm 0.1 \times 10^{-7} \text{ J/m}^2$ to $0.5 \pm 0.05 \times 10^{-7} \text{ J/m}^2$, respectively in the same time period, which is a smaller reduction compared to that on PIPAAm-0.5hr. The average adhesion energy does not truly quantify the adhesive interaction between cell and substrate because it is normalized with the adhesion contact area. To account for the change in adhesion contact area with time, another parameter, defined as the adhesion energy per cell which is the product of average adhesion energy and adhesion contact area, can be used. Fig. 7.3.2.5b shows the adhesion energy per cell for SMC on the series of PIPAAm surfaces during de-adhesion (at 18 °C) after pre-cultured for 3 hrs at 37 °C and 5% CO₂. On all the PIPAAm surfaces, adhesion energy per cell decreases significantly with time during 18 °C incubation. Initially, after 2 min of 18 °C incubation, the adhesion energy per cell on PIPAAm-0.5hr is $4.5 \pm 0.1 \times 10^{-17} \text{ J}$, which is 50 % and 30 % lower than those on PIPAAm-1hr and PIPAAm-1.5hr, respectively. For the first 60 min, the adhesion energy of SMC on PIPAAm-0.5hr is close to that on PIPAAm-1.5hr. However, the overall extent in the reduction of adhesion energy per cell from 0 to 90 min on PIPAAm-0.5hr surface is the largest among the three surfaces (a decrease of nearly two orders of magnitude). These results are related to the different surface nano-topologies of the polymers obtained at different polymerization time to some extent. For instance, the PIPAAm-1hr presents the roughest surface with RMS roughness of 8.4nm and 3.1nm at 18 °C and 37 °C, respectively, which is 5.05 and 2.81 times higher than that on PIPAAm-1.5hr. Our result thus demonstrates that the roughest PIPAAm surface supports stronger cell adhesion and retards the decay of adhesion energy of cells below LCST. A similar result has been reported for osteoblastic cells on nanometric polymer surface¹⁵⁵.

Fig. 7.3.2.6 The fluorescence image of a typical SMC (actin staining) pre-cultured on (a) PIPAAm-1.5hr, (b) PIPAAm-1hr and (c) PIPAAm-0.5hr at 37°C at 0 minutes and 30 minutes after low-temperature incubation at 18 °C. The scale-bar represents 10 μ m.

Previously, it has been shown that SMC go through cytoskeleton remodeling during de-adhesion through the loss of actin stress fibers on thermo-responsive chitosan hydrogel. Fig. 7.3.2.6 shows the immuno-fluorescence images of actin for representative SMC (pre-cultured for 3 hrs) on (a) PIPAAm-1.5hr, (b) PIPAAm-1hr and (c) PIPAAm-0.5hr at 0 min and 30 min after low-temperature incubation at 18 °C. In general, there is significant re-modeling of the actin structure during the course of deadhesion. Initially, the actins are concentrated at the periphery of the elongated SMC on all three surfaces. At the same time, the formation of micro-filaments which isotropically orient along the long axis of cell is detected in the cytoplasm. The extensive spreading combined with the polarization of cytoskeleton indicates that the cells adapt quite well onto PIPAAm surface above LCST. This typical organization of the actin cytoskeleton has been reported in SMC on other biomaterials¹⁵⁶. After 30 min of incubation at 18 °C, the cell on each PIPAAm surface strongly contracts from an elongated to a spherical shape. Moreover, the actin microfilaments originally found in SMC before deadhesion are significantly reduced in length and demonstrate a significant

increase in the anisotropic orientation in the cell cytoplasm. Interestingly, the concentration of actin at the cell periphery after 30 min on PIPAAm-1.5hr is obviously higher than that on PIPAAm-0.5hr. The cytoskeleton transformation during cell deadhesion on PIPAAm-1.5hr is likely to be correlated with the fast initial decay of a/a_0 and a/R of SMC through the increase of traction at the cell boundary. This is consistent with the recently reported role of actin cytoskeleton in the contractility of the cell¹⁵⁷. It would also be of interest to analysis in future the role of microtubule in the process of deadhesion, as the dynamics of microtubule has been shown to affect cell shape changes¹⁵⁸.

7.3.3 SMC De-adhesion from Collagen-Coated PIPAAm Surfaces

Although the contractile phenotype of SMC is maintained when they are spreading on the PIPAAm surfaces, the adhesion kinetics and percentage of adherent cells on this surface are lower than that on normal TCPS surface. Ligand modified surface may not only encourage cells to maintain differentiated state by simulating the *in vivo* micro-environment but also facilitate cell attachment and proliferation. For most types of cells, and especially for cells composed of connective-tissue, the probability for developing anchorage and attachment highly depends on the nature of the surrounding environment. It is known that smooth muscle cells are dispersed in connective tissue throughout the body, where they secrete an ECM that is rich in type I collagen and other proteins. For instance, collagen is a widely known ECM protein for the regulation of cellular behaviors such as adhesion, spreading, proliferation, and migration and thus has been used extensively to enhance cell-material interactions for both *in vivo* and *in vitro* applications¹⁵⁹. There are examples showing that the coating of collagen would not retard cell de-adhesion from TRP. For instance, the detachment of whole cell sheet of human foreskin fibroblasts from the methylcellulose (MC)/PBS/collagen hydrogel matrix only takes around 20 min after temperature drops below LCST¹⁶⁰.

Fig. 7.3.3.1. A series of (a) and (b) are phase contrast images and C-RICM images for a typical SMC on 0.12x-PIPAAm surface, (c) and (d) are phase contrast images and C-RICM images for a typical SMC on 0.3x-PIPAAm1.5hr surface which has been pre-cultured at 37°C for 3 hours at different times during 18°C incubation. The scale-bar represents 10 μ m.

Without the need of chemical cross-linking, collagen *I* self-assembles to form a hydrogel film on the PIPAAm surfaces at neutral pH and 37 °C. PIPAAm-1.5hr was chosen for coating with different concentrations of collagen herein because it leads to sharper decay of a/a_0 , a/R and average adhesion energy during the initial stage of cell de-adhesion compared to other two surfaces. As predicted, the adhesion kinetics of SMC upon seeding on collagen coated PIPAAm-1.5hr surface

above LCST is significantly enhanced compared to that on plain PIPAAm (data not shown). Fig. 7.3.3.1 shows a series of C-RICM and phase contrast images of a typical SMC on 0.12x-PIPAAm (a, b) and 0.3x-PIPAAm (c, d) at different times after 18 °C incubation. The result shows that SMC spontaneously shrinks by retracting its lamellipodium within the initial 10 min of low-temperature incubation on 0.12x-PIPAAm (Fig. 7.3.3.1a, b). The adhesion contact area and projected area on 0.04x-PIPAAm is reduced by 67.2% and 64.3%, respectively, during first 20 min, and is reduced by 71.1% and 69.8% during the entire period. This trend is likely to be the result of a sparse concentration of collagen which triggered cell traction through the activation of integrin-collagen interaction below LCST. In contrast, the recession of adhesion contact and retraction of cell body are significantly delayed under the highest concentration of coated collagen used herein. The cell remains to be elongated towards the end of the deadhesion as shown by the phase contrast image. The adhesion contact area and projected area is reduced by only 30.1% and 39.2% during first 20 min, and 34.2% and 49.1% during the entire period (Fig. 7.3.3.1c, d). A recent study has demonstrated that cell migration is optimized and retarded under moderate and high concentrations of coated collagen, respectively¹⁶¹.

(a)

(b)

Fig. 7.3.3.2. (a) a/a_0 and (b) a/R for SMC pre-incubated on PIPAAm-1.5hr, 0.01x-PIPAAm, 0.04x-PIPAAm, and 0.1x-PIPAAm against time during low-temperature incubation at 18 °C. The error bar is the standard error of at least 60 cells on three identical samples.

(a)

(b)

Fig. 7.3.3.3. (a) Average adhesion energy and (b) adhesion energy per cell for SMC pre-incubated on PIPAAm-1.5hr, 0.01x-PIPAAm, 0.04x-PIPAAm, and 0.1x-PIPAAm against time during low-temperature incubation at 18°C. The error bar is the standard error of at least 60 cells on three identical samples.

Fig 7.3.3.2 shows the plot of (a) a/a_0 and (b) a/R against time for a population of SMC on 0.03x-PIPAAm, 0.12x-PIPAAm and 0.3x-PIPAAm during 18 °C incubation. First, the initial rate of decay a/a_0 of SMC on either 0.03x-PIPAAm or 0.12x-PIPAAm is similar to that on PIPAAm-1.5hr while it is lowest at 0.031 min^{-1}

on 0.3x-PIPAAm (Table 7.3.3). After 40 min, the moderate concentrations of collagen lead to the further reduction of a/a_0 in 0.03x-PIPAAm and 0.12x-PIPAAm than that on PIPAAm-1.5hr. Moreover, a larger extent in reduction of a/R on 0.03x-PIPAAm and 0.04x-PIPAAm are found in comparison with that on PIPAAm-1hr after 20 min (Fig. 7.3.3.2b). Moreover, a/a_0 of SMC on 0.3x-PIPAAm is significantly reduced from 1 to 0.65, but a/R remains nearly as constant during 120 min of deadhesion. The little change in the trend of a/R on 0.3x-PIPAAm is caused by the same rate of retraction of cell body in comparison with the rate of adhesion contact recession. Most importantly, it is demonstrated that the biophysical responses of SMC during deadhesion can be directly tuned by the amount of coated collagen on PIPAAm.

Fig. 7.3.3.3 shows (a) average adhesion energy and (b) adhesion energy per cell against time for a population of SMC on 0.03x-PIPAAm, 0.12x-PIPAAm and 0.3x-PIPAAm during 18 °C incubation. For cells on 0.12x-PIPAAm and 0.03x-PIPAAm, the average adhesion energy is around 1.5 times higher than that on PIPAAm-1.5hr surface between 20-30min after low-temperature incubation. After 2hr of sample cooling, the adhesion energy on 0.03x-PIPAAm becomes $2.6 \pm 0.1 \times 10^{-8} \text{ J/m}^2$ and is only around 43% of PIPAAm-1.5hr surface ($6 \pm 1.0 \times 10^{-8} \text{ J/m}^2$). In contrast, average adhesion energy of cells on 0.3x-PIPAAm remains constant at $1.8 \pm 0.1 \times 10^{-7} \text{ J/m}^2$ with time and is highest among all surfaces. This result strongly indicates that a high density of collagen demolishes the decay of average adhesion energy on PIPAAm surface. The adhesion energy per cell on 0.12x-PIPAAm surface during 2hr de-adhesion is the lowest among the four surfaces (Fig. 7.3.3.3b). Also, the adhesion energy per cell on 0.3x-PIPAAm is slightly reduced during the course of deadhesion. In accordance with the result of average adhesion energy, the highest adhesion energy per cell is found on 0.3x-PIPAAm which is two orders of magnitude higher than those on the other four surfaces after 2 hr of 18 °C incubation, indicating that excess collagen coating on PIPAAm would prevent the cellular activity required to trigger de-adhesion and detachment. Most importantly, a moderate amount of collagen coating improves

the PIPAAm's bio-compatibility without causing apparent undesirable effect on the dynamics of cell de-adhesion. Moreover, the proper amount of collagen can indeed increase the cell de-adhesion efficiency as shown by the fast decay of selected biophysical parameters, such as the normalized contact area and average adhesion energy.

Fig. 7.3.3.4. The fluorescence image of a typical SMC (actin staining) pre-cultured on (a) 0.03x-PIPAAm, (b) 0.12x-PIPAAm and (c) 0.3x-PIPAAm at 37°C at 0 minutes and 30 minutes after low-temperature incubation at 18 °C. The scale-bar represents 10 μ m.

Fig. 7.3.3.4 shows the immuno-fluorescence images of actin for typical SMC (pre-cultured for 3 hr) on (a) 0.03x-PIPAAm, (b) 0.12x-PIPAAm and (c) 0.3x-PIPAAm at 0 min and 30 min after low-temperature incubation at 18 °C. Compared to SMC adhered on PIPAAm-1.5hr surfaces (Fig 7.3.3.4a), the SMC grown on collagen coated PIPAAm-1.5hr surfaces contain higher concentration of actin at cell periphery and lamellipodium at both 0 and 30 min after incubation below LCST. Moreover, the actin filaments of cytoskeleton are more polarized and visible inside the cell cytoplasm on the collagen coated surfaces. The cell on 0.3x-PIPAAm demonstrates the most elongated shape with the highest concentration of isotropically aligned stress fibers in the cell cytoplasm compared to that on 0.03x-PIPAAm and 0.12x-PIPAAm. The high degree of polarization of stress fibers as mentioned above is directly correlated with the slowest rate of

deadhesion on 0.3x-PIPAAm.

Vinculin is another key cytoskeleton protein which is involved in focal adhesion formation which is molecular complex playing critical role in the mechanochemical transduction of cells. Generally, vinculin is involved in the linkage between the intracellular domain of integrin (adhesion receptor) and actin of cytoskeleton^{46, 47}. Vinculin's ability to interact with integrins and cytoskeleton at the focal adhesion appears to be a critical factor for controlling cell spreading and lamellipodia formation. Thus the organization of vinculin for cells during cell detachment from TRP likely affects the kinetics of cell de-adhesion and detachment.

Fig7.3.3.5. The fluorescence image of a typical SMC (vinculin staining) pre-cultured on (a) PIPAAm1.5hr + 0.03x collagen, (b) PIPAAm1.5hr + 0.12x collagen and (c) PIPAAm1.5hr + 0.3x collagen at 37°C at 0 minutes and 30 minutes after low-temperature incubation at 18 °C. The scale-bar represents 10 μ m.

In Fig. 7.3.3.5 shows the immuno-fluorescence images of vinculin for typical SMC (pre-cultured for 3 hrs) on (a) 0.03x-PIPAAm, (b) 0.12x-PIPAAm and (c) 0.3x-PIPAAm at 0 min and 30 min after low-temperature incubation at 18 °C. The result indicates that adhesion plaques composed of large amount of vinculin

appeared on the cell periphery on all collagen coated PIPAAm surfaces. The plague-like structures as mentioned above is the focal adhesion with a typical size of around 2 μm which are commonly found in epithelial cells adhering on ECM coated surfaces. Compared to the cells cultured on 0.03x and 0.12x-PIPAAm, the cell cultured on 0.3x-PIPAAm surface shows brighter vinculin expression before cell deadhesion at 0 min. As shown in a previous study, vinculin synthesis was regulated by the density of seeded cells^{162, 163} and correlated with the degree of focal contact formation^{164, 165}. In our experiment, the densities of cells in the solution used for culturing on these three surfaces are the same but the higher density of vinculin on 0.3x-PIPAAm surface results from the stronger adhesion affinity of cells, which is also consistent with the adhesion energy obtained in Fig 7.3.3.3a. With time increasing from 0 to 30 min, vinculin concentrates inside the cytoplasm of the quasi-spherical cells and the obvious focal adhesions disappear on both 0.03x-PIPAAm and 0.12x-PIPAAm. After 30 min incubation at 18 °C, the cell shrank to a round morphology and vinculin intensities for 0.03x and 0.12x-PIPAAm were still quite high. This is because before detachment, the cell has already synthesized significant amount of vinculin to support the focal contact. After cooling treatment, although the size of the cell body reduce and the contact area decreases, the cells did not have enough time to respond to this focal contact reduction and the vinculin was preserved inside the cytoplasm within 30 min. But for cells deadhering from 0.3x-PIPAAm, focal adhesion at cell periphery remains detectable despite the lowest concentration of vinculin inside the cytoplasm. Again the distinct trend in the transformation of vinculin expression during cell deadhesion on 0.3x-PIPAAm is directly correlated with the slowest cell deadhesion kinetics.

7.4 Conclusion

In summary, we have successfully demonstrated that the polymerization time for PIPAAm grafting directly affects the initial rate of cell de-adhesion. First, it is shown that a complete coverage of PIPAAm film on glass is necessary for

triggering cell deadhesion. Initial deadhesion kinetics is enhanced on the surface with the highest density of grafted PIPAAm chains. This result is likely to be correlated with a particular trend of nano-topographic transformation on PIPAAm-1.5hr. The collagen coating improves the cell adhesion but its effect on cell de-adhesion is highly dependent on the amount of collagen dispersed on PIPAAm surface. Both actin and vinculin staining confirm the cell viability and demonstrates that the difference in deadhesion kinetics on collagen coated PIPAAm is related to the cytoskeleton re-modeling.

Chapter 8

The Influence of Extracellular Matrix Protein Coated PIPAAm on Smooth Muscle Cell Deadhesion Dynamics

8.1 Summary

Cell adhesion has been known to be triggered by the specific interaction between adhesive receptor and extracellular matrix (ECM) molecules such as ECM proteins. However, the exact role of immobilized ECM molecules on the detachment/recovery of cells from thermo-responsive polymer has not thoroughly established. In the current study, the deadhesion kinetics of smooth muscle cells (SMC) from poly(N-isopropylacrylamide) (PIPAAm) modified with various ECM molecules was determined with confocal-reflectance interference contrast microscopy (C-RICM). First, a layer of elastin (EL), laminin (LA), hyaluronic acid (HA) or collagen (CL) with a density of around $1\mu\text{g}/\text{cm}^2$ was coated on PIPAAm surfaces. Then X-ray photoelectron spectroscopy (XPS) and atomic force microscopy (AFM) was used to validate the ECM molecule immobilization and probes the nano-topographic transformation of the ECM molecule modified PIPAAm surfaces across the lower critical solution temperature (LCST), respectively. Our results show that below LCST, EL significantly enhances the initial rates of reduction in both the degree of deformation (a/R) and average adhesion energy of SMC on PIPAAm surface below LCST. In contrast, HA-PIPAAm strongly resists initial SMC deadhesion as shown by the slowest adhesion contact recession and lowest initial reduction rates in a/R and average adhesion energy in comparison with CL-, LA-, EL- and plain-PIPAAm. On the other hand, HA does not retard the rate and extent of adhesion energy per cell compared to other ECM molecules. Moreover, LA is not ideal for effective cell recovery as the average adhesion energy of SMC on LA-PIPAAm is two times and six times higher than that on CL-PIPAAm and EL-PIPAAm, respectively, after 60 min at 18 °C. Interestingly, the enhancement in deadhesion rate is shown to be

correlated with the formation of stress fibers in SMC on EL-PIPAAm.

8.2 Introduction

One of the major challenges in vascular tissue engineering is to preserve the key physiological properties of arteries including mechanical strength and compliance. Generally, most work focuses on the development of biomaterial scaffolds in order to simulate the *in vivo* micro-environment for vascular tissue regeneration and to engineer functional tissue equivalents for subsequent transplantation. However, the potential cytotoxicity and non-ideal biodegradability of biomaterial scaffold hinder the long-term biological functions of embedded vascular tissues. Recently, the use of thermo-responsive polymer (TRP) has led to the realization of non-invasive recovery of intact cells/tissues including cardiac myocytes, hepatocytes, and aortic endothelial cells below its lower critical solution temperature (LCST)^{26, 39}. For example, a confluent layer of cardiac myocyte maintains their pulsatile amplitude during contraction after recovery from a common TRP such as poly(N-isopropylacrylamide) [PIPAAm]¹².

Several groups have demonstrated that the adhesion and regeneration of vascular cells/tissues is affected by the class and concentration of extracellular matrix (ECM) molecules immobilized on biomaterials^{61, 62}. For instance, elastin (EL) is the dominant ECM protein in the arterial wall and contributes up to 50% of the dry weight of blood vessel⁶⁷. It is known that EL induces the migration and contractile phenotype of smooth muscle cell (SMC)^{65, 68, 69} and maintains the compliance of blood vessel⁷⁰. Furthermore, EL has been shown to trigger the incorporation, esterification and accumulation of lipid for SMC seeded on fibrin gels^{71, 72}. While EL is the major component of basement membrane, laminin (LA) is located in the basal lamina of blood vessel. LA is a family of oligomeric glycoproteins which helps to maintain the SMC contractile phenotype. LA has high binding affinity against cell surface receptors, collagen, and other ECM proteins^{77, 78} and regulate growth, differentiation, and wound healing in vascular tissues^{79, 80, 166}. It has been demonstrated that LA was lifted off from PIPAAm

surface after cell detachment at low temperature⁴⁰. Therefore both EL and LA may alter the physiological responses of SMC during non-invasive recovery from PIPAAm surfaces below LCST.

In addition to ECM proteins, hyaluronic acid (HA) which is non-sulfated glycosaminoglycan that regulates the growth and migratory activity of SMC. The binding of HA with small oligosaccharides in culture medium interferes the proliferation and migration of SMC as shown by the altered cell morphology during adhesion⁸¹. Unlike the tension-bearing elements of the ECM proteins such as collagen fibrils, the nonfibrillar HA components are presumably more malleable. This highly malleable substrate exerts less resistance to the cell traction forces against the surrounding ECM. Thus HA facilitates cells to round up and migrate⁸² during cell deadhesion through its role in mechanically coupled signaling^{83, 84, 85, 167}, which makes it attractive to exploit the role of alternative ECM molecule like HA on cell deadhesion upon temperature reduction to below LCST in comparison with ECM proteins like EL and LA.

Until now, there is a lack of design correlation between the surface chemistry, biomolecular recognition and cellular responses for cell regeneration and recovery from TRP. For instance, it has been known that receptor mediated processes are critical to the adhesion and regeneration of cells on biomaterials¹⁶⁸. On the other hand, the role of ligand-receptor interaction between cell and TRP on the detachment of cells is still not thoroughly understood. It has been proven that different types of ECM proteins behave differently during cell recovery from TRP⁴⁰. For instance, collagen is partly left on the PIPAAm substrate while LA is believed to be lifted off with the cell below LCST. Our previous work has demonstrated that exogenous CL layer on PIPAAm not only helps to increase the substrate biocompatibility, but also helps to regulate the cell deadhesion dynamics through adjustment of CL concentration¹²⁷.

In this study, EL and LA which are known to trigger distinct SMC responses are used as model systems for studying the biophysical behavior of SMC during deadhesion from PIPAAm surfaces below LCST. At the same time, HA which is

a non-protein based ECM molecule will provide additional information on the nature of adhesive ligand on cell deadhesion dynamics. The results would provide critical information on the design of highly tailored PIPAAm system for various applications in cell regeneration and recovery through the elucidation on the nature of ligand-receptor interactions on the kinetics of cell deadhesion below LCST.

8.3 Results and Discussion

8.3.1 Micro BCA Assay

Fig. 8.3.1. Standard curve from microBCA assay with crossing (a) EL (0.02x) PIPAAm, (b) CL (0.02x) PIPAAm, (c) PIPAAm EL (absorption) PIPAAm, (d) HA (0.02x) PIPAAm, (e) LA (0.02x) PIPAAm on linear fitted curve.

It is known that cell deadhesion is affected by the concentration of ECM protein on biomaterials¹²⁷. Fig.8.3.1 shows the solution concentrations of ECM molecule recovered from (a) EL-PIPAAm, (b) CL-PIPAAm, (c) EL(ad)-PIPAAm, (d) HA-PIPAAm and (e) LA-PIPAAm surfaces as determined by microBCA assay. Here HA is also detected by micro BCA protein assay. This is because although the molecular structure of HA is a sugar chain, there are also protein specific NH-C=O groups located at the carbon rings beside the main chain. After calculating the surface concentration of adsorbed proteins or HA from the solution

concentration of desorbed molecules, the protein or HA concentration on PIPAAm substrate was determined to be around $1\mu\text{g}/\text{cm}^2$ on all five samples. The theoretical concentration of full protein monolayer coated on PIPAAm surface was determined as $1.25\mu\text{g}/\text{cm}^2$ based on the projected area of the ECM proteins and HA. The slightly lower concentration of ECM molecule on PIPAAm surface in comparison with that of a full protein monolayer is likely caused by the washing step where some ECM molecules may have been removed from the modified PIPAAm surface.

Another interesting point is that the amount of elastin coated on PIPAAm from either protein film casting or chemical adsorption is identical to each other, but the intensity of specific surface groups determined from XPS exhibits minor difference. It has been reported that protein adsorption is strongly dependent on the property of the protein and the surface chemistry of the materials¹³¹. There are alternating hydrophobic and hydrophilic regions in the primary structure of elastin¹⁶⁹. For example, alanine and lysine form the hydrophilic domains¹⁷⁰ and proline, valine and glycine form the hydrophobic motifs of elastin¹⁷¹. Although BCA assay confirms the immobilization of EL on PIPAAm through either film casting or physical adsorption, the molecular orientation of EL on the polymer surface is likely dependent on the distinct mechanisms involved in EL immobilization (which needs further investigation).

8.3.2 C-RICM

Fig. 8.3.2.1. A series of (a) phase contrast images and (b) C-RICM images for a typical SMC on EL (0.02x) PIPAAm surface which has been pre-cultured at 37 °C for 3 hr at different times during 18 °C incubation. The scale-bar represents 10 μm .

C-RICM was applied to probe the deadhesion kinetics of single SMC in real-time. Fig. 8.3.2.1 shows a set of (a) phase contrast images and (b) C-RICM images of a typical SMC in real-time on EL-PIPAAm from 0 to 60 min after incubation at 18 °C and 5% CO_2 . At 37 °C, the rate of SMC adhesion contact formation on EL-PIPAAm is significantly slower than that on CL-PIPAAm which induces strong adhesion contact during the first 30min of incubation (data not shown). The observation coincides with the previous result that a 1 hr lag period before SMC adhesion taking place on EL¹⁷². On the other hand, SMC on EL-PIPAAm adopt highly extended morphology with the formation of lamellipodium at cell periphery (Fig. 8.3.2.1a) and establishes strong adhesion contact (dark region) after 3 hr incubation at 37 °C (Fig. 8.3.2.1b). The initial projected and adhesion contact areas immediately following temperature reduction to 18 °C is 1250 and 1039.7 μm^2 , respectively. From 0 to 5 min, the projected area and adhesion contact area is significantly reduced by 20.3% and 20.6%, respectively. The rate of recession of both areas synchronize well with each other

within 60 min. After 60 min, phase contrast image shows that the lamellipodium disappear from the cell periphery and the cell becomes more spherical due to the active retraction. Overall, the projected and contact areas are reduced to $525 \mu\text{m}^2$ (by 58%) and $390.2 \mu\text{m}^2$ (by 62.3%). The rather larger extent of adhesion contact recession as mentioned above is consistent with the effect of elastin on the delay of growth and phenotypic transition of adherent rabbit arterial SMC in primary culture under serum-deficient condition^{68, 173}. It is because EL only enhances the spreading and adhesion of SMC at later time after cell seeding while only three hrs of cell seeding was applied in our study¹⁶⁸.

Fig. 8.3.2.2. A series of (a) phase contrast images and (b) C-RICM images for a typical SMC on LA (0.02x) PIPAAm surface which has been pre-cultured at 37 °C for 3 hr at different times during 18 °C incubation. The scale-bar represents 10 μm .

Fig. 8.3.2.2 shows a series of (a) phase contrast images and (b) C-RICM images of a typical SMC on LA-PIPAAm at different times after 18 °C incubation under 5% CO_2 atmosphere. SMC maintains an extended triangular morphology immediately following 5 min of 18 °C incubation. The grey circles on C-RICM images are the swelling protein. From 0 to 5 min, the projected area and contact area of the cell are reduced from 1709 to $1357.8 \mu\text{m}^2$ (by 21.6%) and 1596.4 to $1085.7 \mu\text{m}^2$ (by 32%), respectively. After 60 min, the projected and contact areas are reduced to $854.2 \mu\text{m}^2$ (by 50 %) and $718.2 \mu\text{m}^2$ (by 55 %), respectively. Moreover, lamellipodium disappears while filopodium remains extended from the

cell periphery. Although both EL and LA do not prevent cell deadhesion on PIPAAm, LA significantly delays filopodium retraction in contrast to the complete abolishment of the filopodium triggered by EL after 60 min of incubation at 18 °C.

Fig. 8.3.2.3. A series of (a) phase contrast images and (b) C-RICM images for a typical SMC on HA (0.02x) PIPAAm surface which has been pre-cultured at 37 °C for 3 hr at different times during 18 °C incubation. The scale-bar represents 10 μm .

Fig. 8.3.2.3 shows a series of (a) phase contrast images and (b) C-RICM images of a typical SMC on HA-PIPAAm against time after incubation at 18 °C and 5% CO_2 atmosphere. Before 18 °C incubation, the initial projected and adhesion contact area of SMC is 1268.8 and 1107.2 μm^2 , respectively. During the initial 5 min, the cell does not significantly retract on HA-PIPAAm compared to that on EL-PIPAAm or LA-PIPAAm. For instance, both projected area and adhesion contact area are only reduced by less than 10% during the initial period. From 5 to 60 min, cell progressively rounds up but the filopodium remains at the cell periphery. Overall, the projected area and adhesion contact area are reduced by 69.3% and 60.6%, respectively. It has been reported that HA synthesis is high during cytokinesis and is necessary for cell detachment and mitosis¹⁷⁴. However, the exogenous coating of HA used herein does not enhance initial cell deadhesion from TRP.

8.3.3 Geometry transform and adhesive force

(a)

(b)

Fig. 8.3.3.1. (a) a/R and (b) a/R for SMC pre-incubated on EL-PIPAAm, EL(ad)-PIPAAm, HA-PIPAAm, LA-PIPAAm, CL-PIPAAm and plain PIPAAm against time during low-temperature incubation at 18 °C. The error bar is the standard error of at least 60 cells on three identical samples.

(a)

(b)

Fig. 8.3.3.2. (a) Average adhesion energy and (b) adhesion energy per cell for SMC pre-incubated on EL-PIPAAm, EL(ad)-PIPAAm, HA-PIPAAm, LA-PIPAAm, CL-PIPAAm and plain PIPAAm against time during low-temperature incubation at 18°C. The error bar is the standard error of at least 60 cells on three identical samples.

The normalized adhesion contact area a/a_o is applied to quantify the reduction in adhesion contact area during cell de-adhesion below LCST. Fig. 8.3.3.1a shows a plot of a/a_o against time during SMC de-adhesion at 18 °C on EL-PIPAAm, EL(ad)-PIPAAm, HA-PIPAAm, LA-PIPAAm, CL-PIPAAm and

plain PIPAAm. The error bar is the standard error of at least 60 cells on three identical samples. It is found that the initial rate of reduction of a/a_o is highest on CL-PIPAAm among the six samples used herein. In contrast, the extent of reduction of a/a_o (60%) is highest on EL-PIPAAm compared to other samples after 60 min of 18 °C incubation. Despite the difference in the methods of EL immobilization, the similar trend of a/a_o on both EL-PIPAAm and EL(ad)-PIPAAm is brought about the same concentrations of adsorbed EL on both samples. The reduction rate of a/a_o during the initial 5 min is nearly the same on EL-PIPAAm and LA-PIPAAm but it is significantly reduced on LA-PIPAAm after 10 min of 18 °C incubation. After 60 min, the total reduction of a/a_o on LA-PIPAAm is 45% less than that on EL-PIPAAm. All adhesion molecules used herein except HA enhance the recession of adhesion contact on PIPAAm below LCST. The overall reduction of a/a_o for SMC on EL-PIPAAm is nearly 1.1 times, 1.33 times, 1.4 times and 1.5 times higher than that on CL-PIPAAm, LA-PIPAAm, plain PIPAAm and HA-PIPAAm, respectively. Moreover, HA is the least effective substrate for inducing cell deadhesion. It has been indicated that HA triggered the formation of new lamellipodial protrusions with high concentration of actin from cell edges¹⁷⁵. The effect as mentioned above seems to strengthen the adhesion of SMC through the arrest of cytoskeleton retrenchment during cell deadhesion compared to that on EL and LA.

The degree of cell deformation (a/R) is used to define the actual geometry of adherent cells on planar surfaces. Fig. 8.3.3.1b shows the average a/R against time of SMC on EL-PIPAAm, EL(ad)-PIPAAm, HA-PIPAAm, LA-PIPAAm, CL-PIPAAm and plain PIPAAm during 18 °C incubation. The error bar is the standard error of at least 60 cells on at least three identical samples. The result indicates that the initial rate of reduction of a/R is highest (0.09 min^{-1}) and lowest (0.07 min^{-1}) on EL-PIPAAm and HA-PIPAAm, respectively. The trend as mentioned above agrees well with that of a/a_o . Among all six samples, the most significant reduction of a/R (around 40%) which indicates the most significant change in cell shape during the 60 mins of 18 °C incubation is found on

EL-PIPAAm. In contrast, the extent of reduction of a/R on LA-PIPPAm over the 60 min period is lowest and is only half of that on EL-PIPAAm. Moreover, the extent of reduction of a/R on plain PIPAAm, HA-PIPAAm and CL-PIPAAM is 35%, 25% and 27%, respectively. Notably, the extent of geometric transformation and adhesion contact recession for SMC on EL-PIPAAm highly synchronize with each other during cell deadhesion. On the other hand, the extent of adhesion contact recession and geometric transformation for SMC on LA-PIPAAm samples are clearly decoupled with each other.

Based on the measurement of a/R , the average adhesion energy is calculated for characterizing cell-substrate interaction during cell de-adhesion. Fig. 8.3.3.2a shows the average adhesion energy of SMC on EL-PIPAAm, EL (ad)-PIPAAm, HA-PIPAAm, LA-PIPAAm, CL-PIPAAm and plain PIPAAm at 18 °C and 5% CO₂ atmosphere. There is a significant reduction (35% of initial adhesion energy at 37 °C) for SMC on EL-PIPAAm from 0 min ($2.0 \pm 0.1 \times 10^{-7}$ J/m²) to 10 min ($1.3 \pm 0.1 \times 10^{-7}$ J/m²). From 15 min to 60 min, the average adhesion energy of SMC on EL-PIPAAm is further reduced from $6.5 \pm 0.1 \times 10^{-8}$ J/m² to $3.0 \pm 0.1 \times 10^{-8}$ J/m². The method of EL immobilization used herein has no apparent effect on trend of adhesion energy against time on PIPAAm surface. Moreover, the extent of reduction of average adhesion energy on plain PIPAAm surface (from $1.7 \pm 0.1 \times 10^{-7}$ J/m² to $5 \pm 0.1 \times 10^{-8}$ J/m²) during 60 min of 18 °C incubation is similar to that of on EL-PIPAAm and EL(ad)-PIPAAm. In contrast, the extent reduction of adhesion energy is significantly less on CL-PIPAAm, HA-PIPAAm and LA-PIPAAm as shown by the higher adhesion energy of $7 \pm 0.1 \times 10^{-8}$ J/m², $8 \pm 0.1 \times 10^{-8}$ J/m² and $1.1 \pm 0.1 \times 10^{-7}$ J/m², respectively, after 60 min. Generally, it is shown that all adhesion molecules except EL delay the SMC deadhesion from PIPAAm surface. Interestingly, LA provides strongest resistance to the reduction of average adhesion energy which is four times higher than that on EL-PIPAAm. First of all, SMC on LA containing basement membrane have been proved to proliferate slower and express larger quantities of specific contractile proteins of SMC¹⁷⁶. SMC spreading on LA modified surface exhibits high density of

myofilaments¹⁷⁷. Furthermore, myosin expression of SMC cultured on LA modified surface is increased by 3.2 fold compared to that collagen type I¹⁷⁸. These expressions of mechanical markers would cause the highest average adhesion energy of SMC on LA-PIPAAm as observe herein.

Adhesion energy per cell is used to measure the collective adhesive interaction of cell adhering on a planar surface. Fig. 8.3.3.2b shows the adhesion energy per cell against time of SMC on EL-PIPAAm, EL(ad)-PIPAAm, HA-PIPAAm, LA-PIPAAm, CL-PIPAAm and plain PIPAAm at 18 °C and 5% CO₂ atmosphere. At 37 °C, the initial adhesion energy per cell of SMC on CL-PIPAAm ($6.7 \pm 0.1 \times 10^{-14}$ J) and plain PIPAAm ($5.9 \pm 0.1 \times 10^{-14}$ J) are around two times higher than those on the other adhesion molecule modified PIPAAm. From 0 to 5 min, the initial reduction of adhesion energy per cell from $6.7 \pm 0.1 \times 10^{-14}$ J to $1.7 \pm 0.1 \times 10^{-14}$ J on CL-PIPAAm is higher than those on other PIPAAm samples. In contrast, the adhesion energy per cell on other PIPAAm samples except plain PIPAAm (30 min) takes longer time of around 15 min to drop to a steady-state level. From 0 to 60 min, the adhesion energies per cell on EL-PIPAAm, EL(ad)-PIPAAm, HA-PIPAAm, LA-PIPAAm are both reduced by 83% from around $3.0 \pm 0.1 \times 10^{-17}$ J to $5.0 \pm 0.1 \times 10^{-18}$ J. Our experimental results demonstrate that the collective adhesion energy of SMC on PIPAAm is dependent on the type of receptor-ligand interactions. Integrins are the major class of receptors which recognize RGD motif of adhesive ligand¹⁷⁹ for triggering cell-ECM adhesion and cell-cell adhesions¹⁸⁰. RGD domain is a dominant component in CL but it is not found in EL and LA. Therefore EL or LA could only be recognized by SMC through the elastin binding protein (EBP) on cell membrane¹⁸¹. However, EBP is not solely devoted to EL/LA recognition as it also contains lectin binding sites against several glycosaminoglycans commonly found in various ECM molecules. The lack of binding specificity of EBP as mentioned above reduces the affinity of the EBP for elastin and laminin which is reflected in the lower initial adhesion energy per cell observed herein¹⁸².

8.3.4 Immunofluorescence

Fig. 8.3.4. The fluorescence image of a typical SMC (actin staining) pre-cultured on (a) EL (0.02x) PIPAAm, (b) HA (0.02x) PIPAAm, (c) LA (0.02x) PIPAAm, (d) CL (0.02x) PIPAAm at 0 and 60 min after low-temperature incubation at 18 °C. The scale-bar represents 10 μm .

It is known that SMC adheres to ECM protein through the anchoring of several focal adhesion sites along the plasma membrane. Generally, actin microfilaments are always associated to these focal adhesion sites from the cytoplasmic region of the cell¹⁷². Moreover, one single microfilament may be connected to the focal contact linked to ECM molecule and provides the necessary traction forces for carrying cellular functions. Fig. 8.3.4 shows the immune-fluorescence images of a typical SMC stained with actin on (a) EL-PIPAAm, (b) HA-PIPAAm, (c) LA-PIPAAm, (d) CL-PIPAAm after 0 min and 60 min of 18 °C incubation. Before cell de-adhesion, the microfilaments clearly evolve on the SMC adhering on EL-PIPAAm as shown by the highly polarized cytoskeletal structures in the cell cytoplasm along the elongated direction of the cells. This result strongly indicates that the microfilaments lead to the most efficient SMC de-adhesion on EL-PIPAAm. After 60 min of de-adhesion, most microfilaments of the SMC vanish in the cytoplasm, while actin becomes highly concentrated at the cell periphery. Unlike the SMC on EL-PIPAAm, actins which organize in the form of focal adhesion contacts are found at cell periphery of the SMC on CL-PIPAAm. After 60 min, the focal adhesion contacts disappear from

the cell periphery on the CL-PIPAAm surface while the smaller focal contacts emerge in the cell cytoplasm. In contrast, there is a lack of actin-associated structures like microfilament or focal contact in both LA-PIPAAm and HA-PIPAAm at 0 min before deadhesion. Moreover, the transformation of actin organization is absent on both samples as mentioned above. The lack of actin organization and transformation under the influence of LA and HA coincides with the slower adhesion contact recession and cell geometry transformation in comparison with those under the influence of CL and EL. As the staining were done on the same day with the same fluorescence imaging setting, the result indicates that the contractile forces of SMC necessary for triggering cell retraction and deadhesion on LA-PIPAAm and HA-PIPAAm are weaker than those on the other samples.

8.4 Conclusion

The contact between cells and ECM molecule modified substrate would set off a chain of signaling events that lead to long-term changes in gene expression and tissue hemostasis. In our current work, it is shown that EL accelerates the initial rate of SMC deadhesion compared to other ECM molecule modified PIPAAm surface. Despite the fact that LA coexists with EL to support SMC in medial layer of blood vessels, the efficiency of cell deadhesion from LA-PIPAAm is the least satisfactory among all the ECM molecules tested herein. Although LA or HA individually has been demonstrated to facilitate cell migration and proliferation, their existence do not accelerate cell deadhesion from TRP. The cell deadhesion process is a highly intricate event under the coordination between the cell-substrate interaction and mechanochemical signal transduction between cells and the external environment. Our work provides new insights into the role of ligand-receptor interaction on the biophysics of cell deadhesion on a model TRP. The gene expression analysis of SMC triggered by various ECM molecules would be our future research direction to help reveal the signal transduction cascades during cell deadhesion and recovery from TRP for regeneration medicine.

Chapter 9

Engineering Chitosan/Natural Hydrogel Composites for Cell

Patterning

9.1 Summary

Chitosan is a natural cationic biomaterial with promising bio-functional properties in the wound dressing and skin grafting. However, the adhesion of common cell types like fibroblasts on chitosan is still poor without the use of extracellular matrix (ECM) proteins and peptides. With the support of ECM proteins, the cell adhesion on chitosan could be significantly improved. In this part of my thesis research, whey and honey which contain certain types of ECM protein, are used to promote cell adhesion. Our result shows that both whey and honey could accelerate the adhesion of fibroblasts on chitosan. Fibroblasts form strong contact during the first 10min and 30 min seeding on whey sample and honey sample, respectively. The comparison between whey and caseinomacropetides, which is one of the main components of whey which could bind with chitosan was investigated. Caseinomacropetides is effective to improve fibroblasts adhesion but the adhesion rate is slower on caseinomacropetides than that on whey modified chitosan. Neutralized honey sample is also been tested. The results have demonstrated that the immobilized honey is not the dominant factor for fibroblasts adhesion on chitosan. Fibroblasts prefer honey to initiate contact adhesion but they spread faster on neutralized honey after adhesion contact formation compared to that on unmodified honey.

9.2 Introduction

The specific adhesion of cells to biomaterial surface is an important step in the development of cell patterning technology for tissue regeneration. In the previous chapters of my thesis, of the cell deadhesion dynamics on thermo-sensitive hydroxybutyl chitosan, PIPAAm and ECM modified PIPAAm has been

investigated in details. Meanwhile, the extensive exploration of innovative composite of natural chitosan hydrogel remains to be essential. As discussed earlier, chitosan, a copolymer of N-acetyl glucosamine and D-glucosamine, is a natural cationic biomaterial with fantastic properties such as angiogenesis promotion, antimicrobial activities^{183, 184}, biodegradability and cytocompatibility¹⁸⁵, which are crucial in the applications of tissue engineering. Nevertheless, the biofunctionalization of chitosan still requires the presence of ECM proteins to promote the initial cell adhesion and proliferation in resemblance to the case of PIPAAm. ECM proteins such as collagen, gelatin, fibronectin and integrin binding peptides have been applied for chitosan modification in order to promote the adhesion of fibroblasts which otherwise fail to adhere on plain chitosan^{186, 187}. However, increasing concern of mad cow disease (bovine spongiform encephalopathy, BSE) and serious ethical issue involved in using bovine materials limits the use of these ECM proteins in clinical applications. Furthermore, the incorporation of ECM proteins and peptides on chitosan may compromise the antimicrobial properties of chitosan. For instance, common pathogens would adhere to collagen, gelatin and fibronectin coated biomaterials¹⁸⁸. Therefore the exposure of pathogens to the chitosan/ECM protein blended material under ambient conditions during wound healing and skin grafting likely increase the risk of infection¹⁸⁹.

The objective of this part of my research is to exploit new approach for chitosan modification and patterning with the use of natural product as alternative of ECM proteins. Whey and honey are under consideration here based on several considerations. First of all, whey or milk plasma is the liquid remaining in milk after curdling as a by-product of cheese production and is highly abundant from dairy or food industry. Besides, whey is a mixture of various proteins and peptides such as lactoglobulin, alpha-lactalbumin, serum albumin and glycomacropeptides which could promote the growth of intestinal cells, epithelial cells, fibroblasts and osteoblastic cells and stimulates the differentiation of osteoblastic cells¹⁹⁰⁻¹⁹³. In a recent study, the mixture of denatured whey and

diethylene glycol is casted into film for potential application in skin regeneration¹⁹¹. Honey is a highly complex product which contains more than 90% of polysaccharide and water by weight depending on the source. Moreover, other types of constituents such as proteins, organic acids, vitamins, minerals and phytochemicals are found in honey. It has been widely used as a wound dressing reagent for centuries because of its antimicrobial properties originated from its high osmolality, acidity and phytochemical factors¹⁹⁴⁻¹⁹⁶. For example, royal jelly protein and a oligosaccharide from honey are found to be helpful in wound repair process by stimulating immunity^{197, 198}. Although there are a couple of attempts to use honey together with synthetic polymer to form film for wound dressing¹⁹⁹, the origin of the adhesive interaction between cell and the material remains to be unclear. In this part of my thesis, I applied our unique biophysical techniques to reveal the kinetics of cell adhesion on various types of chitosan substrates modified by whey, honey and the corresponding constituents.

9.3 Results and Discussion

Fig. 9.3.1 (a) Phase contrast and (b) C-RICM image of 3T3 fibroblasts after 4 hr of cell seeding on chitosan hydrogel

Chitosan hydrogel is used as a weak cationic exchanger to adsorb anionic components from whey. These anionic extracts from whey immobilized on chitosan have been proven to be efficient in fibroblast adhesion. Moreover, it can

be speculated that anionic proteins and peptides can be readily adsorbed onto the positively charged chitosan hydrogel surfaces. The fibroblasts were bound onto the pure chitosan hydrogel with round shape as shown by phase contrast image but they failed to form any adhesion contact after 4h of cell seeding as shown by C-RICM images (Fig. 9.3.1). In contrast, the cells show distinctive morphology of highly spread fibroblast with the formation of dendritic network on chitosan/whey hydrogel just after 4h of seeding (Fig. 9.3.2). C-RICM in conjunction with phase contrast image shows that chitosan/whey hydrogel (Fig. 9.3.2 a, b) is a quite efficient adhesion surface for fibroblasts.

Fig.9.3.2 A series of (a, c) phase contrast images and (b, d) C-RICM images for typical 3T3 fibroblasts on (a, b) chitosan/whey hydrogel composite and (c, d) chitosan/ caseinomacropeptide hydrogel composite during 37 °C incubation. The scale-bar represents 10 μm.

Fibroblasts start to develop significant adhesion contact after seeding for 10 min and form typical elongated morphology after 30 min. From 10 to 30 min, the adhesion contact area of the SMC was increased from 75.4 μm^2 to 198.6 μm^2 . This dramatic improvement of fibroblasts adhesion induced by whey would be commonly resulted form the presentation of adhesion molecules such as transforming growth factors, insulin-like growth factors, platelet-derived growth factors and fibroblast growth factors²⁰⁰.

(a)

(b)

Fig.9.3.3. (a) a/R and (b) average adhesion energy for 3T3 fibroblasts on chitosan/whey and chitosan/caseinomacropeptide hydrogel composite against time during incubation at 37 °C. The error bar is the standard error of at least 30 cells on three identical samples.

However, whey is a complex mixture of proteins and peptides which are not fully characterized. For instance, it is not clear which proteins or peptides bound to chitosan are responsible for the promoting fibroblast attachment. A water soluble anionic peptide in whey, caseinomacropeptide, has been recently shown to bind with chitosan²⁰¹. Thus the potential role of caseinomacropeptide in the promotion of fibroblasts adhesion is investigated herein. C-RICM result (Fig. 9.3.2 c, d) indicates the delay effect of adhesion contact evolution for SMC on chitosan/caseinomacropeptide complex during the early stage of cell seeding in comparison with chitosan/whey complex. During the first 10 min incubation, no contact adhesion area is formed under C-RICM image. Fig. 9.3.3a shows the degree of deformation (a/R) of SMC against time of cell seeding on the two chitosan based hydrogels. After 80 min, a/R of cells on chitosan/caseinomacropeptide complex approaches that on chitosan/whey complex. Moreover, the adhesion energy of SMC after initial 20 min of seeding on chitosan/whey complex is 20 times higher than that on chitosan/caseinomacropeptide complex (Fig. 9.3.3b). Compared to caseinomacropeptide, the complete mixture of whey exhibits is more effective in enhancing the initial fibroblasts adhesion on chitosan. Interesting, full adhesion contact ($a/R \approx 1$) and high adhesion energy were developed for cells on both samples after 80 min of cell seeding. The faster adhesion contact evolution on chitosan/whey compared to that on chitosan/caseinomacropeptide hydrogel indicates the synergy effect of other proteins and peptides in whey mixture in addition to just a single component of caseinomacropeptides.

In contrast to the previous work in which whey was denatured before it was blended with diethylene glycol to form a homogeneous film¹⁹¹, whey was neither denatured nor blended with polymer solution to form the polymer composite film in this study. Therefore, the composite film for clinical was expected to be two-sided. One side should contain the adsorbed whey layer and another side was pure chitosan. Therefore, this composite film is supposed to be bilayer characteristic in order to maintain the antimicrobial action of chitosan without

compromising the cell adhesive properties. In practice, the chitosan side of the bilayer film will be exposed to the ambient for preventing infection while the whey side exposed to wound will promote the fibroblast adhesion and growth for wound repair. However, this bilayer structure is still far manifestation. Our result so far indicated that fibroblasts can adhere onto the chitosan/whey hydrogel complex even in the absence of serum proteins. However, around 44% of cells formed strong adhesion contact on the substrate and the rest expressed round morphology without adhesion contact formation (data not shown). With serum supplemented to culture medium, all cells bound on the chitosan surface develop strong adhesion contact.

Fig.9.3.4 A series of (a) phase contrast images and (b) C-RICM images for typical 3T3 fibroblasts on chitosan/honey hydrogel composite during 37 °C incubation. The scale-bar represents 10 μm .

Similarly, the chitosan/honey composite was fabricated on glass surface and cell adhesion dynamics was also determined by C-RICM/phase contrast microscope. The transformation in the characteristic morphology of fibroblasts remains similar to cells adhering on whey sample during the course of cell seeding. But the rate of adhesion contact formation for fibroblast on chitosan/honey hydrogel complex is significantly slower than that on the chitosan/whey hydrogel complex. In detail, it took around twenty more mins for SMC to develop a detectable adhesion contact on chitosan/honey hydrogel complex as shown the series of C-RICM images (Fig. 9.3.4). This delay as mentioned above would be

possibly caused by the differential response of cell membrane receptors onto protein containing whey and carbohydrates containing honey¹²⁹.

Honey is also a complex mixture of different compounds. The exact components of honey adsorbed to chitosan hydrogel would be difficult to determine. Glucose, fructose and sucrose are the major components of honey but they can be washed away easily after complexation with chitosan hydrogel because these sugar molecules are highly water soluble and contain no charge group to participate in electrostatic interactions with chitosan hydrogel. Excluding these sugar molecules, gluconic acid is major acidic compound of honey solution (40 mg/mL leads to a pH 4.0 solution)¹⁹⁴. The environmental pH is a sensitive parameter during cell adhesion. In addition, pH directly moderates the surface morphology of chitosan surface. For example, in acidic solution, chitosan hydrogel immobilized on the glass surface would shrink and be etched after 2hr. On the other hand, pH determines the chemical bonds formed in surrounding protein. As environment conditions change, some of the original bonds may be altered to new conformations²⁰². Thus, we are trying to compare the cell adhesion behavior under acidic honey and neutralized honey for modifying chitosan sample.

(a)

(b)

Fig. 9.3.5. (a) Phase contrast and (b) C-RICM image of fibroblasts after 2 h of cell seeding on chitosan hydrogel etched with gluconic acid.

(a)

(b)

Fig.9.3.6. (a) a/R and (b) average adhesion energy for 3T3 fibroblasts on chitosan/honey hydrogel composite against time during incubation at 37 °C. The error bar is the standard error of at least 30 cells on three identical samples.

Firstly, solely gluconic acid failed to promote cell adhesion. No cells were found to form strong adhesion contact on the chitosan hydrogel etched with gluconic acid after 2hr incubation as shown by C-RICM (Fig. 9.3.5). Thus, the enhanced cell adhesion compared to that on chitosan hydrogel should exclude the effect of gluconic acid. Therefore, we could exclude the influence of neutralization

of gluconic acid if there is any possible variation originated from the increase of pH. The honey solution is adjusted to pH 7 to exploit the influence of pH on cell adhesion kinetics. After adding 0.1M NaOH before complexation with chitosan hydrogel, chitosan/neutralized honey hydrogel complex was tested for fibroblast adhesion kinetics. Within 30min, fibroblast start to adhere on to honey modified chitosan surface with apparently adhesion area while cell on neutralized honey doesn't adhere on surface. After 30 min, cells adhesion contact appears on neutralized honey sample and the rate of adhesion is even faster than cells on honey sample (Fig. 9.3.6a) till the adhesion on both samples stabilized after 60min. Increase of pH doesn't significantly change the kinetics of cell deformation and adhesion energy finalized at $2 \times 10^{-7} \text{ J/m}^2$ after 120 min of cell seeding (Fig. 9.3.6b). From these results, we concluded that pH of honey solution conjugated with chitosan is not a dominant factor in fibroblasts adhesion. Fibroblasts adhesion can be effectively stimulated into proliferative phase on honey coated chitosan which is attractive in wound repair progress. It has been known that a 55 kDa royal jelly protein and a 5.8 kDa complex oligosaccharides present in honey induces the immunity by cytokine production, helping the wound repair at inflammation phase¹⁹⁷. In future studies, these biomoleculars would be under investigation.

9.4 Conclusion

The existence of whey or honey is efficient as well as cost-saving for promoting fibroblasts adhesion. The biocompatibility of natural materials chitosan was greatly improved with the support of these two natural products. This method may potentially lead to attractive application in tissue engineering and cell patterning which could avoid controversial ethical issues in using serum and the demands of high cost of materials and technology. As an efficient method to characterize cell adhesion, cell adhesion kinetics provides an effective means to reveal the intrinsic mechanism of those constituents of natural products in the induction of cell adhesion. The detail functional roles of various components in

natural products and the influence of tissue culture environment are always vital issue in tissue engineering. In future work, other constituents would be under investigation and the synergistic effect of various molecular players in cell adhesion would be considered.

Chapter 10

Future Work

Through the research work in this thesis, we establish an efficient biophysical dynamics model to discuss the influence of the thermo-responsive environment on single SMC deadhesion. Even though this research work is focused on single cell, the dynamic response of single SMC to the variation of thermo-responsive environment would help to provide the fundamental research for further application on cell sheet engineering. It has been discussed that several parameters are vital to be considered when designing an appropriate cell generation system. It includes the polymerization period of thermo-responsive polymer, which would influence the sensibility of cells responding to thermo transition of substrate. It also includes the pre-culture period of cells before they are deadhered from thermo-responsive substrate. Longer pre-culture period results in more affinity between cells and substrates. ECM is another non-neglected parameter in designing cell regeneration. The appropriate selection and combination of extracellular proteins would help to trigger the cell intrinsic deadhesion mechanism, as well as improve the substrate biocompatibility. The types of extracellular proteins and the amount are both key elements for cell deadhesion. After all, the fundamental research related to the stimulation of efficient and effective cell generation is not only applied to the single cell, it should also be considered in the process of cell sheet deadhesion. The research work of this thesis is in the same direction of designing an optimistic of cell sheet regeneration system. These works associated with the supporting discussion on surface morphology characterization, surface chemo-groups detection, cytoskeleton remodeling and specific SMC mRNA expression, are dedicated to provide useful information on how to establish an appropriate thermo-responsive system for SMC regeneration.

To move forward, there are still several fundamental issues remain to be resolved if researchers want to optimize and widely apply thermo-responsive polymer in tissue.

The first would be the physical and chemical properties of novel TRP. Physical properties including LCST, molecular weight and chemical functionality could be particular for culturing and harvesting selected anchorage-dependent cells. These physical and chemical properties could be controlled by the surface functional groups, which have been introduced into copolymer with PIPAAm to immobilize various bioactive molecules, such as acrylic acid (AAc) and 2-carboxyisopropylacrylamide (CIPAAm) with carboxyl group, and α -acryloyl- ω -methoxy-PEO. By incorporating these groups into TRP, it not only can improve the bioactivity of materials, but also change its phase transition behavior. For example, AAc will increase the LCST of PIPAAm, thus forbidding BAECs adhesion. CIPAAm can remain the LCST of PIPAAm, but higher CIPAAm concentration will support cell spreading and avoid cells detachment. PEO introduce carboxyl group into TRP, and for its freely mobile chains, it can maintain the hydrophobic aggregation of PIPAAm²⁰³. As well as those hydrophilic AAc, CIPAAm and CNPAAm take effect to cell adhesion, the hydrophobic BMA also play important roles in cell adhesion/deadhesion control. The content of BMA can regulate both surface wettability transition temperatures and the magnitude of wettability changes²⁰⁴. While AAc seems impractical to cell detachment, by controlling the amount, the side group structures of other copolymers with PIPAAm, it can modify the cell detachment.

SMC's structural machinery is comprised of two types of domains that interact with each other: one is the contractile apparatus, with major components SM (smooth muscle)-actin and myosin. The other is cytoskeletal apparatus composed of NM-actin and intermediate filaments. In the contractile apparatus, conformational changes in the actin-attached myosin heads mediate transport of cross-linked arrays of actin filaments. While in cytoskeleton apparatus, a membrane skeleton provides the interface between the contractile machinery and

extracellular matrix. So, from detecting the distribution of these proteins at different period during cell deadhesion/detachment, we may gain new insights into the mechanism of mehcanochemical transduction of SMC on TRP. For example, transfected A7r5 cells exhibited prominent parallel arrays of the polar clustering of vinculin-rich focal adhesions as well as thin actin filaments, and the formation of large, dorsal cups of ruffling membrane¹⁵⁷.

In parallel, two-dimensional gel electrophoresis and mRNA dot-blot hybridization will be used to quantify the cellular functions against the dynamical responses of adherent cells on various types of surfaces. The objective would be probing the changes of biological functions including mRNA and protein expressions of the adherent cells against the kinetic parameters of cell detachment, d.g., half-time, temperature reduction rate, from different thermo-responsive polymers. The more detailed role of ligand-receptor on cell deadhesion from TRP would be help to explain the different regeneration ability of different modified TRP substrate for cell deadhesion.

Our destination is to provide global correlation between polymer physical properties, cell deadhesion/detachment dynamics, cytoskeleton structure and protein/mRNA expressions for designing optimized system for cell sheet engineering.

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