Title
A study of the effect of physical cues on stem cell fate determination

Permalink
https://escholarship.org/uc/item/8hf9k6ht

Author
Chao, Tzu-I

Publication Date
2012-07-12

Peer reviewed|Thesis/dissertation
School of Engineering
University of California, Merced
Doctoral Dissertation

A study of the effect of physical cues on stem cell fate determination

By

Tzu-I Chao

Advisor: Professor Jennifer Lu

June, 2012
A study of the effect of physical cues on stem cell fate determination

By: Tzu-I Chao

Advisor: Professor Jennifer Lu

Abstract

Stem cell research has been fueled by increasing evidence of their great promise in clinical regenerative therapy. Conventionally, stem cell fate determination can be attributed to genetic and biochemical factors. However, the field has started to recognize the importance of the stem cell microenvironments that provide physical cues to influence cell fate decision.

From a tissue engineer’s point of view, introducing physical factors to the differentiation process could be an approach to direct stem cell fate. This concept is supported by a growing body of evidence showing the responsiveness of stem cells to physical stimuli. I thus develop two platforms to study the effect of (a) static environmental cues and (b) dynamic mechanical loading on stem cell fate decisions. Carbon nanotubes (CNTs), which possess relevant features such as (1) dimension analogous to that of natural extracellular matrix (ECM) molecules, (2) large surface area, and (3) ability to serve as nano-heaters to convert absorbed near-infrared (NIR) radiation into heat, were used to fabricate artificial stem cell niches.

I exploit properties (1) and (2) of CNTs to make a biocompatible thin film with large surface area, to promote growth factor adsorption and preferential stem cell differentiation. Enhanced neuron differentiation from human embryonic stem cells (hESCs) was observed in poly(methacrylic acid) (PMAA)-functionalized CNT (PMAA-g-CNT) thin films. Polarized
expression of motor neuron-specific marker, synapsin 1, was also detected in cells differentiated on PMAA-g-CNT surfaces. Cells survive in this platform, with no detrimental effects observed. The improved differentiation can be attributed to the increased surface area created by the nanofibrillar structure, leading to enhanced growth factor adsorption. This is the first study to indicate that increasing surface area by use of CNT substrates leads to enhanced growth factor adsorption and stem cell differentiation.

To shed light on how mechanical stimulation instructs cell fate decision, preliminary work has also been done to develop a remote-controlled nanohybrid actuator system to apply dynamic mechanical stimulation to stem cells. This is achieved by employing the thermal responsive nature of poly(N-isopropylacrylamide) (PNIPAM), and the unique ability of CNTs to absorb NIR. The actuation of PNIPAM hydrogel can be triggered by temperature change, while the cells on the PNIPAM actuator change in cell shape and size upon sensing the mechanical stimulation. CNTs embedded in the polymer matrix convert photon energy into heat and initiate the contraction of PNIPAM gel. The novel device can sense NIR inputs and showed noticeable shrinkage after NIR stimulation. It can therefore be used to apply remotely controlled mechanical loads to stem cells for cell behavior study. Cytosolic calcium fluctuations, which play an important role in cell differentiation and are sensitive to mechanical stimulation, may thus be tuned by using the novel actuator to achieve controlled cell differentiation.

My work described above paves a way for further studies to investigate the effect of mechanical inputs on stem cell fate decision.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abbreviations</td>
<td>10</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>13</td>
</tr>
<tr>
<td><strong>Chapter 1. Controlled stem cell differentiation: current status</strong></td>
<td></td>
</tr>
<tr>
<td>1.1. What are stem cells?</td>
<td>15</td>
</tr>
<tr>
<td>1.2. Cell differentiation induced by biochemical factors (e.g. soluble growth factors, morphogens)</td>
<td>15</td>
</tr>
<tr>
<td>1.3. The physical factors in stem cell differentiation</td>
<td>18</td>
</tr>
<tr>
<td>1.3.1. Static physical cues</td>
<td>19</td>
</tr>
<tr>
<td>1.3.2. Dynamic mechanical loading</td>
<td>21</td>
</tr>
<tr>
<td>1.4. Artificial scaffold for stem cell differentiation</td>
<td>22</td>
</tr>
<tr>
<td>1.4.1. Materials for artificial scaffold</td>
<td>24</td>
</tr>
<tr>
<td>1.4.1.1 Natural materials</td>
<td>24</td>
</tr>
<tr>
<td>1.4.1.2 Synthetic polymers</td>
<td>26</td>
</tr>
<tr>
<td>1.4.1.3 Nanomaterials</td>
<td>27</td>
</tr>
<tr>
<td>1.5. Unsolved problems and unexplored areas</td>
<td>29</td>
</tr>
<tr>
<td>1.6. Scope of my work</td>
<td>31</td>
</tr>
<tr>
<td>1.7. Significance of my work</td>
<td>32</td>
</tr>
<tr>
<td><strong>Chapter 2. Carbon nanotube-based substrates to direct human embryonic stem cells’ fate</strong></td>
<td></td>
</tr>
<tr>
<td>2.1. Carbon nanotubes as scaffold material for neuron regeneration</td>
<td>35</td>
</tr>
<tr>
<td>2.1.1. CNT-based substrates for neuron growth</td>
<td>36</td>
</tr>
<tr>
<td>2.2. Functionalization of CNTs to improve CNT-cell interaction</td>
<td>37</td>
</tr>
<tr>
<td>2.3. Materials and methods</td>
<td>38</td>
</tr>
<tr>
<td>2.3.1. Polymer grafting and substrate preparation</td>
<td>38</td>
</tr>
<tr>
<td>2.3.2. X-ray photoelectron spectra (XPS)</td>
<td>39</td>
</tr>
<tr>
<td>2.3.3. hESC culture</td>
<td>39</td>
</tr>
<tr>
<td>2.3.4. Neuron differentiation</td>
<td>40</td>
</tr>
<tr>
<td>2.3.5. Immunofluorescent staining and image analysis</td>
<td>41</td>
</tr>
</tbody>
</table>
2.3.6. Cell viability analysis  41
2.3.7. Cell attachment assay  42
2.3.8. SEM characterization  42
2.3.9. ELISA assay  43

2.4. Results  43

2.4.1. Grafting polymer molecules onto CNTs  43
2.4.2. Cell adhesion on polymer-grafted CNTs  45
2.4.3. Cell viability on polymer-grafted CNT substrates  47
2.4.4. Neuron differentiation on PMAA-g-CNT thin films: fluorescence intensity assay  47
2.4.5. Neuron differentiation on PMAA-g-CNT thin films: Enzyme-linked immunosorbent assay (ELISA)  48
2.4.6. X-ray photoelectron spectroscopy (XPS) analysis of CNT thin films  50
2.4.7. Direct differentiation of hESCs though monolayer culture on PMAA-g-CNT substrates  51

2.5. Conclusion and discussions  53

Chapter 3. Poly(N-isopropylacrylamide) (PNIPAM) based polymer actuator to apply mechanical stimulation on stem cells for cell behavior study  56

3.1. Mechanical stimulation and cell fate  56
3.2. Approaches to apply mechanical stimulation  57
3.3. Responsive polymer system overview  58

3.3.1. Stimuli-responsive polymers  58
3.3.1.1. pH-responsive material  58
3.3.1.2. PNIPAM, a temperature-responsive material  59

3.4. PNIPAM-based actuators  59
3.5. Cell type of interests  61
3.6. Materials and method  62

3.6.1. Confocal microscopy  62
3.6.2. How confocal microscope works  63
3.6.3. Gain and offset  64
3.6.4. Fluorescence and excitation/emission 65
3.6.5. Gel making procedure 65
3.6.6. Gel swelling kinetics measurement 66
3.6.7. De-swelling kinetics measurement 67
3.6.8. Gel actuation profile 67
3.6.9. Cell membrane staining 67
3.6.10. Cell viability test 68
3.6.11. SEM characterization 68
3.6.12. Excitation and emission wavelength of dyes used 69

3.7. Results 69
3.7.1. Swelling and de-swelling kinetics of the hydrogel actuator 69
3.7.2. Swelling/de-swelling behavior after repeated actuations 72
3.7.3. Cell viability test: cells survive in the PNIPAM-CNT actuator 75
3.7.4. Cells survive in the PNIPAM-CNT actuator after repeated actuation cycles 76
3.7.5. Calibration of heating stage 77
3.7.6. Cells respond to mechanical stimulation: change of cell shape 79
3.7.7. Cell responds to mechanical stimulation: change of cell size 80

3.8. Discussion 82

Chapter 4. Fabrication of a bilayer polymer actuator system 85
4.1. The need to fabricate a bilayer polymer actuator system 85
4.2. Introduction of bilayer hydrogel system: current status 86
4.3. Design of our bilayer system 87
4.3.1. Desired properties and materials selection for the bottom actuation generating layer 87
4.3.2. Desired properties and materials selection of the top cell-seeding layer 89
4.3.3. Stiffness of the substrate 90
4.3.4. Adjusting gel stiffness 91
4.3.5. Surface modification for cell adhesion 92
4.4. Material selection for top cell seeding layer 93

4.5. Materials and methods 94
   4.5.1. Gel making procedure 94
   4.5.2. SEM characterization 96
   4.5.3. Cell membrane staining 97
   4.5.4. Collagen conjugating on PA-PNIPAM-CNT gels 97

4.6. Results 97
   4.6.1. Surface morphology of PA-PNIPAM-CNT bilayer gels 97
   4.6.2. Cells cultured on the two-layer hybrid gel can sense mechanical actuation 99
   4.6.3. UV-Ozone treatment to minimize dye adherence on gels 100

4.7. Conclusion and discussion 103

Chapter 5. Calcium signaling for monitoring cell response to mechanical actuation 105

5.1. Calcium signaling overview 106

5.2. Calcium signaling in development 108
   5.2.1. Calcium signaling in embryogenesis 108
   5.2.2. Role of Ca^{2+} in cell proliferation 109
   5.2.3. Role of calcium signaling in cell differentiation 111
   5.2.4. Role of calcium signaling in liver 111

5.3. Tunable mechanical stimulation to manipulate calcium signaling 112
   5.3.1. Enhanced cytosolic Ca^{2+} concentration upon mechanical stimulation 112
   5.3.2. Controllable mechanical actuation to direct cell differentiation: modulating Ca^{2+} fluctuation frequency 113

5.4. Dye selection for calcium signaling experiments 114
   5.4.1. Development of calcium probes 114
      5.4.1.1. First generation: increased binding specificity and fluorescence intensity 114
      5.4.1.2. Next generation: enhanced probe loading efficiency 116
         5.4.1.2.1. Ratiometric calcium probes 117
         5.4.1.2.2. Non-ratiometric calcium probes 119
5.5. Materials and methods
   5.5.1. Human fetal hepatocyte growth medium composition 124
   5.5.2. Thawing the cells 125
   5.5.3. Subculturing the cells 125
   5.5.4. Calcium probe staining procedure 126
   5.5.5. Calcium signaling measurement 127
5.6. Preliminary results
   5.6.1. Photon emission intensity measurement 127
   5.6.2. Selection of excitation intensity to minimize photobleaching 130
       5.6.2.1. Photobleaching 130
       5.6.2.2. Excitation intensity selection 131
   5.6.3. Effect of temperature 133
5.7. Discussion

Chapter 6. Fabrication of remotely controlled PNIPAM actuator 139
   6.1. Carbon nanotubes as optothermal-triggering materials: an overview 139
       6.1.1. CNTs trigger a volume phase transition of PNIPAM gels 140
       6.1.2. Our ultimate goal: Controlled PNIPAM gel actuation by NIR stimulation 141
   6.2. Materials and methods 142
       6.2.1. Applying NIR stimulation to PA-PNIPAM-CNT composite gels 142
   6.3. Results 142
       6.3.1. NIR triggered gel actuation 142
   6.4. Discussion and future direction 144

Chapter 7. Experimental design to investigate calcium signaling fluctuations upon mechanical stimulation 147
   7.1. Gel actuation triggered by temperature change (30°C to 34°C) imparted by a heating stage 148
       7.1.1. Background 148
       7.1.2. Materials, methods, and anticipated results 149
7.1.2.1. Effect of temperature change on $\text{Ca}^{2+}$ signals
7.1.2.2. Effect of mechanical stimulation on $\text{Ca}^{2+}$ signals
7.1.2.3. Gel making procedure
7.1.2.4. Staining the cells
7.1.2.5. Calcium signaling measurement

7.2. NIR laser stimulation triggers gel actuation and calcium fluctuation
7.2.1. Materials and methods
7.2.1.1. Experimental groups (5 gels will be used in each group)
7.2.1.2. NIR stimulation
7.2.1.3. Calcium signaling measurement

Chapter 8. Conclusion and future directions
8.1. Conclusion of my work
8.2. Future directions

Chapter 9. References
<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>acrylic acid</td>
</tr>
<tr>
<td>ADC</td>
<td>analog-digital-converter</td>
</tr>
<tr>
<td>aFGF</td>
<td>acidic fibroblast growth factor</td>
</tr>
<tr>
<td>AIBN</td>
<td>2,2’-azobis(2-methypropionitrile)</td>
</tr>
<tr>
<td>AM</td>
<td>acetoxymethyl</td>
</tr>
<tr>
<td>AP</td>
<td>ammonium persulfate</td>
</tr>
<tr>
<td>ASC</td>
<td>adult stem cell</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BAPTA</td>
<td>1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinechonic acid</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>BMP4</td>
<td>bone morphogenetic protein 4</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CCK-8</td>
<td>cell counting kit-8</td>
</tr>
<tr>
<td>c-Myc</td>
<td>cellular homolog to the retroviral Myc</td>
</tr>
<tr>
<td>CNT</td>
<td>carbon nanotube</td>
</tr>
<tr>
<td>CRAC</td>
<td>Ca^{2+} release activated Ca^{2+} channel</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>EB</td>
<td>embryoid body</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ELP</td>
<td>elastin-like protein</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ESC</td>
<td>embryonic stem cell</td>
</tr>
<tr>
<td>EthD-1</td>
<td>ethidium homodimer-1</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>GDNF</td>
<td>glial-derived neurotrophic factor</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>hESC</td>
<td>human embryonic stem cell</td>
</tr>
<tr>
<td>HGF</td>
<td>hepatocyte growth factor</td>
</tr>
<tr>
<td>hTERT</td>
<td>human telomerase reverse transcriptase</td>
</tr>
<tr>
<td>HUVEC</td>
<td>human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>ICM</td>
<td>inner cell mass</td>
</tr>
<tr>
<td>IGF1</td>
<td>insulin-like growth factor 1</td>
</tr>
<tr>
<td>IP3</td>
<td>inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>iPSC</td>
<td>induced pluripotent stem cell</td>
</tr>
<tr>
<td>Klf4</td>
<td>krueppel-like factor 4</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>LCST</td>
<td>lower critical solution temperature</td>
</tr>
<tr>
<td>MAA</td>
<td>methacrylic acid</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MSC</td>
<td>mesenchymal stem cell</td>
</tr>
<tr>
<td>MWNT</td>
<td>multi-walled nanotube</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NF-AT</td>
<td>nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NIR</td>
<td>near-infrared</td>
</tr>
<tr>
<td>Oct4</td>
<td>octamer-binding transcription factor 4</td>
</tr>
<tr>
<td>PA</td>
<td>polyacrylamide</td>
</tr>
<tr>
<td>PAA</td>
<td>poly(acrylic acid)</td>
</tr>
<tr>
<td>PAA-g-CNT</td>
<td>poly(acrylic acid)-grafted carbon nanotube</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PEG</td>
<td>poly(ethylene glycol)</td>
</tr>
<tr>
<td>PHEMA</td>
<td>poly(hydroxyl ethyl methacrylate)</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLA</td>
<td>poly(lactic acid)</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PLO</td>
<td>poly-L-ornithine</td>
</tr>
<tr>
<td>PMAA</td>
<td>poly(methacrylic acid)</td>
</tr>
<tr>
<td>PMAA-g-CNT</td>
<td>poly(methacrylic acid)-grafted carbon nanotube</td>
</tr>
<tr>
<td>PMGI</td>
<td>polymethylglutarimide</td>
</tr>
<tr>
<td>PMT</td>
<td>photomultiplier tube</td>
</tr>
<tr>
<td>PNIPAM</td>
<td>poly((N)-isopropylacrylamide)</td>
</tr>
<tr>
<td>PS</td>
<td>polystyrene</td>
</tr>
<tr>
<td>PVA</td>
<td>poly(vinyl alcohol)</td>
</tr>
<tr>
<td>RA</td>
<td>retinoic acid</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cell</td>
</tr>
<tr>
<td>RGD</td>
<td>arginine-glycine-aspartic acid</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>RYR</td>
<td>ryanodine receptor</td>
</tr>
<tr>
<td>SCI</td>
<td>spinal cord injury</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscope</td>
</tr>
<tr>
<td>SEOM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SHH</td>
<td>sonic hedgehog</td>
</tr>
<tr>
<td>SMA</td>
<td>smooth muscle actin</td>
</tr>
<tr>
<td>Sox2</td>
<td>sex determining region Y-box 2</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>Sulfo-SANPAH</td>
<td>sulfosuccinimidyl-6-(4' -azido-2' -nitrophenylamino) hexanoate</td>
</tr>
<tr>
<td>SWNT</td>
<td>single walled nanotube</td>
</tr>
<tr>
<td>TEMED</td>
<td>tetramethylmethylenediamine</td>
</tr>
<tr>
<td>TGA</td>
<td>thermal gravimetric analysis</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
XPS  X-ray photoelectron spectroscopy
Acknowledgements

First and foremost, I would like to express my deepest gratitude to my advisor, Dr. Jennifer Lu. Thank you, Dr. Lu for all of your guidance, motivation, caring, and inspiration during my study at UC Merced. I deeply respect and appreciate all the efforts you put forth to guide and help me, by all means. Your working attitude-refuse to give up, creativity and perseverance have been my inspiration as I hurdled all the obstacles during my graduate study. I will never forget what you said “We will never die of hard working”. I will also never forget that you helped me and treated me as a family member! Thanks!

I would also like to thank Dr. Christopher Viney, Dr. Jinah Choi, and Dr. Meng-Lin Tsao, who generously served on my committee and shared valuable insights. Without the precious suggestions, training and guidance from them, this dissertation would not have been possible. I am really grateful for the time and effort they spent on my monthly review meetings. Special thanks to Professor Viney that guided me during my thesis writing. He spent days and nights providing as much help as possible. His wide knowledge, paying attention to details, and critical thinking have been of great value for me. It is my greatest honor to have the opportunity to work with him. My sincere thanks also go to Dr. Dennis McKean, who has provided me precious experience and knowledge in polymer science. The weekly meeting between him and me has been a wonderful experience for my learning in thinking, writing, and communicating. I am touched by his willingness to devote his time into helping me, such as visiting Merced just for technical inputs. Thanks!

I would also want to thank Dr. Gokce Ugur, Kimball Anderson, Shuhuai Xiang, Yang Liu, Jose Flores, and David Pan for being such wonderful colleagues. In addition, I would like to extend
my sincere thanks to Dr. Ming-Derg Lai for always being there to give me a hand. Deeply appreciated! Moreover, thank you Dr. Chi-Shuo Chen, Dr. Eric Chen, Shelley Wang, Benson Wang, Dr. Wei-Chun Chin, Dr. Kara McCloskey, and Dr. Daniel Hirleman for all the help.

Finally, I want to thank my beloved family, especially my parents and my wife, Shiao-Ya Hong, for the un-conditional and never-ending love. That is the most valuable property I could ever have.

In the end, just as Lou Gehrig said, “I considered myself the luckiest man on the face of the earth”, because of all the experience I have with all of you. Thanks!
Chapter 1. Controlled stem cell differentiation: current status

1.1. What are stem cells?

Stem cells are specialized cells that are able to generate progeny of specific cell types for tissue formation and maintenance. Stem cells possess two important properties: (a) they are able to undergo self-renewal and (b) they are capable of differentiating into different lineages of cells [1, 2]. There are several types of stem cells, including embryonic stem cells (ESCs), adult stem cells (ASCs), and induced pluripotent stem cells (iPSCs) [3, 4] (Figure 1). Due to their potential to generate diverse cell types, stem cells have drawn a lot of interest in basic human development research and the field of clinical regenerative medicine. In developmental biology, stem cells can function as a model system to study organ development and tissue homeostasis. Clinically, stem cells can be differentiated in vitro or in vivo toward specialized cell types to repair tissue damage or treat genetic diseases.

Human embryonic stem cells (hESCs) were originally derived by Dr. Thomson (University of Wisconsin, 1998) from the inner cell mass of blastocysts [5]. The pre-implantation blastocysts were plated on a layer of feeder cells, such as mouse embryonic fibroblasts, and outgrowths of the cells were expanded to establish embryonic stem cell lines. Differentiation of ESCs can be performed by removal of factors that keep ESCs in an undifferentiated state. Then, cells are maintained in suspension culture to form embryoid bodies (EBs), which mimic the structure of embryos and recapitulate early embryonic development. EBs can be plated on substrates and with the addition of growth factors, different lineages of cells can be generated. Compared to ASCs, ESCs can proliferate for long periods of time and are
pluripotent, which means they have the ability to differentiate into almost all the kinds of cells in the human body [3].

ASCs are undifferentiated cells found throughout the adult body to replace dying cells and regenerate damaged tissues. The ASCs are considered as multipotent, which means that they can only be differentiated toward specific lineages of cells. The hematopoietic stem cells were the first type of ASCs to be discovered. [3, 6, 7]. After the discovery of hematopoietic stem cells, other ASCs were identified in different tissues, such as the central nervous system [8], skin [9], and gut [10]. In spite of the discoveries of ASCs in different organs in the human body, for some organs (e.g. pancreatic islets), the existence or the availability of ASCs is still not conclusive [11]. Furthermore, in spite of their efficiency to differentiate into specialized cells, it is not easy to promote ASCs to undergo self-renewal in vitro [12]. Hence, the need for pluripotent, patient-specific stem cells arose.

Reprogramming of somatic cells into the pluripotent state is a major breakthrough achieved by Takahashi and Yamanaka in 2006 [13]. By overexpression of 4 factors, Oct4, c-Myc, Klf4 and Sox2, the adult somatic cells can be reprogrammed and converted into induced pluripotent stem cells (iPSCs). These cells have the morphology similar to that of ESCs, express ESC markers, and can be further differentiated into cells of three germ layers. Although current approaches for reprogramming are still inefficient and time consuming, the derivation of iPSCs makes patient-specific stem cell therapy possible. Concerns of immune rejection during allotransplantation may also be bypassed.
Figure 1. Differentiation potential of hESCs, iPSCs, and ASCs. (Adapted from Human stem cell research and regenerative medicine—present and future [3], Volarevic et al., 2011)

Nowadays, many of the diseases that create a burden on society are due to the shortage of critical cell types that our body is unable to replace. The access to a variety of stem cells leads to an increasing effort to use them with the intention of treating these diseases, such as diabetes, neurodegenerative diseases, hematopoietic disorders, and heart failure. With the plasticity and multiple-lineage potential offered by stem cells, there comes the need to direct differentiation towards specific lineages. Most of the effort can be categorized into two directions: (a) biochemical factor-induced differentiation, and (b) physical factor-induced differentiation.
1.2. Cell differentiation induced by biochemical factors (e.g. soluble growth factors, morphogens)

Cell differentiation is a biological process where less determined cells adopt the functions and characteristics of specialized cells. Over the last decades, progress has been made in using soluble factors, such as growth factors and small molecules, to induce cell differentiation [1, 14, 15]. These soluble factors interact with receptors on the cell membranes, or translocate into the cytoplasm, to turn on signal cascades. Thus, gene expression can be changed and the differentiation process can be turned on. A large number of differentiation protocols have been developed with an attempt to recapitulate the development processing [14]. For example, vascular endothelial growth factors (VEGFs), which play important roles in hematopoietic development and angiogenesis [16], can be added along with bone morphogenetic protein 4 (BMP4), to induce erythropoietic differentiation from hESCs [17]. Addition of hepatocyte growth factors (HGFs) and acidic fibroblast growth factors (aFGFs) triggers the expression of hepatocyte-specific genes and promotes hepatic differentiation [18]. Supplementing of basic fibroblast growth factors (bFGFs) and brain-derived neurotrophic factors (BDNFs) induces neuron differentiation of embryonic stem cells toward motor neurons [19, 20].

Biochemical inductions have long been the main approaches to direct stem cell fate. However, with the increasing awareness that biochemical stimulation is by itself insufficient to generate functional cells with high purity, the application of physical inputs along with biochemical induction may be the key to unlock the full potential of stem cells and achieve functional differentiation.
1.3. The physical factors in stem cell differentiation

1.3.1. Static physical cues

Traditionally, stem cell fate determination can be principally attributed to genetic and biochemical factors. However, there is increasing recognition that a variety of environmental factors can also be involved controlling cell differentiation. It has been shown that substrate physical properties, such as stiffness [21], roughness [22], and topography [23-26], can direct stem cell fate [27].

The effect of substrate stiffness on stem cell fate decision has been revealed in a landmark study reported by Engler et al. showing that human mesenchymal stem cells cultured on substrates with different stiffness adopt different fates [21]. When cultured on compliant substrates (Young’s modulus 0.1~1 kPa), cells undergo neuron differentiation. Moderate stiffness (8~17 kPa) facilitates muscle differentiation, while a stiff substrate (25~40 kPa) promotes osteogenic differentiation.

On the other hand it has been demonstrated that cells display a preference for rough or smooth topography, according to their origins [28]. Fibroblasts were shown to prefer smooth surfaces while osteoblasts favor rough ones. In the field of bone engineering, increased roughness has been shown to promote bone differentiation [22, 28, 29]. For instance, compared to smooth titanium surfaces, human bone-marrow derived cells cultured on surfaces roughened with HF/HNO₃ etching displayed higher expression of osteopontin and accelerated differentiation [29].
In vivo, cells are exposed to micron or submicron-scaled topographic cues presented by the extracellular matrix (ECM). Natural stem cell niches also have specific topographies determined by the spatial arrangement of ECM molecules and surrounding cells. Experiments performed in vitro further highlighted the importance of niche topography on cell fate determination. For example, increased expression of neuron marker and increased cell alignment were observed when rat hippocampal progenitors were cultured on patterned polystyrene substrates [24]. Micropatterned grooves (width: 16 μm, spacing: 13 μm, depth: 4 μm) were created by photolithography and expression of neuron differentiation markers was higher on patterned substrates, compared to planar substrates. These results suggest that substrate topography is involved in controlling neuron differentiation. It has also been shown that the ability of cells to sense geometry cues can extend to nano scale features. Human mesenchymal stem cells cultured on synthetic nanogratings of 350 nm expressed higher amounts of neuron-specific marker, compared to micropatterned or unpatterned control groups [25]. In addition, reports have indicated that osteogenic differentiation was increased on vertically aligned TiO2 nanotubes with a diameter of 15 nm [26]. In this report, substrates of TiO2 nanotubes with different diameters (15, 20, 30, 50, 70, and 100 nm) were created and their effect on bone cell differentiation was compared. The highest expression of osteocalcin, a marker of bone differentiation, was observed on substrates consisting of 15 nm tubes, compared to smooth substrates and substrates consisting of TiO2 nanotubes with other diameters. The evidence noted above indicates that static physical factors, such as substrate stiffness, roughness, or topography, can play an important role in stem cell fate determination.
1.3.2. Dynamic mechanical loading

The importance of static physical cues has started to receive considerable attention and is summarized above. On the other hand, cells *in vivo* are constantly receiving dynamic mechanical loads such as (1) shear stress generated by blood flow, (2) muscle stretching, (3) compressive loads at joints and (4) strain due to body movements. Mechanical strain coming from continuous cell division or dynamic tissue remodeling can also contribute to the loads experienced by the cells. These dynamic mechanical loads have been shown to regulate developmental processes [30]. Hence, from a tissue engineering point of view, exerting dynamic mechanical loads on undifferentiated stem cells could be another method to facilitate stem cell differentiation. This concept is built on the premise that the mechanical loads that regulate stem cell differentiation *in vivo* would also promote differentiation *in vitro*.

Different types of mechanical load commonly used to stimulate stem cells are listed below.

Compression: Applying compressive loads (10% strain magnitude, 1Hz, 4 hours a day for 3 or 7 days) to mesenchymal stem cells encapsulated in agarose gels has been shown to promote chondrogenic differentiation [31].

Stretching: MSCs subjected to 10% uniaxial strain at 1Hz for 7 days increased the expression of smooth muscle actin (SMA) and underwent myogenic differentiation [32]. ESCs cultured on flexible silicon substrates were also subjected to cyclic strain (4-12% strain, 1 Hz, 24hrs) and increased smooth muscle differentiation was observed [33].
Shear stress: Shear stress is the amount of tangential force (force parallel to the cross section) per unit area applied by flowing fluid (e.g. blood flow). Exposure of endothelial progenitor cells to shear stress (0.01-0.25 pascal, Pa) induced endothelial differentiation and formation of tube-like structures [34]. Shear stress stimulation (0.5 Pa, 48hrs) can also induce the expression of hematopoietic markers [35]. Compared to static culture, mESCs cultured under shear (1 Pa) showed increased expression of cardiovascular markers [36].

Taken together, it can be summarized that physical cues, can be employed to influence stem cell behavior and direct cell fate.

1.4. Artificial scaffold for stem cell differentiation

Responsiveness of stem cells to physical factors as mentioned above suggested that the mechanical properties of the cell microenvironment can direct stem cell fate. Indeed, one of the rapidly growing fields in stem cell research is to create an artificial microenvironment, a stem cell scaffold, that can provide physical cues to regulate cell behavior [1, 37]. In general, scaffolds are designed to provide structural stability for tissue development. Scaffolds can also function as “cell-instructive information templates” to provide physical and chemical cues [38]. Furthermore, for stem cell-based cell therapy, artificial scaffolds can serve as a “shelter” for transplanted cells to survive in a harsh injury microenvironment [39]. The scaffolds can also be used to reconnect the damaged tissues [40].
Additionally, in some cases, incorporation of differentiated cells in scaffolds is important for the implants to be functional. For example, the locomotion of scaffold-implanted rats was better than that of rats transplanted with stem cells only (Figure 2) [41]. Another report indicated that, when stem cells are co-implanted with scaffolds, the injured animals can regain partial coordinated use of their limbs [40]. On the other hand, animals implanted with only stem cells without scaffold showed very minimal behavior improvement with scar tissue formed around the injury site.

The examples mentioned above highlight the importance of physical factors provided by the stem cell scaffolds.
Figure 2. Effects of scaffold on animal behavior after spinal cord injury. Rats suffering from spinal cord injury were transplanted with neural stem cells alone, neural stem cells with scaffolds, and scaffolds alone. The percentage of animals responding to toe pinching (pain reflex) was significantly higher in the group transplanted with neural stem cells with scaffolds, compared to the other groups. (Adapted from Functional recovery following traumatic spinal cord injury mediated by a unique polymer scaffold seeded with neural stem cells [41], Teng et al., 2002)

1.4.1. Materials for artificial scaffolds

Depending on its application, a stem cell scaffold should possess some of the following properties:

- biocompatibility, and the ability to support cell adhesion;

- optimal mechanical strength; and

- allow the transport of oxygen, nutrients, and waste.

Thus, the scaffold material selection is of great importance.

1.4.1.1. Natural materials

Natural materials, such as extracellular matrix (ECM) molecules, matrigel, alginate, or even decellularized tissue matrices, have been used as scaffold materials for stem cell differentiation [1]. Collagen is one of the main components of ECM and has expressed low immunogenicity. Collagen gels have been shown to support neurite outgrowth [42].
Incorporation of fibronectin, which is another type of ECM molecule, in collagen gels promotes ESCs differentiating into endothelial cells [43]. On the other hand, addition of another ECM molecule, laminin, induces differentiation of cardiomyocytes [43]. Hyaluronic acid is another type of ECM molecule and it has been shown to promote chondrocyte differentiation [44-46]. Matrigel is a gelatinous protein matrix secreted from mouse sarcoma cells. It is used extensively in angiogenesis studies [47, 48] and has been shown to promote endothelial differentiation [49]. However, the composition of matrigel is not well-defined and may differ from batch to batch. Hence, matrigel may not be an appropriate material for cells used for human therapy. Alginate, a polysaccharide that is anionic due to carboxyl groups, is derived from algae [50]. Alginate is biocompatible and non-immunogenic, and forms crosslinked hydrogels in the presence of Ca$^{2+}$. Gels composed of alginate have been shown to be conducive for hepatic differentiation [51]. Decellularized tissue matrices are biodegradable and generate 3D anatomical structure with native ECM molecules [52]. Reports indicate that decellularized hearts support cardiac differentiation of hESCs [53], and seeding of cardiac cells as well as endothelial cells in the decellularized hearts [54] can generate pump function under electric stimulation.

In general, scaffolds composed of natural materials provide good cell-matrix interaction and efficient cell adhesion can be achieved [1]. However, concerns still exist, such as (a) quality may differ for each batch, (b) manufacturing/isolation difficulties and (c) lack of mechanical strength.
1.4.1.2. Synthetic polymers

Many of the materials used to fabricate bioscaffolds are synthetic polymers. Synthetic polymers provide several advantages over natural materials:

• they can be mass-produced;

• the quality of the products can be controlled;

• the size and functionality of the polymer can be tailor-made; and

• less immunogenic response can be expected.

There are several synthetic polymers that are commonly used as scaffold materials, such as poly(ethylene glycol) (PEG), and poly(lactic acid) (PLA) [1]. PLA-based scaffolds are being used extensively due to their biodegradability [55, 56]. PEG is not biodegradable, but it can be functionalized easily and PEG hydrogels have displayed mechanical properties similar to those of soft tissues, such as cartilage [55, 57, 58]. Poly(hydroxyl ethyl methacrylate) (PHEMA) and poly(vinyl alcohol) (PVA) are some other frequently used synthetic polymers [1, 2].

An ideal scaffold should also provide good cell-matrix interaction. Covalently attached bioactive moieties — such as Arg-Gly-Asp (RGD) which is derived from fibronectin — to the lysine residue of poly(L-lactic acid-co-L-lysine) copolymer has been shown to increase cell adhesion [55, 59, 60]. Enhanced cell-matrix interaction due to the incorporation of RGD ligands has been shown to promote chondrogenic differentiation of human embryonic stem cells [61]. Another peptide fragment, Ile-Lys-Val-Ala-Val
(IKVAV) which is derived from laminin, can also be incorporated into PEG scaffold to enhance cell adhesion [62, 63].

Synthetic polymers can be easily tailored with different functional moieties, and can thus be chemically crosslinked to form scaffolds with different structures. Hence, in addition to the physical factors (stiffness, roughness, topography) mentioned previously, other physical inputs, such as pore size of the synthetic polymer scaffolds, can also be involved in cell fate determination [2, 37, 64]. Pores of the scaffold are important in bone engineering because they allow proliferation, migration, and vascularization of osteoblasts or mesenchymal stem cells [65]. A pore size between 100-500 \(\mu\)m is recommended for osteogenic differentiation, while larger pores lead to the generation of fibroblastic tissue [2, 66, 67].

1.4.1.3. Nanomaterials

Natural extracellular matrix (ECM) is composed of a complex network of nanofibrillar proteins. For example, the most abundant ECM molecules in humans, collagen, consists of fibrous bundles with diameters in the range 50-500 nm [55, 68]. The diameter of laminin, another ECM molecule, is also at the nanometer scale [69]. It has been shown that cells indeed sense nano-scaled topography and alter their behavior, such as differentiation [26, 70-72], in response. For instance, human osteoprogenitors cultured on disordered nanopits (approximately 100 nm in diameter) produce bone mineral, while cells cultured on smooth substrates and ordered nanopits do not [23]. As a result, a growing body of research has focused on incorporating nanomaterials into stem cell scaffolds to mimic the nano-scaled spatial cues of the extracellular matrix.
Carbon nanotubes (CNTs) are graphite sheets existing either as single walled nanotubes (SWNTs) or multi-walled nanotubes (MWNTs) [73]. SWNTs usually have a diameter of approximately 1 nm [74], while the diameter of MWNTs is in the range of 2-50 nm [75]. CNTs are light with a size comparable to that of ECM molecules, such as collagens and laminins, and have been used in scaffold fabrication [75]. CNTs have excellent mechanical strength while also being flexible. Therefore, CNTs are able to maintain the structural integrity of scaffolds during cell growth. They can also be incorporated in scaffolds to adjust the mechanical (e.g. tensile) properties of the scaffolds [76]. CNTs are electrically conductive, so a conductive scaffold can be fabricated [77] by incorporating CNTs. It has been shown that functionalized CNT substrates are biocompatible and can promote neurite outgrowth [78]. CNT scaffolds have also been shown to promote bone healing, compared to control polymer groups [79].

Titanium oxide (TiO$_2$) nanotubes are another commonly used nanomaterial for bioscaffolds [72, 80]. For example, it has been noted that rat MSCs cultured on vertically aligned TiO$_2$ nanotubes with 15 nm diameter displayed accelerated integrin clustering and formation of focal contact, which is the cell junction by which cells attached to the underlying matrix [80]. Osteogenic differentiation is also elevated on nanotubes with 15 nm diameter, compared to smooth substrates or substrates with TiO$_2$ nanotubes of other diameters. Nano-scaled topography has been proven to be an important physical cue for cell fate determination. As a result, the incorporation of nanomaterials in scaffolds, to mimic the spatial dimensions of natural ECM, should be taken into consideration.
A summary of some scaffolds is listed below (Table 1)

Table 1. Bioscaffolds of different materials.

<table>
<thead>
<tr>
<th>Scaffold material</th>
<th>Cultured cell type</th>
<th>Scaffold features</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen I-matrigel mixture</td>
<td>Neonatal rat cardiomyocytes</td>
<td>Applying uniaxial stretch (10%, 2 Hz, 7 days) to collagen scaffolds leads to mature muscle bundles.</td>
<td>Tissue engineering of a differentiated cardiac muscle construct [81].</td>
</tr>
<tr>
<td>Decellularized rat hearts</td>
<td>Neonatal rat cardiomyocytes and endothelial cells</td>
<td>Cells survive in the scaffold and the constructs generate pump function under electric stimulation</td>
<td>Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart [54].</td>
</tr>
<tr>
<td>Mixture (50:50) of poly(lactic-co-glycolic acid) and poly(lactic-co-glycolic acid)-polylysine</td>
<td>Mouse neural stem cells</td>
<td>Rats transplanted with stem cells and scaffolds displayed better locomotion then rats transplanted with stem cells only.</td>
<td>Functional recovery following traumatic spinal cord injury mediated by a unique polymer scaffold seeded with neural stem cells [41].</td>
</tr>
<tr>
<td>Chitosan and MWNTs</td>
<td>C2C12 myoblastic mouse cells</td>
<td>Cells seeded on this scaffold displayed osteogenic activity.</td>
<td>Multiwall carbon nanotube scaffolds for tissue engineering purposes [82].</td>
</tr>
</tbody>
</table>

1.5. Unsolved problems and unexplored areas

Although chemical induction through soluble factors is the major trend to trigger stem cell differentiation, there is growing awareness that physical factors may also facilitate stem cell differentiation. I focused on investigating how physical factors can regulate the differentiation process. There are two specific topics that I am interested in:
(a) the effect of CNT-based substrates on stem cell differentiation (static physical cues);

(b) the effect of mechanical load on stem cell behavior (dynamic physical inputs).

(a) Previous reports indicate that functionalized carbon nanotube substrates are biocompatible and can support neuron growth. However, it is not clear what mechanisms are operating for neuron differentiation of hESCs on CNT substrates. Results from other groups suggested that CNTs support neuron outgrowth possibly due to (a) their tubular morphology being analogous to that of neural processes and (b) an ability to interact with neurons. However, no other possible mechanisms were provided to explain why CNT substrates may promote neuron differentiation. To address this question, I fabricated 2D CNT films as substrates to investigate the neuron differentiation of hESCs.

(b) Tissue regeneration is a dynamic process that requires chemical and mechanical stimulations applied at specific timing. However, most of the traditional scaffolds used in stem cell research are unable to exert dynamic mechanical stimulation with spatial and temporal control. Specifically, although chemical induction has been shown to trigger hepatic differentiation, few articles have mentioned how mechanical load can be exerted through dynamic scaffolds to regulate stem cell behavior. To address how mechanical stimulation can affect fetal hepatocytes function and fate decision, I fabricated a remotely-controlled polymer actuator. This new device can be used to exert dynamic mechanical stimulation for stem cell behavior studies.
1.6. Scope of my work

I fabricated two different types of functional scaffolds for stem cell research by harnessing unique attributes of nanomaterials:

1. CNTs possess a large surface area that can provide a better cell-matrix interface and enhance growth factor adsorption.

2. CNTs have dimensions analogous to those of natural ECM molecules, creating a biomimetic stem cell niche.

3. CNTs exhibit strong absorption in the NIR range to convert photon energy into heat and trigger remote controlled mechanical actuations.

My work can be divided into two aspects:

(a) I fabricated 2D CNT thin films and used them as scaffolds to investigate neuron differentiation of human embryonic stem cells. To the best of our knowledge, this is the first report to show that functionalized CNTs can promote neuron differentiation from human embryonic stem cells. I also provide evidence showing that the roughness of CNT substrates leads to higher growth factor adsorption. In agreement with reports published later from other groups, I propose a possible mechanism to explain why CNT substrates promote neuron differentiation. Increased surface area on CNT-based substrates leads to more growth factor adsorption and promotes neuron differentiation. Increased protein adsorption on this substrate also leads to better cell adhesion. Monolayer attachment of hESCs can thus be achieved on our CNT substrates, due to enhanced protein adsorption and increased surface
area. Neuron cells directly differentiated from hESC monolayers, bypassing a 3D aggregate stage (embryoid body stage), in which heterogeneous differentiation often occurs, can yield cells with higher purity. In addition, more matured neuron cells, which exhibited polarized expression of synaptic vesicles, were also observed on our functionalized CNT substrates. No significant detrimental effect was observed on our CNT substrates, indicating that this thin film is a biocompatible platform for further stem cell research.

(b) I also investigated the effect of dynamic mechanical stimulation on fetal hepatocytes. A dynamic polymer actuator was fabricated by using a temperature-responsive polymer, PNIPAM. This actuator swells when the temperature is below 32°C and de-swells when T>32°C. Cell shape and size changed in response to gel actuation. The effect of mechanical load on cytoplasmic Ca^{2+} concentration, which plays important roles in the differentiation process and is sensitive to mechanical stimulation, was also investigated. Furthermore, CNTs were incorporated in this dynamic actuator to convert absorbed NIR into localized heat and trigger gel actuation. A remotely-controlled polymer actuator was fabricated to exert dynamic mechanical stimulation on stem cells. The outputs (e.g. frequency) of the mechanical loading can thus be tuned to initiate desired calcium fluctuations for cell fate determination.

1.7. Significance of my work

1. To the best of our knowledge, my work in fabrication of functionalized CNT thin films is the first report to show that polymer-grafted CNT substrates can promote neuron
differentiation from human embryonic stem cells. A possible mechanism has been provided indicating that increased substrate surface area due to nanofibrillar topography leads to enhanced growth factor adsorption and promotes differentiation. Previous reports indicated that the cell/CNT contact may facilitate neuron outgrowth. My findings provide another perspective and direction for further research in stem cell differentiation.

2. Despite increasing recognition of the importance of dynamic mechanical stimulations, the underlying mechanisms of how mechanical inputs modulate the differentiation process are not yet fully understood. My work in fabrication of remotely controlled nanohybrid polymer actuators is capable of generating the full dynamic mechanical range that is provided by nature and beyond. This novel platform paves a road for further studies to investigate the regulation mechanism of mechanical signals on stem cell fate decision. Controlled mechanical stimulation can be exerted to trigger desired cell response (e.g. cytosolic calcium fluctuations with desired frequency) to manipulate cell fate.
The need: Differentiation of stem cells to generate functional tissues to repair damaged ones

Obstacles: Biochemical induction is not enough to generate functional tissues

My strategy: Supplement biochemical induction with physical factors to promote differentiation

Figure 3. Diagram of my work. Supplement biochemical induction approaches with static physical cues (increased surface area) and dynamic mechanical load (hydrogel scaffold actuation) to promote functional differentiation of stem cells.
Chapter 2. Carbon nanotube-based substrates to direct human embryonic stem cell fate

Restoration of damaged nerve tissue, such as spinal cord, represents a major challenge in the biomedical field because adult neurons have limited capacity to regenerate [83, 84]. Take spinal cord injury as an example, there are about 250,000 to 400,000 patients in the US suffering from spinal cord injury (SCI), mostly due to trauma or traffic accidents, that could lead to death or life-long paralysis. The derivation of neurons from stem cells holds promise to treat neurological pathologies of the central and peripheral nervous system such as Parkinson’s disease, and spinal cord injury [85-87].

Human embryonic stem cells (hESCs) are pluripotent cells derived from the inner cell mass (ICM) of the blastocysts [15, 88]. They are able to differentiate into almost all cell types according to regulated spatial and temporal signals, thus, potentially providing a nearly unlimited supply of cell types and holding great promise for regenerative medicine and cell-based transplantation therapy in many neurological and neural degenerative diseases [89].

There is increasing evidence indicating that the extracellular matrix (ECM) which cells inhabit is critical in controlling stem cell differentiation [90-93]. In order to achieve control of cell fate determination, constructing a microenvironment, a scaffold, that mimics the physical and chemical characteristics of natural ECM has been the central strategy in tissue engineering and regenerative medicine [94, 95]. Natural ECM is composed of a complex network of nanofibrillar molecules [1, 68, 69, 96]. Thus, the emergence of nanomaterials provides opportunities to design biomimetic scaffolds for directing hESC differentiation.
2.1. Carbon nanotubes as scaffold material for neuron regeneration

Carbon nanotubes (CNTs) with size comparable to ECM molecules such as collagens and laminins, both of which have been reported to favor neuron growth [97-100], are extensively explored for biomedical applications [101-103].

The following unique characteristics make CNTs a highly promising material system in the neural regeneration field, creating a supportive environment for the differentiation of hESCs’ into neural cells.

- CNTs with dimensions (diameter and length) analogous to collagens and laminins, major components in the ECM, capable of providing a topographic cue.

- CNTs with mechanical compliance analogous to that of structural proteins in natural ECM, can maintain structural integrity during cell growth.

- Electrical conductivity: Electric stimulation has been used to promote neuron growth [104-106]. Instead of allowing implanted cells to grow randomly and haphazardly, aligned neuron growth to bridge the injury site can be achieved by electric stimulation [106]. Hence, it is expected that a scaffold incorporating CNTs will enable electrical stimulation and promote neuron growth.

- Protocols for facile chemical modifications of CNTs using natural and synthetic biocompatible polymers have been developed [107-111]. Therefore, chemical cues can be incorporated onto CNT surfaces providing enhanced biocompatibility and appropriate cell-matrix interaction to facilitate cell growth and differentiation.
All these unique characteristics of CNTs hold great technological promise for neuron related medical applications.

2.1.1. CNT-based substrates for neuron growth

It has been reported that substrates prepared using CNTs are biocompatible and can support neuron growth and differentiation [112-114]. It has also been observed that neurons grown on a CNT network have exhibited better signal transmission [115], possibly due to the fact that CNTs form tight contacts with neuron membranes leading to electrical shortcuts [116]. These reports suggest that CNTs may be a promising scaffold material for directing hESC differentiation toward neuronal lineage.

2.2. Functionalization of CNTs to improve CNT-cell interaction

CNTs are often functionalized to improve their biocompatibility and promote interaction with biomolecules [113, 117, 118]. Different approaches have been developed to graft polymers onto CNTs [110, 111, 119-122]. We have developed a method [107] to graft CNTs with biocompatible polymers via in-situ polymerization using acetone, which is a good solvent for the monomer but a poor solvent for the corresponding polymers [123]. Poly(methacrylic acid) with poly(acrylic acid), PMAA and PAA hereinafter, were two biocompatible polymers grafted on CNTs using this approach, respectively forming poly(methacrylic acid)-grafted carbon nanotube (PMAA-g-CNT) and poly(acrylic acid)-grafted carbon nanotube (PAA-g-CNT).
Both PMAA and PAA are negatively charged in neutral or slightly basic media. The reported pKa value (6-7) of methacrylic acid (MAA) is higher than that (4-5) of acrylic acid (AA) [124-128]. Due to the presence of –CH₃ groups on the polymer backbone, PMAA is more hydrophobic than PAA. Nanofibrillar surfaces formed by PMAA-g-CNT and PAA-g-CNT, along with surfaces coated by their corresponding polymers were used to study the effect of chemical properties and physical topographical cues on cell adhesion, viability and differentiation. Poly-L-ornithine (PLO), conventionally used as a substrate for neuron growth, was used as reference.

2.3. Materials and methods

2.3.1. Polymer grafting and substrate preparation

To graft CNTs with PAA, CNTs were first treated by refluxing in 4M nitric acid for 12 hours to render CNT surfaces more hydrophilic and also remove residual uncapped catalysts. After excessive rinsing using deionized water to remove acid, CNTs were dried in vacuum. The treated CNTs were then dispersed in a solution of a mixture of acrylic acid and acetone by sonication. After 30 min of de-oxygenation, 2,2’-Azobis(2-methylpropionitrile)¹ (AIBN) was then added. The solutions were then heated to initiate and maintain polymerization. The polymerization was stopped after 10 hrs. Acetone, unreacted monomers, and unbound polymers were removed by filtration using deionized water. The solutions of PAA-g-CNT were centrifuged at 8000 rpm for 15 minutes. The supernatant which contains CNTs grafted with polymer was collected and deposited onto

¹ 2,2’-Azobis(2-methylpropionitrile) is also known as Azobisisobutyronitrile.
glass coverslips and dried at 45°C. The substrates were then sterilized by ethanol before they were used for cell growth.

2.3.2. X-ray photoelectron spectra (XPS)

X-ray photoelectron spectra were obtained using a Physical Electronics Quantum 2000 scanning XPS system with a focused monochromatic Al-Ka X-ray source for excitation and a spherical section analyzer. A 1 mm diameter X-ray beam was used for analysis. The X-ray beam was incident normal to the sample, and the X-ray detector was centered on a direction at 45° away to the normal. The pass energy was 23.5 eV, giving an overall energy resolution of 0.3 eV.

2.3.3. hESC culture

hESC lines H9 from Wicell (Madison, WI) (passage 32-55) were cultured in a 20% knockout serum replacement medium [129, 130] on mitomycin C (Sigma-Aldrich) treated mouse embryonic fibroblasts (MEFs) feeder layers. The standard 20% knockout serum replacement medium contained 20% KO serum replacement (Invitrogen), 1% nonessential amino acids (Invitrogen), 1 mM L-glutamine (Invitrogen), Dulbecco’s modified Eagle’s medium (DMEM/F12) (Invitrogen), 0.1 mM β-mercaptonethanol (Sigma-Aldrich), and 4 ng/ml FGF-2 (Sigma-Aldrich). The medium was changed every day and hESCs were passaged every 7 days.
2.3.4. Neuron differentiation

Embryoid Body attachment and differentiation: The neuron differentiation process was modified from procedures reported by Li et al. [19]. Human embryonic stem cell colonies were treated with dispase (0.5 mg/ml, Invitrogen) to remove colonies from MEF feeder layers. The colonies were cultured in ultra-low attachment dish (Costar) for 4 days in N2 medium consisting of DMEM/F12, nonessential amino acids, sodium pyruvate (Invitrogen), N2 supplement (Invitrogen), and FGF-2 (8 ng/ml) to form EBs. At day 5, EBs were attached by using N2 medium supplement with laminin (1 μg/ml, Invitrogen) on following substrates: PLO (15 μg/ml, Sigma-Aldrich) coated surfaces as control; PAA coated surfaces and PAA-g-CNT coated surfaces. Cells were then cultured in laminin-containing N2 medium for another 7 days. Samples were then fixed for immunofluorescent staining or SEM analysis.

Monolayer hESC attachment and differentiation: hESCs were isolated manually and directly attached onto the substrates using N2 medium supplement with laminin. Motor neuron induction was then performed by using a modified protocol reported by another group [131]. At the 7th day, 0.1 μM of Retinoic Acid (RA) (Sigma-Aldrich), 1 μM of cAMP (Sigma-Aldrich) and 2 μM of heparin (Sigma-Aldrich) were added to the medium. After another 7 days of culture, sonic hedgehog (SHH) (100 ng/ml, R&D systems) was added to the culture medium. After an additional week, 10 ng/ml of insulin-like growth factor 1 (IGF1), glial-derived neurotrophic factor (GDNF) and brain-derived neurotrophic

---

2 N2 Supplement is a 100X concentrate of Bottenstein's N2 formulation. It can be used to support the growth of post-mitotic neurons.
factor (BDNF) were supplemented in the medium and cells were cultured for another 4
days before performing immunocytochemical staining.

2.3.5. Immunofluorescent staining and image analysis

After culturing for another 7 days following EB attachment, the differentiated cells were
stained with antibodies against neuron marker β-Tubulin III (1:500) (Chemicon). Cell
nuclei were stained by DAPI (1:5000). A fixed exposure time was used for image
acquisition from 30 randomly picked fields in each kind of substrate. Images were
acquired with a Nikon Eclipse TE2000-U fluorescent microscope (Nikon Eclipse TE2000-
U, Tokyo, Japan). Quantitative fluorescence analysis was performed by a modified
protocol [112]. The results were obtained using the image analysis software, SimplePCI
(Compix Inc., Imaging Systems, Sewickley, PA, USA) by calculating fluorescence
intensity of immunopositive cells against DAPI-stained cells. Results of β-tubulin III
fluorescence intensity against DAPI intensity in each randomly picked field were
calculated and served as an indicator of differentiation efficiency.

Direct hESC differentiation: The differentiated cells using monolayer attachment were
fixed with 4% paraformaldehyde for 20 minutes and treated with Triton X-100 for 20
minutes. Samples were blocked in 2% goat serum and then stained by antibodies against
synapsin I (Millipore) followed by treatment with secondary antibodies.

2.3.6. Cell viability analysis

Cell viability of different substrates was analyzed using Cell Counting Kit-8 (CCK-8)
(Dojindo Laboratories, Tokyo, Japan). This kit measures the metabolic activity of
dehydrogenases by employing a tetrazolium salt. In the presence of dehydrogenases, the tetrazolium salt produces a yellow-colored water-soluble formazan. The amount of the formazan is directly proportional to the number of living cells and can be measured by a thermo multi-scan EX plate reader (Thermo Multiskan EX plate reader, VWR, CA, USA). EBs were attached onto each kind of substrates at day 0, and the absorbance was measured at day 1, 3 and 5. The viability of cells was assessed at day 1, 3 and 5 using day 1 as reference.

2.3.7. Cell attachment assay

EBs in each test group were incubated with CCK-8 reagent for 1 hour and absorbance was measured. These cells were then attached using N2 medium supplement with laminin (1 μg/ml, Invitrogen) on the aforementioned substrates. Four hours later, the numbers of attached cells were determined by using CCK8 reagent. The percentage of cell attachment in different groups was compared to the PLO control.

2.3.8. SEM characterization

Samples for SEM were fixed using 4% paraformaldehyde, washed with phosphate buffered saline (PBS), and rinsed with deionized water. Dehydration was completed by using 30%, 50%, 75%, 95%, 100% ethanol. CO2 critical point drying was used to remove any residual solvents. A thin layer of Au/Pd was finally deposited on these substrates. Images were then acquired with an FEI Quanta 200 ESEM system.
2.3.9. ELISA assay

Lysates of differentiated cells were collected and total proteins were quantified by Micro BCA\(^3\) kit (Thermal Scientific). The standard ELISA protocol was used to determine the expression of \(\beta\)-Tubulin III and Oct4 by measuring the absorbance at 450 nm.

2.4. Results

2.4.1. Grafting polymer molecules onto CNTs

Polymers can be wrapped onto CNTs by van der Waals, ionic and covalent linkages [108, 109, 122, 132, 133]. Therefore polymer encoded with chemical and biological cues can be attached onto CNT surfaces. We graft CNTs with biocompatible polymers via in-situ polymerization using acetone, which is a good solvent for the monomer but a poor solvent for the corresponding polymers. As the polymerization reaction progresses, the interaction between growing polymer chains and solvent becomes increasing unfavorable. When the living polymeric radicals reach critical chain length, they are no longer soluble in acetone. Further propagation of the radical chains in solution is prohibited. One possible path is to graft onto CNTs [134].

The thermogravimetric analysis (TGA) of PMAA-g-CNT and PAA-g-CNT is displayed in

---

3 Bicinchoninic acid (BCA) assay is an approach used to quantify the concentration of a protein solution. First, the Cu\(^{2+}\) ions from cupric sulfate were reduced to Cu\(^+\) by the peptide bonds in protein. The amount of produced Cu\(^+\) is proportional to the concentration of total protein. After that, two bicinchoninic acid molecules bind with one Cu\(^+\) ion and form purple complexes that absorb light at 562 nm. The concentration of the protein solution can thus be determined by absorbance reading at 562 nm.
Figure 4. TGA measurement was carried out on a Perkin-Elmer Pyris-1 thermal analysis system under nitrogen atmosphere at a scan rate of 10 °C/min. The weight loss as a function of temperature suggests that there are about 10 wt% polymers grafted onto CNTs.

Figure 5 is a SEM image showing smooth tube surfaces, supporting the claim that polymer molecules, PAA in this case, have been grafted on CNT surfaces.

Figure 4. TGA analysis of polymer grafted CNTs, PMAA-g-CNTs (left) and PAA-g-CNTs (right)

Figure 5. SEM image of PAA-g-CNTs,4 (field of view: 1 μm by 0.5 μm).

---

4 The SEM image was acquired by our collaborator using a Phillips XL30 FEG SEM.
2.4.2. Cell adhesion on polymer-grafted CNTs

One of the desired features for scaffold material is that, it should provide a good cell-material interface and enable cell adhesion. Furthermore, for scaffolds intended for injury repair, their ability to retain the cells near the injury site and not allow migration to healthy regions needs to be examined. Injured tissues may not provide the suitable environment for stem cell occupancy [12], so good cell-matrix interaction provided by the scaffold to retain the stem cells are desired.

To test the effectiveness of cell adhesion on different substrates, a comparison experiment was carried out. Figure 6a is a histogram showing EB adhesion. Comparing PAA to PMAA, more EBs were observed on PMAA surfaces (p<0.05), suggesting that PMAA surfaces provide a more appropriate environment for hESC attachment. This result highlights the influential role of surface chemistry in cell adhesion. Cell attachment was greatly improved using their corresponding polymer grafted CNTs, and significantly better than the widely used smooth PLO surface. This result corroborates recent reports indicating that roughened surface regulates adhesion of neuronal cells [135].
Figure 6. (a) Histogram of EB attachment efficiency on different types of surfaces. Results were collected and calculated from three different experiments. Values were shown as mean ± SEOM\(^5\). (p<0.05\(^6\)); (b) Cell viability evaluation. Results were collected and calculated from three different experiments. Values were shown as mean ± SD. (p<0.05); (c) Representative cell morphology images of differentiated neural lineage cells from hESCs on different types of surfaces, scale bars indicate 100 μm; (d) Neuron differentiation efficiency histogram. Results of β-tubulin III fluorescence intensity against DAPI intensity were collected and calculated from at

\(^5\) SEOM represents the standard error of the mean.
\(^6\) P values were calculated using the Student’s t test. * indicates significance level of 0.05.
least 15 randomly picked fields. Values were shown as mean ± SEOM. (p<0.05); (e) SEM image of the surface morphology of PMAA-g-CNTs.

2.4.3. Cell viability on polymer-grafted CNT substrates

Results of the cell viability evaluation are displayed in Figure 6b. The overall cell survival on polymer-g-CNT and PMAA surfaces is comparable to that on the standard PLO stratum. This result indicates that CNT scaffolds where CNTs are immobilized on surfaces, unlike CNTs floated in the culture medium that will enter the cells, have no detrimental effect on cell growth. The reduced cell death on PAA-g-CNT surfaces, compared to PAA surfaces, suggests that CNT-based thin film scaffolds may have a positive influence on cell survival, thus making them attractive as long-term shelters for transplanted cells to inhabit and differentiate in.

2.4.4. Neuron differentiation on PMAA-g-CNT thin films: fluorescence intensity assay

After EB attachment and continuous culturing for another 7 days, the differentiated cells were stained with antibodies against neuron marker β-Tubulin III. Figure 6c is a set of representative fluorescence images acquired by using confocal microscopy to show the morphology of differentiated cells. Figure 6d is the histogram quantitatively revealing the effects of nanotextured surface topography and polymer surface chemistry on neuron differentiation. The fluorescence intensity ratio of neuron-specific marker, β-tubulin III, over DAPI, was used as an indication of neuron differentiation efficiency. Among all the substrates, CNT-based surfaces mimicking the topography of natural ECM, exhibit
enhanced neuron conversion when compared to their pure polymer counterparts. In particular, PMAA-g-CNTs (Figure 6e) produced the highest yield, considerably higher than PLO, a standard substratum commonly used for neuron culture and studies. PAA, which has already been reported to inhibit neuron growth and differentiation [136], not surprisingly sustained the lowest yield of neuron differentiation.

2.4.5. Neuron differentiation on PMAA-g-CNT thin films: Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed to confirm the enhanced neuron differentiation observed on PMAA-g-CNT surfaces by studying the expression of neuron and hESC-specific markers, β-Tubulin III and Oct4, respectively. Figure 7 is the histogram showing the expression of β-Tubulin III, normalized by PLO. PMAA-g-CNT substrates indeed provide the highest neuron conversion efficiency. Figure 7b summarizes the expression of hESC-specific marker, Oct4, collected from different substrates. The significant decrease of Oct4 expression on PMAA-g-CNT surfaces confirms that those hESCs have lost hESC traits. The data indicate that PMAA-g-CNT surfaces can simultaneously offer physical cues provided by CNTs and proper surface chemistry provided by PMAA. Acting together, enhanced neuron differentiation has been achieved. Figure 7c is a group of SEM images of differentiated hESCs. The transformation of round-shaped cells into a more elongated shape, with neurites branching into many directions on the PMAA-g-CNT surface, is another indication of successful differentiation. In particular, enhanced axonal extensions can be clearly seen in the SEM images, further supporting the claim that PMAA-g-CNT
surfaces offer a desirable physical and chemical interfacial environment for the neuronal differentiation of hESCs.

Figure 7. 7a, 7b: Histograms of ELISA using Abs against, β-Tubulin III and Oct4 respectively. Results were collected and calculated from three different experiments. Values were shown as mean ± SEOM. (p<0.05); 7c, SEM images of differentiated neurons on (a) PLO, (b) PMAA-g-CNT, (c) PMAA, (d) CNT. The scale bar indicates 10μm.
2.4.6. X-ray photoelectron spectroscopy (XPS) analysis of CNT thin films

Results showed above indicate that CNT-based substrates promote neuron differentiation from hESCs. I hypothesized that the increased surface roughness created by nanofibrillar CNT substrates may lead to enhanced adsorption of growth factor, which in turn results in enhanced neuron differentiation. To investigate the protein adsorption on each kind of substrate, XPS was performed. This technique is well suited for studying protein and peptide adsorption [137, 138]. Table 2 summarizes the molar percentage change of nitrogen (N) before and after soaking each substrate in the culture solution followed by excessive rinse. There is no N detected initially on any of the substrates before soaking except PLO. After soaking, comparison of all polymer substrates reveals that PMAA surface has the highest N content, indicating more adsorption of proteins. As can be seen, more N was detected on polymer grafted CNT surfaces, PAA (7.6%) vs. PAA-g-CNTs (9.9%); PMAA (10%) vs. PMAA-g-CNTs (13.4%). This result further indicates that proteins and peptides such as growth factors from the culture medium have been readily sequestered and immobilized onto CNT nanofibrillar surfaces, analogues to growth factors present in natural ECM [139]. The greater amount of growth factors adsorbed on CNT-based surfaces may contribute to higher differentiation yield. Among all the surfaces tested, the surface coated with PMAA-g-CNTs has the highest N content. The strong affinity to proteins can be explained by the combination of large surface area and nanoscale grooves created by CNTs, a physical topographical cue, and more hydrophobic environment (compared to PAA) rendered by PMAA functionalization, a chemical cue. Proper physical properties together with desirable surface chemistry enable PMAA-g-CNT surfaces to exhibit higher adsorption of growth factors. Larger numbers of adsorbed
proteins on a thin film scaffold, growth factors in particular, promote hESC differentiation. The scanning electron micrograph of PMAA-g-CNTs shown in Figure 6e represents a typical morphology of a polymer grafted CNT surface. Results published later at 2011 by Park et al [140] fit in with our findings that they claimed CNT network patterns facilitate selective adsorption of laminin. The selective laminin adsorption on CNT patterns was confirmed by staining with antibodies against laminin. This enhanced adsorption of laminin resulted in increased cell adhesion and polarized differentiation of human neural stem cells.

Table 2. Summary of XPS analysis result.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>PLO</th>
<th>CNT</th>
<th>PMAA-g-CNT</th>
<th>PMAA</th>
<th>PAA-g-CNT</th>
<th>PAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change in N (molar %)</td>
<td>5.1 %</td>
<td>12.2 %</td>
<td>13.4 %</td>
<td>10%</td>
<td>9.9%</td>
<td>7.6%</td>
</tr>
</tbody>
</table>

2.4.7. Direct differentiation of hESCs though monolayer culture on PMAA-g-CNT substrates

Current approaches to differentiate hESCs can be divided as either including a 3D cell aggregates (EB) stage, or directly differentiating the cells as monolayer culture on substrates composed of ECM molecules or supportive stroma [14]. Differentiation of hESCs by the monolayer approach, while bypassing the EB culture step, reduces the possibility of generating cells at different developmental stages [1, 141, 142]. Employing

7 For each kind of substrate, two samples were analyzed, with four different spots were analyzed on each sample.
the unique feature of CNT-based substrates, enhanced cell adhesion, we found that nanotextured surfaces created by CNTs facilitate monolayer attachment. The monolayer attachment can be attributed to larger surface area, better cell-substrate interface, and enhanced protein adsorption provided by nanofibrillar substrates. In general, monolayer attachment of hESCs is difficult to achieve even with the aid of fetal bovine serum (FBS) addition.

Synapsins are neuron-specific proteins and comprise the most abundant synaptic vesicle proteins [143]. Synapsin I, which interact with small synaptic vesicles, and cytoskeleton components is often used as a motor neuron specific marker [131, 144, 145]. Figure 8 is a set of fluorescence images of differentiated hESCs taken after staining with antibodies against synapsin I. Abundant and polarized expression of synapsin I was clearly seen in cells grown on the PMAA-g-CNT surface, while at the same stage, faint expression of synapsin I was barely detected in cells on the PLO surface. A plentiful amount of synaptic vesicles suggests that neuronal cells on the PMAA-g-CNT surface are more mature. This is the first demonstration that relatively matured neuron cells have been formed by direct differentiation of hESCs on a polymer functionalized CNT surface. Deriving neuron cells directly from hESC monolayers, bypassing the EB culture step in which heterogeneous differentiation often occurs, will yield cells with higher purity. Furthermore, compared to EBs, which are cells wrapped in layers of ECM molecules and have already started the process of differentiation, hESCs are considered “softer” and thus more susceptible to external signals and more easily coerced into specific lineages [146].
Figure 8. Representative cell morphology images of differentiated neural lineage cells from hESCs. Cells were stained with antibodies to detect the expression of synapsin I. Scale bars indicate 10 μm.

2.5. Conclusion and discussions

In conclusion, I fabricated a novel platform consisting of PMAA-g-CNTs to investigate the effect of CNT-based substrates (static physical cues) on stem cell differentiation. Enhanced neuron differentiation from hESCs was observed in PMAA-g-CNT substrates. Polarized expression of motor neuron marker, synapsin 1, was also detected in cells differentiated on PMAA-g-CNT thin films. Cells survive in this novel platform, with no detrimental effects
observed. The improved neuron differentiation can be attributed to the increased surface area created by CNT’s nanofibrillar structure, leading to enhanced growth factor adsorption.

It has been reported that implanting scaffolds that contains stem cells into injury sites can promote axonal growth [41]. The vital role of scaffolds is also apparent from the fact that neural precursors can be incorporated into host tissues with the aid of scaffolds [147], and that partially coordinated motility can be gained after implanting scaffolds that contain neural precursors [40]. Our findings suggest that CNT-based scaffolds provide a new platform in which co-transplanted cells can anchor, survive, and differentiate.

The in-situ polymerization method to graft polymers on CNTs reported herein can be extended to graft other biocompatible polymeric materials onto CNTs to modulate hydrophobicity, surface charge and protein adsorption. It is expected that combining the topological cues offered by CNTs with the desirable biological cues which can be achieved by polymer grafting, will lead to further enhancement in selective differentiation of hESCs.

This investigation provides solid evidence that nanofibrillar surface morphology generated by CNTs, mimicking the characteristics of ECM with enhanced cell adhesion and growth factor adsorption, promote the differentiation process. PMAA is more hydrophobic and less acidic than PAA. Consequently, greater amounts of proteins and peptides can be localized onto the PMAA surfaces. Since cell membranes are mostly composed of negatively-charged phospholipids, there is more repulsive interaction between cells and the PAA-based surfaces than between cells and the PMAA-based surfaces. Therefore less negatively charged and more hydrophobic PMAA surface will provide a more appropriate niche. This work further supports the importance of surface chemical properties to cell behavior. As shown, PMAA-
g-CNT provides a desired microenvironment with physical and chemical cues enabling direct monolayer attachment of hESCs. This is the first demonstration that relatively matured neuronal cells can be derived directly from hESCs using a new scaffold system, polymer grafted CNT thin film scaffolds. Coupled with the electrical stimulation ability, polymer grafted CNTs hold great potential in nerve injury repair, neural tissue engineering and neuron prosthesis.
Chapter 3. Poly(N-isopropylacrylamide) (PNIPAM) based polymer actuator to apply mechanical stimulation to stem cells for cell behavior study

3.1. Mechanical stimulation and cell fate

Virtually every cell in vivo is constantly exposed to external mechanical stimuli, from matrix stiffness of the cellular niche, cell division or tissue remodeling-induced strain, and strain imposed by body movement, to tissue-specific dynamic forces, such as muscle stretch, joint movements, and shear stress [148-150]. Hence, other than appreciate the biochemical signaling regulatory mechanism, much attention have focused on understanding the effect of mechanical stimulation on cell behavior regulation. Mechanical forces are known to affect many basic biological processes including: cell shape regulation, cell migration, cytoskeleton organization, cell growth, apoptosis, and tissue morphogenesis [151-153]. Cells alter their behavior and function in response to mechanical forces. Aberrant mechanical regulation has been found to cause abnormal axis formation, defects in embryo development, and organ dysfunction [154-157]. Stem cells and partially-differentiated progenitor cells are especially susceptible to mechanical manipulation [158, 159], and a growing body of evidence reveals that mechanical stimulation such as shear stress, stretching, and compression, plays an important role in cell fate determination [160-162]. Given the growing evidence showing that biochemistry and genetics alone are not sufficient to explain all biological processes [163], such as cell morphology and cell fate decision, applying mechanical stimulation in addition to biochemical induction may be the key to achieving preferential and functional differentiation for clinical therapy.
3.2. Approaches to apply mechanical stimulation

Different approaches have emerged for applying mechanical cues to cultured cells [164-169]; they include stretching flexible substrates, computer-controlled piston systems, glass probe-mediated mechanical stimulation, magnetic bead-mediated local stress, optical tweezers, and polymer actuators.

Cyclic strain was applied by using a device equipped with an air vacuum system to stretch a deformable membrane on which the cells are resident [32]. When rat bone marrow derived progenitor cells were stimulated in this way with 10% strain at 1Hz for 7 days, the stimulus led to alignment of the cells and expression of markers for smooth muscle differentiation. In another study, air-pressure driven motion of compression rods exerted a compressive force on mesenchymal stem cells encapsulated in hydrogel scaffold [165]. Stimulation at the frequency of 1Hz, 1-4 hours per day for up to 3 weeks led to increase of cartilage-specific markers. Mechanical forces were also applied to cells cultured on polyacrylamide substrate by using a microneedle with blunted tip mounted on a micromanipulator to push the substrate toward or pull it away from the fibroblasts [170]. Magnetic beads coated with Arg-Gly-Asp (RGD) peptides twisted in a magnetic field at a frequency of 0.75 Hz resulted in mechanical stress that promoted cell spreading and differentiation of embryonic stem cells [166]. Induction of focal adhesion was observed for cells subjected to pulling forces. Laser tweezers can also be used to apply mechanical force to cells [168]. A laser at 847 nm constrained the movement of fibronectin-coated beads and led to accumulation of vinculin, a sign of focal complex formation. The approaches mentioned above provide different ways to mechanically stimulate the cells and further unveil how mechanical signals participate in
biological processes. However, most of the available systems so far are economically expensive (like optical tweezers that can stimulate one or only a few cells at a time) and/or rely on complicated set-up (like home-made computer-controlled piston systems).

3.3. Responsive polymer system overview

Among these systems, dynamic actuators provide several advantages such as ease of setup and the ability to stimulate the entire culture simultaneously [171]. Different materials have been considered as candidates to provide mechanically-stimulatory actuations. Actuators made of piezoelectric materials displayed fast response, but the provided strain is usually less than 0.1% [172], unless complicated linkages were setup. Alloys with shape memory effect provide significant deformation, but long reaction time and limited life cycles are the major drawbacks. Stimuli-responsive polymer actuators, on the other hand, display fast response and large enough deformation to provide the desired actuations.

3.3.1. Stimuli-responsive polymers

3.3.1.1. pH-responsive material

Reports have been published describing the use of poly (acrylic acid) (PAA) or poly (methacrylic acid) (PMAA) based compounds as pH-responsive materials due to their ability to swell reversibly in response to pH change [173]. When pH > its pKa, the –COOH groups of PAA are ionized. Hence, the polymer chain acquires negatively charged functional groups, leading to repulsion and swelling. However, the response
usually takes place at a pH significantly lower than that of normal physiological conditions. Polymer actuators made from materials that exhibit volume changes under physiological conditions are more desirable.

3.3.1.2. PNIPAM, a temperature-responsive material

Poly(N-isopropylacrylamide) (PNIPAM) is a widely used stimuli-responsive material that has a lower critical solution temperature (LCST) in the range of 32-33 °C [174-178]. Below its LCST, PNIPAM swells, existing in a well-hydrated condition where the favorable enthalpy of amide-water hydrogen bonding stabilizes the swollen state of PNIPAM. As the temperature increases towards the LCST, the unfavorable hydrophobic solvation entropy of isopropyl hydrophobic groups of PNIPAM dominates, causing the PNIPAM to collapse and de-swell, releasing water. This reversible property of PNIPAM renders it useful in many biomedical and biotechnological applications such as (i) controlled release systems or on-off switches for controlling the activity of enzymes [179, 180], (ii) a substrate for cell attachment/de-attachment and growth studies [171, 181-183], (iii) in microfluidic devices [184], and (iv) in membranes [185].

3.4. PNIPAM-based actuators

Pelah et al. demonstrated that reversible cell shape deformation can be induced by exerting stretching and compression forces using a PNIPAM gel actuator [169]. Red blood cells (RBCs), which are highly deformable, were embedded in PNIPAM gel matrix and were compressed to adopt a sickle-shaped morphology at temperatures above the LCST. RBCs
were also sandwiched between PNIPAM gels and coverslips or glass slides; upon gel swelling/de-swelling, cells can be deformed. PNIPAM-based scaffolds have also enabled dynamic cell culture [186] on micro-patterned (grooves of 10 μm width, 20 μm depth, and ridge with 2 μm width) PNIPAM film. Dynamic culture was performed by changing the temperature between 29°C (for 10 minutes) and 37°C, 10 cycles per day for 5 days. Adsorption of an elastin-like protein (ELP) on the film surface promotes initial cell adhesion and maintains cell attachment during repeated dynamic culturing. This dynamic culture system promotes proliferation but inhibits differentiation of rat bone marrow mesenchymal stem cells. Another research group used fibronectin–immobilized PNIPAM-based material to exert mechanical stimulation and stretch the cells [187]. Cell morphology changes and extracellular signal-regulated kinase (ERK) activation was observed 20 minutes after temperature change. The examples mentioned above suggested that PNIPAM-based materials can be used as polymer actuators to induce cell stretching or contraction. However, these mentioned approaches rely on manually changing temperature and subsequent gel deformation. Also, the magnitude and frequency of the actuation can not be well controlled. Hence, a new approach to remotely switch on the actuation with better control of frequency and magnitude is desired.

Recently, a PNIPAM-based actuator that can be folded into desired shapes has been fabricated [178]. Water loss when T>LCST creates mechanical strain and leads to subsequent folding of the device into the desired shape. The authors also showed that by incorporation of single wall nanotubes (SWNTs) at 0.75 mg/ml, this device is also optically responsive. Although there is no actuation of the device, an opaque spot can be generated due to the heating upon near-infrared (NIR) laser (785 nm) stimulation. The report
mentioned above further highlights the promise of PNIPAM-based polymer actuators, but further experiments need to be performed to fabricate a polymer actuator that can generate desired actuation and motion, upon remote stimulation with NIR.

3.5. Cell type of interests

Human telomerase reverse transcriptase (hTERT)-reconstituted human fetal hepatocytes that have been proven to maintain their proliferation and differentiation ability [188], two important features commonly observed in stem cells, were chosen to be cultured on our novel actuator and subjected to mechanical stimulation. Hepatocytes in vivo are in the close vicinity of sinusoid space that receives blood flow from the portal vein and hepatic artery [189]. Considering that cells near vasculature constantly receive stretching force due to blood flow [150], the stretching force exerted by our actuator during gel swelling can be analogous to the stretch that hepatocytes experienced in vivo. Previous studies have suggested that mechanical stimulation may be beneficial for improving hepatocyte-specific function, including enhanced albumin production [190], increased urea synthesis [191], and up-regulation of detoxification genes [192]. Achieving functional hepatic differentiation through chemical induction remains challenging [193]. Our novel approach, by using PNIPAM actuator to apply mechanical stimulation on fetal hepatocytes provides an opportunity to investigate how cells respond to mechanical loading and ultimately achieve functional hepatic differentiation to cure chronic liver diseases, in which the regenerative ability of hepatocytes has been disabled [194].
3.6. Materials and methods

3.6.1. Confocal microscopy

Confocal microscopy is an approach that uses point illumination and a pinhole to eliminate out-of-focus light for improving contrast and resolution of optical images. It provides several advantages over traditional wide field epi-fluorescence microscopy, such as the capability to eliminate out-of-focus light from the focal plane, and thus the ability to acquire images of optical sections from thick samples. With the introduction of a pinhole placed in front of the detector, emitted light from below the focal plane will be focused behind the pinhole and so excluded by the pinhole. On the other hand, light emitted above the focal plane will be focused on a plane before the pinhole, and is also excluded by the pinhole from reaching the detector. As a result, the out-of-focal-plane light no longer overlaps desired emission from the sample, and sharp images can be acquired. Moreover, signal averaging can be performed by taking the sum of each corresponding pixel value and dividing by the number of scans to obtain the final value of that pixel in the image. Since noise fluctuates randomly, a zero net contribution from noise can be approached via such an averaging process. On the other hand, pixel values of specific signals are usually persistent and so can be preserved. The capability of averaging raises the signal to noise ratio of a confocal system and hence improves image quality. Finally, for thick tissues or samples, optical sections can be performed and images can be stacked. Thus, 3D images and other information regarding the samples can be obtained for further investigation.
3.6.2. How confocal microscope works

The point-like light source emitted from the excitation laser system (point illumination) passes through a pinhole aperture before being reflected by a dichromatic mirror. Then, the reflected light is made to scan across the sample, in a defined focal plane. Emitted fluorescence from the sample will then pass the dichromatic mirror and will be concentrated as a confocal point at the pinhole aperture of the detector. For conventional epi-fluorescence microscopy, the whole sample is evenly illuminated at the same time. Emitted light from each illuminated spot interferes with the light emitted from other illuminated spots located within or below or above the objective focal plane, leading to the formation of Airy disk images. Point illumination ensures that only one spot on the sample is lighted at a time, so the lateral interference of illuminated dots that occurs in wide field microscopy is avoided. Also, with the deployment of the pinhole aperture, which serves as a spatial filter, in front of the photomultiplier tube (PMT), out-of-focus light from the Z axis will be excluded. The combination of aperture-mediated point illumination and the use of the pinhole spatial filter at the conjugate image plane in front of the PMT results in improved image quality. The PMT converts collected photons into electrons, and the signals can be further adjusted by tuning the gain (voltage) and offset (threshold) to ensure that strong signal/noise ratio can be achieved. Output electrons from the PMT are further processed in an analog-digital-converter (ADC), turning analog current to digital signals, and the intensity of the collected fluorescence can be converted to a corresponding number before being sent to the digital image processor. The processor reconstructs the signal, spot by spot, into a line, and line by line into the frame, before the image is shown on the screen.
In brief, the point light source, the light focused on the sample, and the pinhole aperture in front of the detector, are optically conjugated in the same plane in a confocal system. As a result, lateral interference of light, as well as out-of-focal-plane light, will be minimized to improve image quality.

3.6.3. Gain and offset

The fluorescence emissions from the sample are collected and measured by the PMT, which captures photons using its photo-sensitive surface. When exposed to photon influx, the photocathode of the PMT generates electrons through the photoelectric effect, and the electrons flow through electron multipliers and the anode to complete the circuit before being processed by the analog-to digital converter. Due to the existence of the pinhole aperture to exclude the out-of-focus fluorescence, the collected photons are limited in number and the generated electric signals are usually weak. As a result, other than electron multipliers, additional options can be used to raise and adjust final output signals from the PMT. Gain and offset are two controls for adjusting image intensity so that the largest number of grey levels can be exported from the output signal of the PMT.

By adjusting the gain value, the output voltage can be multiplied so the maximum signal can be set to a value right below saturation. Raising the gain value results in the conversion of a dim image to one with stronger fluorescence intensity. However, it is not only the signal that is increased, but also the noise. Increasing the gain will also lead to an increase in the voltage differential between high and low voltage amplitude (in a wave).
On the other hand, the offset control can be used to make sure that the background is black (0 volt output). By adding a voltage (positive or negative) to the output signal, the noise can be depressed to below the PMT detection threshold while the signals can still be above the threshold. In an alternative interpretation, adjusting the offset can be viewed as being equivalent to adjusting the threshold of what is a noise. The voltage differential between high and low voltage amplitude (in a wave), in this case is not changed.

3.6.4. Fluorescence and excitation/emission

Before being stimulated with light, the electronic configuration of the fluorophore is in its ground state. After being stimulated with excitation light, the electrons are raised to a higher energy. Then, the excited electrons may lose some of the energy to the surroundings and return to their lowest excited single state. After that, the electrons are able to relax back to their ground state while automatically emitting fluorescent light. When the electrons move from a high energy state to their ground state, loss of vibrational energy occurs, so the emission spectrum moves to longer wavelengths (the longer the wavelength, the lower the energy) compared to the excitation spectrum. This is called the Stokes shift.

3.6.5. Gel making procedure

**PNIPAM and PNIPAM-CNT gels:** Gel precursor solution consisting of NIPAM (monomer; 0.7M), SR-415 (crosslinker; 0.0035M), and Tetramethylethylenediamine (TEMED) (0.023M) were dissolved in DI water was mixed, purged with nitrogen for 30 minutes, and then mixed with purged ammonium Persulfate (AP) (initiator; 0.0045M) solution. The
reaction was performed at room temperature in 12 wells or molds for 18 hours. Then, gels were exposed to air for 1 hr to stop the reaction. After that, gels were washed with PBS for 15 cycles (room temperature for 15 minutes and then 37°C for 15 minutes per cycle). Then gels were autoclaved before used in cell culture-related experiments.

For PNIPAM-CNT gels, 0.01% (wt) of carboxylated MWNTs were dissolved in DI water before being sonicated for 24 hrs. Then, CNTs were centrifuged at 5000 rpm for 25 minutes and supernatant that contained dispersed CNTs was collected. The centrifugation process was repeated again. After that, monomer and crosslinker were added to the dispersed CNT solution, same as the procedure for making PNIPAM gels, before being mixed with initiator to form gels. The gelation was performed at room temperature for 18 hours. Then, the gels were washed in the same way as the PNIPAM gels.

3.6.6. Gel swelling kinetics measurement

Investigation of gel swelling kinetics was done by using a protocol modified from the literature [195]. Gels were incubated in PBS at 37°C in a water bath overnight, and water was wiped off from the samples by using paper towels so that the dry weight of the gel (W_d) could be determined. Then, the gels were incubated in PBS at RT (approximately 22°C) and the swelling kinetics of the gels were investigated gravimetrically by recording the weight of the samples at predetermined time (W_t). The time points that weight of the samples recorded were at 0, 2, 4, 6, 8, 10, 15, 20, 30, 60, and 90 minutes. Weight change was calculated from the formula:

\[
\text{Weight change (\%)} = 100 \% \frac{(W_t - W_d)}{W_d}
\]
3.6.7. De-swelling kinetics measurement

Dry weight of the gels was measured after gel incubation in PBS at 37°C overnight. Then, gels were exposed to RT in PBS for 24hrs and the weight of liquid ($W_s$) in the swollen samples was recorded after wiping off the water from gel surface. After that, gels were incubated at 37°C and the weight of the gels at predetermined time ($W_t$) was recorded. The time points that weight of the gels recorded were at 0, 5, 10, 15, 20, 30, 40, 60, and 120 minutes. Liquid retention (%) was calculated from the formula:

$$\text{Liquid retention} = 100\% \frac{(W_t - W_d)}{W_s}$$

3.6.8. Gel actuation profile

Dry weight ($W_d$) of the gels was measured. Then gels were incubated in PBS at RT for 10 minutes before being exposed to 37°C for another 40 minutes. The 10 min (RT) and 40 min (37°C) process of temperature change is defined as an actuation cycle. Gels were subjected to a predetermined number of cycles. Then, the weight of the gels was measured again at predetermined time points ($W_t$) and the weight change of the gels in this cycle were calculated and plotted. The swelling/de-swelling behavior in the actuation cycle was plotted and termed the actuation profile.

$$\text{Weight change} \% = 100 \% \frac{(W_t - W_d)}{W_d}$$

3.6.9. Cell membrane staining

hTERT cells were seeded at a density of 4x10^3 per cm^2 on tissue culture dishes (or fabricated hydrogels) in 37°C Dulbecco’s modified Eagles medium containing 10% fetal
bovine serum, 5μg/ml insulin, 2.4μg/ml hydrocortisone and standard antibiotics [196]. 24 hours later, cells were washed with 37°C PBS for 3 times. Then, cells were stained with DAPI (20 μg/ml) and CellMask™ cell membrane dye (2.5 μg/ml) in PBS for 2 hours and 10 minutes, respectively. Then, cells were washed with 37°C PBS for 5 times to remove residual dyes on the substrate. After that, cells were immersed in PBS and imaging was performed by confocal microscopy (Eclipse C1 Plus, Nikon).

3.6.10. Cell viability test

Cells were seeded using 37°C cell culture medium at a density of 4x10^3 per cm^2 on polyacrylamide-PNIPAM composite gels. Cells were cultured on the gels for 10 days in 37°C incubator and cell culture medium was changed every other day. After 10 days, cells were washed with PBS for 3 times and stained with DAPI (20 μg/ml) at 37°C for 2 hours. Then, cells were stained with cell viability dyes, calcein AM (2 μM) and EthD-1 (4 μM), at 37°C for 30 minutes. Live cells metabolize calcein AM to calcein and appear green, while dead cells appear red due to EthD-1 passing through damaged membranes and binding to DNA. After 30 minutes, cells were washed with PBS for 5 times and images were acquired by using a Nikon Eclipse C1 Plus confocal microscope.

3.6.11. SEM characterization

After actuation cycles, gels were first immersed in liquid nitrogen for freezing and preserving their internal structure. Then, gels were freeze-dried overnight. A thin layer of Au/Pd was finally deposited on these substrates before SEM characterization. Images were then acquired by FEI Quanta 200 ESEM system.
3.6.12. Excitation and emission wavelength of dyes used

Cell membrane dye: 554 nm/567 nm

Cell viability dye component A (Calcein AM): 494 nm/517 nm

Cell viability dye component B (EthD-1): 528 nm/617 nm

DAPI: 364 nm/454 nm

3.7. Results

3.7.1. Swelling and de-swelling kinetics of the hydrogel actuator

Swelling kinetics of both PNIPAM and PNIPAM-CNT gels were investigated to compare swelling behavior of the gels. Figure 9 shows the swelling behavior of gels at RT. PNIPAM gels increase around 60% in weight within 10 minutes while PNIPAM-CNT gels reach around 30% weight increase. At 15 minutes, PNIPAM gels increased around 75% in weight, while PNIPAM-CNT gels raised around 38%. After 1.5 hours, almost 174% weight increase was observed for PNIPAM gels while PNIPAM-CNT gels increased about 96%. Data here shows that PNIPAM hydrogels displayed a faster swelling response compared to PNIPAM-CNT gels. The rates differ by approximately a factor of two.

De-swelling kinetics of PNIPAM and PNIPAM-CNT gels are shown in Figure 10. 10 minutes after incubating the hydrogels at 37°C, PNIPAM gels lost around 36% of liquid (PBS) content while PNIPAM-CNT gels lost around 18%. At 15 minutes, PNIPAM lost
around 49% of liquid content, compared to 22% in the case of PNIPAM-CNT gels. Both types of gels reach stable liquid retention in around two hours. At this time point, PNIPAM gels have lost around 74% of liquid content, while PNIPAM-CNT lost 38%. Again, the rates differ by approximately a factor of two. The results here indicate that PNIPAM gels display a faster actuation response, whether swelling or de-swelling. The relatively gentle swelling and de-swelling kinetics of PNIPAM-CNT gels could be due to the relatively immobile nature of chains covering CNTs. Since collapse or expansion of the network is limited by robust CNTs, PNIPAM-CNT gels cannot shrink or swell as much as pure PNIPAM gels.
Figure 9. Swelling kinetics of PNIPAM and PNIPAM-CNT gels. Weight changes of the gels were measured at predetermined time points. Values are shown as mean ± SD. (n=5) (**p<0.01); P values were calculated using the Mann-Whitney U test.

Figure 10. De-swelling kinetics of PNIPAM and PNIPAM-CNT gels. Liquid retention of the gels were measured at predetermined time points. Values are shown as mean ± SD. (n=5) (**p<0.01); P values were calculated using the Mann-Whitney U test.
3.7.2. Swelling/de-swelling behavior after repeated actuations

Insufficient fatigue life of a polymer actuator usually leads to deficiencies in safety, performance and reliability. To investigate if our polymer actuators can provide consistent performance after repeated actuations, PNIPAM and PNIPAM-CNT gels were subjected to 10 swelling/de-swelling cycles per day, RT 10 minutes and 37°C 40 minutes per cycle, for 5 consecutive days.

As shown in Figure 11(a), the responsiveness of PNIPAM actuators at the 51st cycle was diminished in comparison to the responsiveness at the 1st cycle. The significant drop in swelling and de-swelling kinetics observed in PNIPAM actuators after 50 actuation cycles suggests that the polymer actuators fatigue over time, which may lead to insufficient and inconsistent mechanical stimulation to cells.

In contrast, PNIPAM-CNT actuators exhibited similar actuation profiles at the 1st and 51st cycles, although less weight change occurred, as shown in Figure 11(b). The absence of a significant decrease in the swelling and de-swelling kinetics of PNIPAM-CNT gels suggests that incorporation of CNTs in PNIPAM actuators may prevent polymer actuator fatigue over time. One possible reason for this “fatigue-resistant” effect is that CNTs incorporated in the gel matrix may strengthen the internal structure and prevent formation of defects after repeated actuation.

As shown in Figure 12(a), more defects and cracks in the actuator internal structure were observed in the case of pure PNIPAM gels. A significant increase in pore size was also observed as a consequence of cycling this material (5.76 μm at 1st cycle vs 8.35 μm at 51st cycle).
cycle\(^8\)). The results here suggest that the deformation of PNIPAM actuators in response to repeated mechanical loading leads to changes in the internal microstructure and thus to bulk property changes. In contrast, similar internal structure was observed in PNIPAM-CNT actuators at the 1st and 51st cycles (Figure 12b).

This phenomenon should be taken into account for next generation polymer actuator design. The integration of nano-materials in polymer actuator systems may increase their life cycle and ensure reliable mechanical inputs applied to cells for long term biological studies. Thus, for further study regarding cell behavior on the actuator system, I used PNIPAM-CNT gels as the platform.

---

\(^8\) SEM images of PNIPAM and PNIPAM-CNT gels were taken and pore size was measured by using Analysis Pro software (Olympus). 300 pores were measured for both PNIPAM and PNIPAM-CNT gels.
Figure 11. Comparison of (a) PNIPAM gel and (b) PNIPAM-CNT gel actuation between the 1st and 51st cycles. Gels were swelled for 10 minutes before being de-swelled for 40 minutes in each cycle. Values of weight change are shown as mean ± SD. (n=7) (**p<0.01); P values were calculated using the Mann-Whitney U test.
Figure 12. SEM images (10 kV, 5000X, 6.8 mm working distance) of internal pore structures in (a) PNIPAM and (b) PNIPAM-CNT actuators at the 1st and 51st cycles. Defects and cracks in internal pores are shown in red rectangles. Scale bar indicates 20 μm.

It is noteworthy that after 10 minutes of swelling during the first cycle, PNIPAM gels displayed a 47% increase in weight (Figure 11), compared to the 61% increase shown in the swelling kinetics data (Figure 9). The difference may due to the continuous exposure to air during weight measurement for the swelling kinetics study. Gels were taken out from beaker for weight measurement at 2 min, 4 min, 6 min, 8 min, and 10 min. For actuation profile measurement, the gels were originally immersed in PBS before moving them from 37°C and exposing them to RT for consecutive 10 minutes. Then gels were taken out from PBS just once at t=10 min for weight measurement. Since the gels in the swelling kinetics experiment were exposed to air more frequently and for longer duration, it is not surprising that these gels swelled more.

3.7.3. Cell viability test: cells survive in the PNIPAM-CNT actuator

To investigate if cells can survive in this nano-hybrid polymer actuator for further study, cell viability tests were performed. Figure 13(a) is an image of a control group, for which cells were cultured in a non-responsive tissue culture dish and treated with 20% ethanol for 30 seconds before performing the cell viability test. Live cells metabolize calcein AM to calcein and appear green. Dead cells appear red, due to EthD-1 passing through damaged cell membranes and binding to DNA. In Figure 13(b), cells cultured on PNIPAM-CNT gels for 5 days all appeared green, which is an indication of live cells that metabolize calcein AM to fluorescent calcein. None of these cells appeared red, which is
an indication of cells remaining alive. This result shows that cells really can survive in this novel nano-hybrid polymer actuator.

![Image of cells cultured on regular polystyrene tissue culture dish and PNIPAM-CNT gel](image1.png)

**Figure 13.** (a) Cells cultured on a regular polystyrene tissue culture dish and treated with 20% ethanol for 30 seconds before performing the cell viability test. Dead cells appeared red, while live cells were green. (b) Cells cultured on PNIPAM-CNT gel for 5 days before the cell viability test was performed. Cells appeared green, indicating that they were alive. Scale bar indicates 50 μm.

### 3.7.4. Cells survive in the PNIPAM-CNT actuator after repeated actuation cycles

In order to know if cells can survive in this platform for a long time, I performed a cell viability test after cells were subjected to repeated mechanical stimulation. Gels were swelled and de-swelled for 51 cycles (10 cycles per day for 5 days), and were stained with
cell viability test dyes. As shown in Figure 14, green fluorescence was observed, demonstrating that cells are still alive. No red cells were observed, even after repeated mechanical actuation, which is a promising indication that cells can reside in this device and survive after repeated actuation cycles. Results here indicate that this PNIPAM-CNT actuator system provides a platform for investigating the long term effect of physical input on cell behavior change.

Figure 14. Cells cultured on PNIPAM-CNT gels were subjected to repeated actuation cycles, 10 cycles per day for consecutive 5 days, before the cell viability test was performed. Cells appeared green indicating that cells were alive. Scale bar indicates 50 μm.

3.7.5. Calibration of heating stage

The actuation behavior generated by our PNIPAM-CNT gels is driven by temperature change. Thus, the control of temperature is critical for my experiments. In order to (a)
have precise control of temperature inside the heating chamber and (b) minimize the possibility of overheating, calibration of the heating stage (LiveCell\textsuperscript{+}, Pathology Devices) was performed. PNIPAM, a well-documented temperature responsive polymer with its LCST at around 32°C, was selected as a temperature indicator. Below its LCST, PNIPAM gels are hydrophilic and transparent. However, when temperature is increased to above 32°C, the hydrophobic interaction dominates and PNIPAM turns white in appearance. The color change at 32°C can thus be used to calibrate the actual temperature inside the heating chamber. Temperature on the heating stage display was adjusted to 29°C and maintained at this temperature for 10 minutes to reach equilibrium. Then, the displayed temperature was increased by 1 degree every 5 minutes until it reached 33°C. As shown in Figure 15, when the displayed temperature is at 29°C, PNIPAM gel is transparent. However, when the displayed temperature reaches 30°C, the gel starts to turn milky white in appearance, especially at the edge. The change in color becomes more obvious, even in the center of the gel, when the temperature reaches 31°C on the display. It is reasonable that the color change initiates at the periphery of the gel, since this is the portion of the gel that has more interaction with the heated water. PNIPAM gel is reported to have its LCST at around 32°C, which means that it should change color at 32°C. The fact that our PNIPAM hydrogel starts to change color when the temperature is displayed as 30°C suggests that there may be a 2 degree difference between temperature inside the heating chamber and temperature displayed on the monitor. To minimize the risk of overheating and subsequent membrane blebbing of cells, I hence calibrate the true temperature inside the heating temperature as $T_t$, while the displayed temperature on the monitor is $T_p$. $T_t$ is defined as
Tp + 2 (with temperatures expressed in °C). All the temperatures in experiments mentioned hereafter are Tt, the true temperature inside the heating chamber.

Figure 15. Calibration of the heating stage. PNIPAM gel was immersed in PBS and incubated in the chamber of the heating stage for 10 minutes with the displayed temperature set at 29°C. Then, the displayed temperature was increased by 1°C every 5 minutes until it reached 33°C. The color change of PNIPAM gel was observed and marked with red arrowhead.

3.7.6. Cells respond to mechanical stimulation: change of cell shape

I observed that live cells cultured on PNIPAM-CNT actuator indeed sensed the mechanical stimulation. Cells were cultured on our PNIPAM-CNT gels before staining with calcein AM. Then, gels were exposed to RT and cell morphology was observed by confocal microscopy. As shown in Figure 16, cells were stretched and extended after the gels swelled. Two minutes after the gels were exposed to RT, the cells appeared to be more stretched and elongated. The bent cell shape observed at time 0 was replaced by a straighter, more elongated shape when gels were swelled for 10 minutes. These
preliminary results suggest that cells can indeed survive in our novel PNIPAM-CNT scaffold and change cell morphology in response to mechanical stimulation.

Figure 16. Cells respond to gel swelling by stretching and a change in morphology. Gels were exposed to RT for 15 min and cells cultured within the gel were stained with calcein AM. Cell images were taken at 0 min, 2 min, and 10 min. Cells were stretched and shape changes are marked with red rectangular boxes. Scale bar indicates 50 μm.

3.7.7. Cell responds to mechanical stimulation: change of cell size

To further confirm that cells did sense the mechanical actuation, cells are labeled with cell membrane dye to highlight the outline of cells. The cell membrane is a commonly used marker of cell boundaries, and can be used to determine the size of cells. The cell membrane was labeled with an amphipathic probe, which contained a hydrophilic dye conjugated on a lipophilic moiety for cell membrane anchoring. As shown in Figure 17a, cells stretched and cell size increased when gels were exposed to room temperature. When gels de-swell at 37°C, cell size decreased. Moreover, the perimeter of the cells increased with gel swelling, while the mean intensity of fluorescence from the cells decreased with
gel swelling (Figure 17b). The increase in cell area and perimeter during gel swelling indicated that cells were stretched in coherence with gel swelling. The decrease in mean intensity of fluorescence from the cells can possibly be explained by the decrease in the amount of membrane dye per unit area when the cells are larger: since cells are stretched when the gel swells, the increased cell size leads to a decreased ratio of amount of dye/cell area.

Figure 17. Cells respond to gel swelling by changing their size. (a) Gels were exposed to RT for 15 minutes and cells labeled with cell membrane dye were imaged. Change in area, perimeter,
and mean intensity was measured by using NIS-element software (Nikon, Japan). (b) Summary of change in area, perimeter, and mean intensity of a fluorescence labeled cell during gel actuation.

The preliminary results presented above indicate that (a) cells can survive in our PNIPAM-CNT actuator, and (b) cells can sense the mechanical stimulation provided by our PNIPAM-CNT actuator, resulting in cell shape and size changes. The phenomenon of cell size increasing with gel swelling at RT was not observed when cells were cultured on polystyrene dishes, as shown in Figure 18.

![Image of cell size measurement on polystyrene dishes after temperature change.](image)

Figure 18. Cell size measurement on polystyrene dishes after temperature change.

3.8. Discussion

Smart materials, such as stimuli-responsive polymers, are materials that alter their properties in response to minute external stimuli. PNIPAM-based hydrogels, which exhibit reversible
swelling (when T< 32°C) and de-swelling (when T>32°C), are thus capable of exerting mechanical actuations on cells due to dimensional change of the material. However, few papers have discussed the long-term actuation behavior of PNIPAM bio-mimetic actuators. We reported here that PNIPAM gel actuators show signs of fatigue after repeated cycles, and the amplitude of swelling/de-swelling diminish over extended tests. Investigation of the microstructure of PNIPAM gels using SEM showed that more cracks and defects were generated after repeated deformation. On the other hand, addition of less than 0.02% (wt%) of CNTs to a PNIPAM matrix prevents the formation of defects (Figure 12b) and provides similar actuation kinetics after repeated actuation cycles (Figure 11b). Using polymer actuators to investigate how mechanical input can affect cell behavior and influence cell fate, the consistent actuation kinetics provided by this nano-hybrid system is critical and essential.

Telomerase reverse transcriptase (hTERT)-reconstituted human fetal hepatocytes are capable of indefinite replication and maintain the differentiation potential [196], while being an ideal vehicle to investigate hepatic differentiation and drug metabolism. It has been shown that mechanical stimulation leads to increased expression of detoxification genes in primary human hepatocytes [197]. Reports also suggest that PNIPAM-based material can induce activation of hepatic stem cells in a liver injury rat model system [198]. Ductular cells overlaid with PNIPAM gel showed signs of hepatic differentiation, expressing albumin and C/EBPα, markers of differentiated hepatocytes. Hence, applying mechanical stimulation to hTERT-reconstituted fetal hepatocytes by using our PNIPAM nano-hybrid actuator may provide an opportunity to derive differentiated functional hepatocytes for liver failure therapy and drug screening.
In this preliminary study, I demonstrated that our PNIPAM and PNIPAM-CNT actuators are capable of exhibiting swelling/de-swelling behavior in response to temperature change. Faster swelling and de-swelling kinetics were observed in PNIPAM gels, compared to CNT-incorporating counterparts. However, when subjected to repeated actuations, the responsiveness of PNIPAM-CNT polymer actuators does not diminish over time, compared to PNIPAM actuators. Incorporation of CNTs in PNIPAM actuators increases the life cycle of the actuator; it acts as a “fatigue-resistant” material, providing better performance and reliability for usage. I also demonstrated that cells can survive in this novel platform, even after experiencing repeated actuation cycles. Results also suggested that cells changed shape in response to mechanical stimulation initiated by gel swelling/de-swelling. We expect that insight will be gained by using this polymer actuator to investigate the role of dynamic mechanical loading on hepatic differentiation.
Chapter 4. Fabrication of a bilayer polymer actuator system

4.1. The need to fabricate a bilayer polymer actuator system

Our goal is to apply mechanical stimulation to cells residing on the PNIPAM hydrogel actuators. In order to generate a fast and strong swelling/de-swelling response, a porous structure of the PNIPAM gel matrix is required, for the following reason:

For PNIPAM gels to swell, it requires water to diffuse into the gel through the pores and form hydrogen bonds with PNIPAM when the temperature is below the LCST. When T>LCST, water is expelled through the pores, resulting in gel de-swelling. Since the diffusion of water is through the pores in the PNIPAM gel matrix, faster and stronger actuation can be expected to correlate with larger pore sizes.

However, a large pore size is like a double-edge sword. On one hand, it facilitates the diffusion of water and provides the desired fast and strong actuation. On the other hand, large pores at the surface often can trap cells. As a result, the edges of different cells may not be in the same focal plane, and so the acquired images may not be well-focused. This problem may hinder the correct measurement of cell behavior, such as shape change or calcium signaling change. Adjusting the crosslinking density or monomer concentration can be used to decrease the pore size, but the swelling/de-swelling capability of PNIPAM gel will be sacrificed if pore size is decreased.
To overcome this problem, a “hybrid bilayer gel system” is desired, combining two sought-after features that (1) provide fast and strong actuation for cell stimulation and (2) provide a flat substrate with small pores for cell behavior analysis.

4.2. Introduction of bilayer hydrogel system: current status

Hydrogels are composed of polymer chains that are cross-linked by either covalent, ionic or physical interactions. These gels have become one of the fast-emerging and physiologically relevant type of material that have been used extensively in different fields, from drug delivery, actuator systems, and microfluidic systems, to tissue engineering [173, 199-203]. Attempts have been made to create hybrid hydrogel systems that integrate the properties of each hydrogel component within the system to increase their versatility and application. For example, a bilayer system composed of gels with different swelling behaviors, in which one layer (NIPAM-acrylic acid) swells in response to ionic strength or pH change, while the other layer (poly-ethylene oxide diacrylate) remains static, can be fabricated to create stimuli-sensitive folding actuators [204]. Stress formed at the gel interface due to differential swelling leads to hinges folding in coherence with pH change. Combination of two hydrogel layers, PNIPAM and poly(dimethyl acrylamide) respectively, with different patterns can generate surfaces with different properties at different locations on those surfaces [205]. A bilayer constructed with gels of different composition (a top collagen gel layer and a bottom PEGylated fibrin layer) to engineer the cell-matrix interaction has also been developed, to direct different cell fates from a single population [206]. The PEGylated fibrin layer promotes vascular differentiation from adipose-derived mesenchymal stem cells, while the
collagen layer specifies the dermal connective tissue fate of cells from the same population. Reports also describe how multiple layers of cross-linked PA and PAA gels have been fabricated as an electro-responsive, muscle-mimic actuator [172]. Palladium electrodes were embedded in this composite gel, and injection of $\text{H}^+$ at the anode led to shrinkage of the PAA gels, with released water being absorbed by the PA gels. Moreover, deformation of multilayered PA gel (lower elastic modulus) and PAA gel (higher elastic modulus) resulted in a linear contraction, rather than bending. These examples indicate that hydrogels with different properties can be integrated into one platform to increase their versatility or provide new functions.

4.3. Design of our bilayer system

Our bilayer composite construct consists of a bottom actuation-generating layer and a top cell-seeding layer that are cross-linked with each other. The actuation-generating layer is composed of PNIPAM-based material that swells and de-swells with temperature change. A highly porous structure is desired in this layer to ensure that significant and fast gel deformation can be generated. On the other hand, the designed top layer is the cell seeding layer, with the properties of small (less than 50 μm) pore size, a coherent interface with the bottom layer, and good cell adhesion.

4.3.1. Desired properties and materials selection for the bottom actuation generating layer

This layer is made of PNIPAM or PNIPAM-CNT-based matrix. PNIPAM is a thermo-responsive polymer that will collapse when the temperature is above its LCST, due to the
hydrophobic interactions. When temperature is below the LCST, PNIPAM forms hydrogen bonds with water and swells. The larger the pore size, the more it is possible for water to diffuse in and out rapidly. Hence, the gel deformation that generates mechanical actuation can be fast and strong. To manufacture hydrogels with a large pore size, the concentrations of monomer and crosslinker are critical factors [207-209]. Increases in initial monomer concentration lead to smaller pore size [207]. If the same amount of monomer is used, a lower crosslinker concentration leads to larger pores [208, 210]. Furthermore, it has also been suggested that a larger size of the crosslinker translates into larger pores [211]. SR415 (Figure 19), which is a triacrylate polyethylene glycol crosslinker and has long 3-armed structure [209, 212, 213], can thus be used in this layer to increase the spacing between the crosslink points. Therefore, a larger pore size can be expected in the actuation-generating layer.

Figure 19. Chemical structure of SR415. $x+y+z \approx 20$. 
4.3.2. Desired properties and materials selection of the top cell-seeding layer

In a porous single-layered PNIPAM hydrogel system, cells may be trapped in the large pores (>100 μm) and so make imaging and cell retrieving difficult. Hence, we can incorporate another layer of hydrogel, which has smaller pore size (<50 μm), on top of the actuation generating layer. Cells can thus reside on the top layer, which has smaller pore size, (a) can still sense the mechanical stimulation and (b) facilitate for imaging. The top cell-seeding layer should possess the following desired properties: (1) flat with small pores for imaging and cell retrieving purpose, (2) stiffness matches that of hepatocytes, and (3) good cell adhesion.

Since a small pore size is required in this layer, the concentration of crosslinker can be elevated to increase crosslinking density and decrease pore size. Moreover, crosslinkers like bisacrylamide (Figure 20), which is shorter in length and smaller in size, can be used to decrease the spacing between crosslink points.

Figure 20. Chemical structure of bisacrylamide.
Thickness of top layer is another important factor. A top layer that is too thick will minimize the mechanical stimulation transferred from the bottom layer. On the other hand, if the top layer is too thin, it will be conformable to the bottom layer. This will lead to a wrinkled surface, and thus interfere with the imaging process.

4.3.3. Stiffness of the substrate

Cell function and fate decisions can be regulated by physical cues from their substrate [27, 150]. Hence, it is desired to have the mechanical properties, such as matrix stiffness, of our platform to mimic the properties of the in vivo microenvironment of the cells. It has been shown that cell characteristics, such as morphology, migration, and fate decision, can be influenced by substrate stiffness [21, 214-220]. Cells cultured on a compliant substrate tend to adopt a round morphology, and migrate toward stiffer substrate. Reports have also been published that human mesenchymal stem cells (MSCs) cultured on soft matrix acquire neuronal fate, while they are directed toward bone lineage when cultured on stiff substrates [21]. The fact that the response of cells to substrate stiffness echoes the nature of the native tissue from which they are derived, highlights the importance of making our substrate stiffness match that of human liver.

Young’s modulus (E), an indicator of substrate stiffness, is defined as resistance to elastic deformation, and can be determined from the slope of a stress versus strain plot. The Young’s modulus of normal liver, which is a relatively soft organ, is between 400 and 600 Pascal (Pa; = newtons/m²) [163, 214]. However, the Young’s modulus of liver can increase to around 15k Pa following liver diseases, such as fibrosis and cirrhosis, or liver injury [216, 221]. Cells cultured on soft substrates generally show signs of growth arrest,
poor cell adhesion, and apoptosis [221, 222]. Nevertheless, hepatocytes, which are considered “soft” and are the main cell type in liver, respond to stiff substrates by spreading and proliferating more rapidly, although they lose their differentiation traits [163, 216, 221, 223, 224]. Moreover, hepatocytes in their soft in vivo microenvironment exhibit spherical-aggregated morphology, while culturing in vitro using stiff tissue culture plates causes them to adopt an elongated shape, and they are sometimes mal-functioned. Several lines of evidence have shown that stiffness of the substrate plays a fundamental role regulating behavior and function of cells in a cell-type-specific manner that probably correlates with their function in vivo [21, 225, 226]. As a result, the Young’s modulus of our device and that of the cell itself should be taken into consideration.

4.3.4. Adjusting gel stiffness

To fine-tune the stiffness of the polymer substrate, the types of monomer, and the concentration of crosslinker and monomer can be adjusted. PA gels have been commonly used as a platform to investigate the role of substrate stiffness on cell behavior. By simply adjusting the concentration of crosslinker, the stiffness of the gel can be tuned across a wide range [21, 217, 220]. PA gels are also bio-inert, so unlike other protein gels or polysaccharide gels that will react directly with cell surface receptors, the chemical cues sensed by the cells can be decoupled from physical cues.

Reports published in 2006 demonstrated that by keeping monomer concentration constant and adjusting crosslinker concentration, the Young’s modulus of PA gels can be tuned between 0.1 kPa to 40 kPa [21]. Similarly, by adjusting the ratio of total concentration of acrylamide + bisacrylamide over concentration of bisacrylamide, the Young’s modulus of
the gel can be adjusted between 1 to 42 kPa [227]. Using 10 % acrylamide with different concentration of bis-acrylamide, ranging from 0.26 to 0.03%, the Young’s modulus of the PA gel can be tuned between 80 Pa and 10 Pa [217]. Another research group, which maintains acrylamide concentration at 8 %, fabricates gels with Young’s modulus ranging from 14000 to 30000 Pa, by manipulating bis-acrylamide concentration from 0.03 % to 0.06 % [220]. Other examples of controlling gel stiffness by tuning crosslink density have also been reported. The Young’s modulus of PA gel at 20°C, with molar ratio of bis-acrylamide to acrylamide between 0-5%, can increase from approximately 50 to 350 kPa [228]. At the same temperature and same range of bis-acrylamide/monomer ratio, the Young’s modulus of PNIPAM gels can be adjusted between 0 to 20 kPa [176, 228]. Increasing the temperature to 40°C can increase the Young’s modulus of PNIPAM gel to values ranging from 15 to 120 kPa, with a molar ratio of bis-acrylamide to NIPAM between 0-5% [228].

4.3.5. Surface modification for cell adhesion

Although a top layer with small pores may be good for imaging and cell retrieving purpose, the lack of roughness on smooth gel surfaces may lead to poor ECM molecule adsorption. This may ultimately result in poor cell adhesion. Conjugation of arginine-glycine-aspartic acid (RGD) motifs [57, 229, 230], plasma treatment[9] [231], or incorporation/grafting of extracellular matrix molecules [217, 232] are some common ways used to promote cell adhesion. Furthermore, materials with bio-inert nature that cells do not attach well to should be avoided. A proper balance of hydrophobicity, as well as incorporation of

---

[9] Plasma treatment, such as argon plasma, is a common way to modify the hydrophobic polystyrene surface and promote cell adhesion. The possible mechanism is that by performing plasma treatment, oxygen-containing functional groups may be incorporated on the hydrophobic polystyrene surface and increase wettability.
functional groups on the gel surface, are important to supporting cell adhesion [231, 233, 234], and should be taken into consideration.

4.4. Material selection for top cell seeding layer

**PNIPAM:** A combination of higher NIPAM concentration and crosslinker concentration can be used to make a top layer with smaller pores. Since the same kind of monomer, crosslinker, and solvent are used, with the only difference compared to the bottom layer being the concentration, a better interface between top layer and bottom layer can be expected. The top cell-seeding layer can be crosslinked and thus connected well to the bottom actuation generating layer. The possibility that the two layers will separate after repeated cycles, or that mechanical stimulation cannot be transferred efficiently from the bottom layer to the top layer, can be minimized. However, there is a concern regarding this design. If the top layer was made with PNIPAM-based material, cells might be detached from the PNIPAM surface when the temperature is lower than the LCST of PNIPAM during our experiment to swell the gels. The detachment of cells from PNIPAM surfaces when T<LCST is a common phenomenon due to the hydration of the gel surface. To overcome this issue, ECM molecules such as fibronectin, can be conjugated on the PNIPAM surface to minimize cell detachment from hydrated PNIPAM gel [235].

**Polyacrylamide:** A top cell seeding layer that is not composed of PNIPAM can minimize the possibility of cells detaching from a PNIPAM gel surface at T < LCST [236-239] due to the hydration of the gel surface at low temperature. PA gels are commonly used in biology
to investigate the relationship between gel stiffness and cell migration [220], focal adhesion formation [217], and cell fate decision [21]. The cost for PA gel is low, so it can be mass-produced. The stiffness of PA gel can be easily tuned [217, 220, 227] to match the stiffness of tissues *in vivo*. Considering the ease of making PA gels and adjusting their properties, and the potential cell-detaching effect from PNIPAM gel, I selected PA gel as the top layer material.

4.5. Materials and methods

4.5.1. Gel making procedure

**PA-PNIPAM-CNT hybrid gels:** PA gel precursor solution was prepared by dissolving acrylamide (1.3M), bisacrylamide (0.019M), and TEMED (0.03M) in DI water. The solution was mixed and purged with nitrogen for 30 minutes before mixing with purged ammonium persulfate (AP) (0.02M) solution. The gel formation reaction was performed at room temperature in 12 wells for 5 minutes. Then, PNIPAM-CNT gel precursor solution (same recipe and procedure as mentioned in chapter 3, 3.6.5.) and AP solution were added on top of the PA gels to make the PNIPAM-CNT gel layer. Gelation was performed at room temperature for 18 hours, after which the PNIPAM-CNT gels had formed and bound with the PA gels, generating bilayer hybrid gels. The gels were then washed with PBS for 15 cycles (room temperature for 15 minutes and then 37°C for 15 minutes per cycle) before being autoclaved.
Alternatively, the PA-PNIPAM-CNT bilayer gels can be made in molds with a flat surface and a defined shape (rectangular), if necessary. A thin layer of polymethylglutarimide (PMGI) can be coated on the substrate, functioning as a sacrificial layer. Removing the sacrificial layer can produce hydrogels with a flat surface and minimize the possibility of distortion while the gels are collected.

The fabrication procedure is shown below (Figure 21). A thin layer of PMGI is spin-coated (3000 rpm for 1min) on silicon wafer to function as a sacrificial layer. Then, the wafer is baked at 150°C for 20min before the Teflon mold is placed and fixed on the wafer by using binder clips. Top layer gel precursor solution is then poured on the wafer for polymerization. Five minutes later, the bottom layer gel precursor solution (actuation-generating layer) is added on top of the first layer and polymerization is performed at RT for 18hrs. After that, gels are soaked in 1M NaOH at RT for 20 seconds to dissolve the PMGI sacrificial layer. Finally, the gels become detached from the wafer. Gels are collected and washed for 10 times in PBS to remove residual monomer before being used in cell culture.
4.5.2. SEM characterization

Bilayer gels were manufactured and freeze-dried overnight. Then, a thin layer of Au/Pd was deposited on the gels. SEM images were then acquired with an FEI Quanta 200 ESEM system.
4.5.3. Cell membrane staining

Cells were seeded at a density of $4 \times 10^3$ per cm$^2$ on PNIPAM-CNT gels. Twenty-four hours later, cells were washed three times with PBS. Cells were then stained with CellMask™ cell membrane dye, at a concentration of 2.5 $\mu$g/ml in PBS for 10 minutes at 37°C in an incubator. After that, cells were washed in PBS for 5 times and imaged using confocal microscopy.

4.5.4. Collagen conjugating on PA-PNIPAM-CNT gels

PA-PNIPAM-CNT gels were made, washed and autoclaved. Then, the side with polyacrylamide gels was immersed in 10mM of N-Sulfosuccinimidyl-6-(4'-azido-2'-nitrophenylamino) hexanoate (Sulfo-SANPAH) (pH 8.5 in PBS) (Pierce) and was exposed to the UV light (13.4 watts) of a biosafety cabinet for 5 minutes. After that, suction was performed to remove Sulfo-SANPAH solution. This process of photo-activation was repeated once. Then, gels were washed in PBS at RT for 5 times before being soaked in collagen I (1mg/ml) solution overnight at 4°C. The reacted gels were washed completely with 37°C PBS before cells were seeded.

4.6. Results

4.6.1. Surface morphology of PA-PNIPAM-CNT bilayer gels

In order to fabricate a bilayer hydrogel system, which consists of top cell-seeding layer with small pores and bottom actuation-generating layer with large pores, the concentration
of acrylamide and bisacrylamide in the top layer was increased. The desired feature of the top layer, small pores (less than 50 μm), can minimize the possibility of cells being trapped in large pores (> 100 μm) and so hindering the acquisition of useful images. As shown in Figure 22, large pores (approximately 100 μm in diameter) were observed on the surface of both PNIPAM and PNIPAM-CNT single layer gels. On the other hand, a smoother surface was observed on PA-PNIPAM-CNT gels (Figure 22c). Cells trapped in large pores may not appear in the same focal plane, and thus will lead to unfocused images. The smoother surface provided by PA-PNIPAM-CNT gels can thus provide a better platform for cell imaging and behavior study.

![Figure 22. Surface morphology of (a) PNIPAM, (b) PNIPAM-CNT, and (c) PA-PNIPAM-CNT gels. Scale bars indicate 100 μm.](image-url)
4.6.2. Cells cultured on the two-layer hybrid gel can sense mechanical actuation

I have shown that cells cultured on PNIPAM-CNT single layer gels can sense mechanical actuation (chapter 3, 3.7.6. and 3.7.7.). However, the question now arises as to whether cells can sense the actuation on the new platform, a bilayer PA-PNIPAM-CNT gel. As shown in Figure 23, cells cultured on the hybrid bilayer actuator exhibited a size change with gel swelling and de-swelling. When temperature was fixed at RT, the gel swelled and the cell size was observed to increase. Conversely, if temperature was set at 37°C, the de-swelling of the gel resulted in decreased cell size.

![Figure 23. Cells cultured on the PA-PNIPAM-CNT bilayer gels sensed mechanical stimulation and responded by a change in cell size. Scale bar indicates 50 μm](image)

Furthermore, this novel hybrid gel actuator system is also biocompatible, and cells can survive in this platform. Ten days after cells were seeded on the bilayer gel, a cell viability test was performed as shown in Figure 24. Live cells undergo cell viability tests metabolize calcein AM to calcein and appear green. The human fetal hepatocytes shown in Figure 24 were stained green, indicating that cells were still alive.
Figure 24. Cells can survive on a PA-PNIPAM-CNT composite gel system for at least 10 days. Scale bar indicates 50 μm.

4.6.3. UV-Ozone treatment to minimize dye adherence on gels

So far, the new bilayer system seems promising since (a) it can generate mechanical actuation to stimulate cells, and (b) cells can survive in this bilayer actuator for at least 10 days. However, we found that the dyes used to label our cells will adhere to the gel surface, possibly due to the formation of hydrogen bonds between dye molecules and polymer gels. The phenomenon of dye adherence and binding on gels was observed on PNIPAM gel, PNIPAM-CNT single layer gel and PA-PNIPAM-CNT bilayer gel. Figure 25 is an example of dye adherence on PA-PNIPAM-CNT gel surface. The residual dye adhered on the gel surface created a strong background and may interfere with our data analysis. Thus, an approach to minimize dye adherence is desired.
UV-Ozone surface treatment was originally developed for the removal of organic contaminants and surface cleaning of semiconductor substrates [240]. By using a mercury lamp that generates two wavelengths of interest, 184.9nm and 253.7nm, hydrocarbons on substrates can be oxidized and decomposed. In general, substrates are exposed in an oxygen-filled chamber, and light output at 184.9nm from the mercury discharge tube is absorbed by oxygen molecules, leading to the production of ozone. Meanwhile, light output at 253.7nm breaks up the generated ozone molecules and continuously generates atomic oxygen molecules that can oxidize the hydrocarbons on substrates. While it has been shown that UV-Ozone treatment can be used to oxidize and remove organic contaminants on different substrates, little is known about whether this process can be used to oxide functional groups on polymer substrates for minimizing hydrogen bond formation between dyes and substrates. I performed a series of tests, by manipulating the temperature and treatment time, to investigate whether UV-Ozone treatment can minimize dye adherence on gels.
Figure 25. An example of dye adhered on gel surface (PA-PNIPAM-CNT gels), leading to increased background fluorescence. Cell membrane dye (red) and DAPI (blue) were used to label the cell membrane and the cell nucleus. Residual cell membrane dye was observed on gel surface. Scale bar indicates 50 μm

First, PA gels were made and washed in PBS for 5 times. Then, gels were UV-Ozone treated (at 25°C, 50°C, 75°C for 20, 40, and 60 min, respectively) and washed with PBS for 5 times before staining with cell membrane dye (2.5 μg/ml) for 10 minutes at 37°C in an incubator. As shown in Figure 26, a higher temperature (chosen from 25°C, 50°C, and 75°C) during UV-Ozone treatment correlates with less cell membrane dye adherence on the gel surface. In addition, longer UV-Ozone treatment times correlate with lower fluorescence intensity being observed on the gel surface. Hence, treating PA gel surfaces
with UV-Ozone at 75°C for 1hr was chosen to minimize dye adherence on those surfaces during cell staining and imaging.

Figure 26. Effect of UV-Ozone treatment on cell membrane dye adherence on PA gels. Constant exposure time was applied when photographing images. Scale bar indicates 50 μm.

4.7. Conclusion and discussion

Cells respond to physical stimulation, such as mechanical manipulation and substrate stiffness, by a series of biological process, including cell shape change. The shape of cells is
believed to have a significant impact on cell growth and fate determination [241]. For example, human mesenchymal stem cells spreading over large areas are directed toward osteocytes while cells allowed to spread in confined areas differentiate into adipocytes [242]. To analyze cell shape change, cells can be stained with cell membrane dyes to highlight the outline of the cells. Then, change in cell shape and size can be acquired.

So far, I showed that (a) cells can survive in the bilayer composite gel actuator for at least 10 days, (b) cells cultured on this new platform can sense mechanical actuation, and (c) dye interference can be minimized by UV-Ozone treatment.

However, I found out that after UV-Ozone treatment, cells do not attach to the gel. It may due to the alteration of surface chemistry after UV-Ozone treatment. Chemical cues required for ECM binding or cell attachment may be damaged. Other approaches such as measuring cytosolic calcium concentration change, which (a) is sensitive to mechanical inputs and (b) is specific that requires Ca^{2+}-probe binding to fluoresce, may provide another way to demonstrate that cells are influenced by gel swelling and de-swelling.
Chapter 5. Calcium signaling for monitoring cell response to mechanical actuation

One of the approaches to observing cell behavior changes upon mechanical stimulation is to monitor calcium signaling change [170, 243-245]. Calcium influx can be induced by mechanically stimulating cells [246, 247]. The increase in cytosolic Ca\(^{2+}\) concentration which is mediated by stretch-activated channels on the cell membrane can be monitored by loading calcium-specific probes into cells. Upon binding with Ca\(^{2+}\), the fluorescence intensity of calcium probes increases more than 100 fold and the calcium fluctuation can be observed easily by recording fluorescence intensity variation. Calcium signal profiles, including frequency, rate of rise and decay, and magnitude, can be decoded in spatial and temporal manners.

The advantages of this approach are (a) quick and real-time monitoring, (b) high sensitivity, (c) high specificity and (d) excellent spatial resolution. The change of calcium profile can be as fast as mini-seconds after receiving stimulation, as opposed to hours or days for gene expression. In addition, it has been shown that a fluid shear stress as minute as 0.1 dynes/cm\(^2\) (or approximately 10 piconewton per cell) can trigger a transient calcium increase in bovine aortic endothelial cells [248]. Moreover, calcium concentration spatial profile can be monitored in real-time, so the intracellular signal relay and cross talk between organelles can be revealed. The intracellular calcium gradient can be mapped by the loading of calcium-specific indicators. As a result, mechanically induced response between different organelles can be observed, which may not be easily approached by other known methods. Other than intracellular communication, elevation of calcium signals can be propagated between cells [249, 250]. Hence, the propagated calcium waves can be monitored for investigating cell-cell communication in response to mechanical stimulation. Finally, the preferential calcium-probe binding makes this approach highly specific.
Indeed, there are numerous advantages studying the effect of mechanical stimulation by monitoring calcium signaling profiles. However, there are also some limitations and shortcomings. For example, the autonomous calcium fluctuations inside a cell may interfere with calcium signals that result from mechanical stimulation. Moreover, a rapid calcium signal profile change does not necessarily herald the alteration of gene expression. As a result, more work needs to be done to identify and carefully investigate the interaction of molecules that link Ca^{2+} oscillations to gene expression change. When studying the changes in calcium signaling upon mechanical stimulation, these considerations should be taken into account carefully.

5.1. Calcium signaling overview

Calcium ions participate in almost every aspect of life, mediating a wide variety of cell functions, including cell morphology regulation, metabolism, secretion, apoptosis, and proliferation [251-254]. An ion as simple as Ca^{2+}, can be coded as complex signals that differ in frequency, amplitude, space, and time to serve different roles in maintaining cell function. For example, calcium ions bind to calmodulin and regulate cell morphology by controlling actin-myosin interaction [255]. Releasing calcium ions from the sarcoplasmic reticulum (SR) and recruiting calcium sparks activate myocyte contraction [256, 257]. Inhibition of calcium-binding protein, calmodulin, slows proliferation [258, 259]. Disruption of calcium signals may cause permeability changes in mitochondria, activation of endonuclease, or disruption of cytoskeleton organization, eventually leading to cell death [260].
Cells mobilize their calcium signaling by allowing calcium influx across the cell membrane and/or by releasing calcium storage from the endoplasmic reticulum or sarcoplasmic reticulum [261, 262]. When calcium signals are activated, they activate effector proteins and propagate the signals throughout or between the cells. Cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_c\)]) in the resting state is retained at around 100 nM, but upon stimulation, it can rise sharply to more than 1 \(\mu\)M [263, 264]. An increase in [Ca\(^{2+}\)\(_c\)] can serve as a signaling messenger to regulate many biological processes, such as cell growth, cell migration, exocytosis, and cell death (Figure 27) [254, 260, 263, 265, 266].
Figure 27. Calcium signaling mechanism. (Adapted from “Calcium – a life and death signal”, Berridge et al., 1998 [266]) Cytosolic Ca\(^{2+}\) concentration can be up-regulated by calcium influx from extracellular sources or calcium storage inside the endoplasmic reticulum (ER). The elevated Ca\(^{2+}\) can regulate cell functions, including fertilization, proliferation, secretion, metabolism, and contraction. Mitochondria will uptake some of the Ca\(^{2+}\) released from the ER and then return the Ca\(^{2+}\) to the ER. If, there is Ca\(^{2+}\) overload in mitochondria, cells will undergo apoptosis.

5.2. Calcium signaling in development

5.2.1. Calcium signaling in embryogenesis

The role of Ca\(^{2+}\) signaling in development can be traced back to as early as egg activation and axis patterning [267, 268]. After fertilization, an increase in cytosolic calcium concentration triggers the release of cortical granule materials and blocks polyspermy. During later stages of development, a ventral to dorsal gradient of Ca\(^{2+}/\) inositol 1,4,5-trisphosphate (IP3) has been suggested to specify dorsal-ventral axis patterning in *Xenopus* [269]. Disrupting this Ca\(^{2+}/\) IP3 gradient leads to re-specification of the dorsal-ventral axis [270, 271]. It has also been indicated that during left-right development, motile cilia generate a leftward fluid flow while the mechanosensing cilia on the left side of the embryo sense the stimulation and trigger influx of calcium. The elevation of calcium concentration on the left side then up-regulates the expression of nodal, and pitx2, which
are important factors for left-right asymmetry establishment [272]. All the aforementioned examples indicate that Ca$^{2+}$ ions are actively involved in cell development.

5.2.2. Role of Ca$^{2+}$ in cell proliferation

Calcium ions have been acknowledged to play an important role in regulating cell proliferation. For example, it has been shown that proliferative activities of normal cell lines and tumorigenic cells lines increase with the concentration of extracellular calcium [273]. Later, it was revealed that Ca$^{2+}$ ions not only are involved in early G$_1$, and G$_{1}$/S, G$_{2}$/M transitions of the cell cycle [274], but also are integrated into signaling pathways that govern cell proliferation, such as mitogen-activated protein kinase (MAPK) signaling pathways. [258, 275-277].

One of the better understood examples is during T cell activation following antigen binding [258]. Antigen binding leads to activation of phospholipase C (PLC), which generates diacylglycerol (DAG) and IP3. DAG activates protein kinase C (PKC) which in turn activates MAPK signaling cascades, a well-known signal pathway that promotes cell proliferation. IP3 binds to the IP3 receptor Ca$^{2+}$ channel on the ER membrane, leading to calcium release from intracellular calcium storage. Then, Ca$^{2+}$ release activated Ca$^{2+}$ channels (CRACs) on the cell membrane will be activated resulting in calcium influx that is necessary for T cell activation (Figure 28). Once activated, T cells leave the quiescent G$_0$ phase, enter the cell cycle, and proliferate.
Figure 28. Function of Ca\textsuperscript{2+} during T cell activation and proliferation. Adapted from “The versatility and universality of calcium signaling”, Berridge et al., 2000 [275]. Antigen binding to T cell receptors recruits as well as activates PLC, generating DAG and IP\textsubscript{3}. IP\textsubscript{3} releases the Ca\textsuperscript{2+} storage from ER. Increased Ca\textsuperscript{2+} concentration activates transcription factors including NF-κB, CREB, and NF-AT, inducing cell proliferation.
5.2.3. Role of calcium signaling in cell differentiation

Calcium ions can also function as versatile carriers of information that ultimately direct cell fate. Calcium spikes driven by ryanodine receptors (RYRs) trigger the differentiation of myocytes toward somites in *Xenopus* [275, 278]. Calcium/calcineurin-dependent activation of a transcription factor, nuclear factor of activated T cells c1 (NFATc1), turns on the expression of osteoclast-specific gene and induces osteoclast differentiation [279]. Carey et al reported that cultured mouse neural crest-derived cells exhibit transient intracellular calcium increase during the process of neuron differentiation [280]. 50% of the active cells, which showed increased calcium concentration, generated clones containing neurons. On the other hand, none of the inactive cells yielded clones containing neurons. When the differentiation activity declined, the frequency of calcium spikes and the percentage of active cells also decreased. Blocking calcium transient activity by using Mg$^{2+}$ also inhibits neurogenesis of the mouse neural crest-derived cells. Differentiation is the process that generates different cell types for specific functions. One of the key steps in the differentiation process is to control spatial and temporal calcium signaling profiles for cell fate regulation.

5.2.4. Role of calcium signaling in liver

Calcium ions, through interacting with calcium-binding proteins, or binding directly with proteins to change enzyme activity, play a critical role in many biological processes in hepatocytes including cell proliferation and bile secretion [281-283]. For example, calcium signals promote the secretion of organic anions into bile by insertion of the transporter protein, multidrug resistance associated protein 2, into the plasma membrane [281]. In a
liver injury model reported by Sung et al, it is reported that mechanical wounds trigger calcium propagation [284]. Blocking this calcium propagation can inhibit the cell proliferation and wound healing along the edge of the wound. Reports also indicate that nucleoplasmic calcium is required for hepatocyte proliferation [282].

5.3. Tunable mechanical stimulation to manipulate calcium signaling

5.3.1. Enhanced cytosolic Ca$^{2+}$ concentration upon mechanical stimulation

It has been acknowledged that intracellular calcium concentration is highly sensitive to mechanical stimulation [170]. Studies have been conducted showing that shear stress-stimulated cells exhibit calcium influx [243, 285]. Applying mechanical stress by using micropipettes induces calcium waves in human keratinocytes [245]. Stretch-induced intracellular calcium increase has also been observed in mouse primary urothelial cells, and the response is stretch distance- and speed-dependent [247]. Reports have been published that stretching forces applied by using a microneedle lead to an increase in cytosolic calcium concentration and traction forces in fibroblasts [286]. Moreover, it has also been demonstrated that mechanically vibrating the elastic polyacrylamide gels to stimulate human umbilical vein endothelial cells (HUVECs) causes an intracellular calcium concentration increase via influx of calcium ions through stretch-activated ion channels [170]. The influx of calcium activates phospholipase C and leads to more calcium release from the endoplasmic reticulum. Upon mechanical stimulation, the
concentration of cytosolic Ca\(^{2+}\) increases and transmits the signals to regulate cell functions.

Mechanical stimulation of rat hepatocytes by using micropipettes led to a calcium signal increase, not only in stimulated cells but also in non-stimulated neighboring cells [246]. This intercellular crosstalk is mediated by the release of adenosine triphosphate (ATP) into the extracellular space, leading to calcium signal propagation. The field of using mechanical stimulation to induce desired Ca\(^{2+}\) fluctuations in hepatocytes for cell function regulation is still largely unexplored. Although calcium signaling plays an important role during cell differentiation and proliferation, limited work has been done to examine the effect of mechanical stimulation on calcium signal transduction in hepatocytes.

5.3.2. Controllable mechanical actuation to direct cell differentiation: modulating Ca\(^{2+}\) fluctuation frequency

One of the important types of information contained in Ca\(^{2+}\) fluctuations is the frequency of spikes. It has been observed commonly in different cell types that the frequency of [Ca\(^{2+}\)]\(_c\) spikes encodes information to regulate gene expression, mitochondrial metabolism, and kinase signaling [287-291]. In astrocytoma cells, although the same amount of agonist was applied, significantly more gene expression was observed when stimulants were delivered at one minute intervals – compared to 0.5 or 2 min intervals, as a sustained plateau, or as a single pulse [290]. In Jurkat T cells, rapid Ca\(^{2+}\) oscillations were shown to stimulate 3 transcription factors, NF-κB, NF-AT and Oct/OAP, while infrequent fluctuations stimulated NF-κB only [287].
As mentioned above, differences in the frequency of $[\text{Ca}^{2+}]_c$ spiking have been linked to the regulation of gene expression and cell function. I expect that using controlled mechanical stimulation, with adjustable frequency appropriately tuned, can evoke a desired temporal calcium fluctuation pattern. This would allow us to modulate hepatocyte function and direct cell fate. Hence, I planned to use PNIPAM-based polymer actuators to investigate the mechanically-triggered calcium oscillation patterns of human fetal hepatocytes. In the long run, with the incorporation of CNTs, the actuation can be modulated by remote NIR. The spatial and temporal $\text{Ca}^{2+}$ signal profile can be adjusted, and thus preferred gene expression may be achieved.

5.4. Dye selection for calcium signaling experiments

5.4.1. Development of calcium probes

5.4.1.1. First generation: increased binding specificity and fluorescence intensity

In the late 1970s, the development of first modern artificial fluorescent calcium indicator, quin-2, by Tsien et al. was a breakthrough for calcium signaling research [292]. The new generation of probe is derived from a common calcium chelator, ethylene glycol tetraacetic acid (EGTA) (Figure 29a), with some modifications, such as two methylene groups of EGTA replaced by benzene rings. This modification lowers the $pK_a$s of nitrogen from 8~9 to 5~6, so less proton binding to the probe can be achieved. Then, addition of a methyl group (Figure 29b, red circle) enhanced the quantum yield of fluorescence. The excitation peak of this probe is at 339 nm. Once $\text{Ca}^{2+}$ binds to this
probe, the conformation change of the probe (Figure 29b) leads to a change of emission wavelength, from 510 nm to around 525 nm. A five-fold increase in the quantum yield of fluorescence is also observed once this probe binds with Ca$^{2+}$. This invention overcame some significant limitations of previously-used dyes, for example the low light intensity displayed by the calcium-binding bioluminescent dye, aequorin [293].

Figure 29 (a) chemical structure of EGTA. Adapted from “New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures”, Tsien 1980 [292]. (b) Chemical structure of Quin 2. Adapted from Quin 2 user manual (Dojindo molecular technologies, Inc., Japan). Addition of a methyl group (red circle) enhanced the quantum yield of Quin 2 fluorescence.
5.4.1.2. Next generation: enhanced probe loading efficiency

Subsequently, incorporation of the membrane permeable acetoxymethyl ester (AM ester) group into the probes (Figure 30) further improved the loading efficiency of these calcium probes [294]. The charge-neutral, lipophilic, cell membrane-permeable AM ester group facilitates dye incorporation into the cell membrane and dye loading. Thus, loading of dyes into the cells can be more efficient, compared to time-consuming forced-introduction approaches such as microinjection. Once it enters the cells, the probe will be hydrolyzed back to the hydrophilic, non-membrane-permeable form. Thus, the calcium probes are retained inside the cell.

![Chemical structure](image)

Figure 30. Chemical structure of the first AM ester-incorporated calcium probes. (a) the original compound; and (b) it’s AM ester form. (Adapted from “A non-disruptive technique for loading calcium buffers and indicators into cells”, Tsien et al., 1981 [294].)
5.4.1.2.1. Ratiometric calcium probes

Currently, most calcium probes have been classified as ratiometric probes and non-ratiometric probes. The excitation/emission spectrum of ratiometric probes, for example indo-1 AM (Figure 31) and fura-2 AM (Figure 32), differ according to whether or not there is calcium binding. As a result, the calcium concentration can be obtained by calculating the ratio between two fluorescence values acquired at the two different wavelengths [295].

For ratiometric probes, Ca$^{2+}$ concentration can be calculated using the formula:

$$[\text{Ca}^{2+}] = K_d \times \left( \frac{R - R_{\text{min}}}{R_{\text{max}} - R} \right) \times \frac{S_{\lambda 2}}{S_{\lambda 2}} [295].$$

$K_d$ is the Ca$^{2+}$ dissociation constant of the probe. $R$ is the fluorescence intensity ratio $F_{\lambda 1}/F_{\lambda 2}$, while $\lambda 1$ is the detection wavelength for the calcium-bound probe and $\lambda 2$ is the wavelength for calcium-free probe. $R_{\text{max}}$ is the $R$ value at saturating calcium concentration and $R_{\text{min}}$ represents the $R$ value at zero calcium concentration. $S_{\lambda 2}$ is the intensity value at zero calcium concentration for $\lambda 2$, and $S_{\lambda 2}$ is the intensity value at calcium saturation for $\lambda 2$. Ratioing can minimize the effect of calcium probe leaking or uneven probe loading during the experiment.

Considering Indo-1 as an example, a unique property of this probe is that the emission shifts from ~475 nm (without calcium binding) to ~400 nm (Ca$^{2+}$-saturated), when it is excited at 338 nm. Indo-1 also exhibits strong binding affinity to calcium, with a dissociation constant of 230 nM.
However, many of the ratiometric probes are excited at UV region. Probes excited by UV light generally have some disadvantages such as low absorption of the dye, or autofluorescence. Also, exposing cells to UV is undesirable. Indo-1, specifically, is known for rapid photobleaching, which may lead to a diminished signal/noise ratio [296, 297].

Figure 31. (a) Chemical structure of Indo-1 AM. (b) Absorption (excitation) and emission (excited at 338 nm) spectra of Indo-1 at pH 7.2. A: absorption (excitation) spectrum, Ca\(^{2+}\)-saturated; B: absorption (excitation), Ca\(^{2+}\)-free; A’: emission spectrum, Ca\(^{2+}\)-saturated; B’: emission spectrum, Ca\(^{2+}\)-free. (Adapted from Indo-1 AM user manual, Invitrogen)
Figure 32. (a) Chemical structure of Fura-2 AM. (Adapted from Fura-2 AM user manual, Invitrogen) (b) Absorption (excitation) and emission (excited at 340 nm) spectrum of Fura-2 at pH 7.2. The absorption (excitation) spectra is detected at 510 nm. A: absorption (excitation) spectrum, Ca$^{2+}$-saturated; B: absorption (excitation), Ca$^{2+}$-free; A’: emission spectrum, Ca$^{2+}$-saturated; B’: emission spectrum, Ca$^{2+}$-free. (Adapted from user Fura-2 AM user manual, Invitrogen)

5.4.1.2.2. Non-ratiometric calcium probes

On the other hand, non-ratiometric calcium probes are mainly excited in the visible range. The single excitation spectrum enables easier data interpretation and instrumentation. Calcium indicators that can be excited by visible light provide several advantages over indicators that are excited by UV light:

1. Stronger absorption of the dye so a lower dose of the dye can be used and less phototoxicity can be expected.
2. Many modern fluorescence facilities use lasers as light sources, and it is much cheaper to produce light in the visible range, compared to the cost of lasers operating in the uv range.

3. Less autofluorescence\textsuperscript{10} from the sample [296, 298-300].

There are several visible light-excitable calcium indicators commercially available, with indicators modified from rhodamine and fluorescein are more frequently used [298].

Rhodamine-based indicators (Red): For example, Rhod-2 acetoxyethyl (AM) ester (Figure 33), a long-wavelength calcium indicator developed in 1989. It has excitation and emission maxima at 552 nm and 581 nm respectively. Conjugation of rhodamine with (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid) (BAPTA), which is a tetracarboxylic salt with a calcium-specific binding site, produced a valuable calcium dye (Rhod-2) for calcium signaling studies. Once binding with calcium has occurred, a more than 100-fold increase in fluorescence is observed. The dissociation constant of this indicator, in the absence of Mg\textsuperscript{2+}, is 570 nM. Some concerns still exist for Rhod-2 AM related studies. First, it still binds to cations like Mn\textsuperscript{2+} and Pb\textsuperscript{2+} with a higher binding affinity than Ca\textsuperscript{2+} (Rhod calcium indicators manual, Molecular probes).

Second, the non-uniform cytosolic distribution of this dye\textsuperscript{11} [296] leads to a punctate staining pattern.

\textsuperscript{10} When excited in the UV range, some intracellular components (for example, NADH, which has excitation and emission peaks at around 340 nm and 440-470 nm respectively) emit fluorescence that may interfere with calcium signaling observation.
Figure 33. (a) Chemical structure of Rhod-2 AM. (b) Absorption (excitation) and emission spectra of Ca$^{2+}$-saturated Rhod-2 at pH 7.2. A: absorption (excitation) spectrum; B: emission spectrum. (Adapted from Rhod-2 AM user manual, Invitrogen)

Fluorescein-based indicators (Green): For example, Fluo-4, analogs of the parent product, Fluo-3, with two chlorines replaced by fluorines (see Figure 34 for chemical structures of each probe). This replacement leads to a faster loading rate of the dye, compared to Fluo-3. Also, the wavelength of maximum absorption is 12 nm less than that of Fluo-3, leading to higher excitation intensity at 488 nm and stronger fluorescence signals. Fluo-4 has its maximum excitation/emission at 494 nm / 506 nm, and a dissociation constant for Ca$^{2+}$ of 345 nM. Compared to Rhodamine-derived

11 Due to its positively charged nature, Rhod-2 AM accumulates in the mitochondria, which are more negatively charged at their inner membrane.
calcium probes, which are positively charged in nature, negatively-charged fluorescein-derived indicators have a higher affinity for calcium. The AM ester form of Fluo-4 is almost non-fluorescent, but after cleaving by esterases and binding with calcium, the fluorescence intensity increased more than 100-fold. Another Fluo-4 derivative, Fluo-4 NW (No-Wash), has a modified formula containing surfactants that help to disperse and aid the solubility of the esters. Hence, improved loading speed and elimination of washing steps can be achieved. A chemical that inhibits the activity of organic anion transporters, Probenecid, can also be added with the probe, inhibiting extrusion of the probe by the organic anion pumps.

A list of some commonly used calcium probes is listed in Table 3.
Figure 34. (a) Chemical structures of Fluo-3 and Fluo-4. (b) Absorption (excitation) and emission spectra of Ca$^{2+}$-saturated Fluo-4 at pH 7.2. A: absorption (excitation) spectrum; B: emission spectrum. (Adapted from Fluo-4 user manual, Invitrogen)
Table 3. A comparison of some commonly used calcium probes:

<table>
<thead>
<tr>
<th></th>
<th>Absorption (excitation) peak (nm)</th>
<th>Emission peak (nm)</th>
<th>Ratiometric</th>
<th>( K_d ) for Ca(^{2+} ) (nM)</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluo-3</td>
<td>506</td>
<td>526</td>
<td>No</td>
<td>390</td>
<td>1. 100-fold increase in fluorescence intensity upon calcium binding.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2. Two fluorines replace two chlorines (Fluo-3), leading to stronger fluorescence signals and faster loading.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3. Fluorescein (negatively charged) derived probe with higher affinity for calcium ions, compared to rhodamine derivatives.</td>
</tr>
<tr>
<td>Fluo-4</td>
<td>494</td>
<td>506</td>
<td>No</td>
<td>345</td>
<td>1. 100-fold increase in fluorescence intensity upon calcium binding.</td>
</tr>
<tr>
<td>Rhod-2</td>
<td>552</td>
<td>581</td>
<td>No</td>
<td>570</td>
<td>1. 100-fold increase in fluorescence intensity upon calcium binding.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2. Rhodamine (positively charged) derived probe.</td>
</tr>
<tr>
<td>Indo-1</td>
<td>No Ca(^{2+} ): 346</td>
<td>No Ca(^{2+} ): 475</td>
<td>Yes</td>
<td>230</td>
<td>1. Can use single laser for excitation and monitor two emissions.</td>
</tr>
<tr>
<td></td>
<td>High Ca(^{2+} ): 330</td>
<td>High Ca(^{2+} ): 401</td>
<td></td>
<td></td>
<td>2. Less compartmentalization compared to Fura-2.</td>
</tr>
<tr>
<td>Fura-2</td>
<td>No Ca(^{2+} ): 363</td>
<td>No Ca(^{2+} ): 512</td>
<td>Yes</td>
<td>140</td>
<td>1. Very limited sensitivity to ([Ca^{2+}]) above 1μM.</td>
</tr>
<tr>
<td></td>
<td>High Ca(^{2+} ): 335</td>
<td>High Ca(^{2+} ): 505</td>
<td></td>
<td></td>
<td>2. Slow calcium dissociation from the probe may hinder observation of rapid Ca(^{2+}) fluctuations.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3. Absorption shift upon Ca(^{2+}) binding.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4. Less photobleaching compared to Indo-1.</td>
</tr>
</tbody>
</table>

5.5. Materials and methods

5.5.1. Human fetal hepatocyte growth medium composition

Human telomerase reverse transcriptase (hTERT)-reconstituted fetal hepatocytes were a gift from Dr. Mark Zern [196]. The growth medium for hTERT-reconstituted fetal
hepatocytes is composed of Dulbecco’s Modified Eagles’ Medium (DMEM) (Invitrogen) containing 10% fetal bovine serum (FBS) (Invitrogen), 2.4 μg/ml hydrocortisone (Sigma), 5 μg/ml insulin (Sigma), 50 units/ml penicillin and 50 μg/ml streptomycin (Invitrogen) as described [196].

5.5.2. Thawing the cells

A cryo-preserved vial was incubated in a 37°C water bath for 2 minutes, while ensuring that the cap and O-ring were above water level. The vial was then sprayed with 70% ethanol and moved into a biosafety cabinet. Operations after this step were performed in strict aseptic conditions. To mix with cells in the vial, an equal amount of cell culture medium were added to the cell freezing medium, drop by drop. The cell suspension was transferred to a 15 ml centrifuge tube and spun at 125 g for 5 minutes. After removal of the supernatant, the sediment was re-suspended with 4 ml of cell culture medium. Cells were pipetted gently. The cells were then dispensed on regular 10 cm tissue culture plates at a density of 1 x10⁴ per cm². The cell culture medium was changed every other day.

5.5.3. Subculturing the cells

When cells reached 80% confluence, cell culture medium was removed by suction. Then, cells were rinsed and washed with phosphate buffered saline (PBS) for 3 times to remove serum, which contains trypsin inhibitors. After that, PBS was removed before 6 ml of 0.25% (w/v) trypsin solution was added to the plate. The plate was then incubated for 5 minutes at 37°C with trypsin solution to detach the cells from the plate. Following cell detachment, cells were collected and mixed with an equal volume of cell culture medium.
Cells were transferred to a 15 ml centrifuge tube and spun at 125 g for 5 minutes. Supernatant was removed and cells were re-suspended in 4ml of cell culture medium. Cell numbers were counted afterwards by using a hemocytometer, and cells were seeded on regular polystyrene tissue culture plates (BD, Falcon) at a density of $1 \times 10^4$ per cm$^2$. The cell density may be slightly different when cultured on polymer actuators. Finally, cells were cultured in an incubator with 5% CO2, and culture medium was changed every other day.

5.5.4. Calcium probe staining procedure

Cells were seeded at a density of $1 \times 10^4$ per cm$^2$. Twenty-four hours later, cells were washed with PBS for three times. Then, 250 mM of probenecid solution (Gibco, Invitrogen), a commonly used inhibitor to prevent anions such as Ca$^{2+}$ probes from being transported out of a cell, was prepared. 1 ml of Fluo-4 Direct$^\text{TM}$ calcium buffer was added to 77 mg of probenecid to form the recommended 250 mM probenecid stock. After complete mixing, 200 μl was taken out from the probenecid stock and mixed with 10 ml of Fluo-4 Direct$^\text{TM}$ calcium buffer. The mixture was poured into a bottle of Fluo-4 Direct$^\text{TM}$ calcium reagent. Thus, a 2X calcium probe solution with a final concentration of 5 mM probenecid was made. Then, cell culture medium was mixed with the 2X calcium probe solution at 1:1. The derived solution was labeled as 1X calcium probe solution. Finally, cells were stained in 1X calcium probe solution for 1hr at 37°C before calcium signal measurements were performed.
5.5.5. Calcium signaling measurement

Calcium signaling experiments were performed using a Nikon eclipse TE-3000 inverted microscope (Nikon, Japan). Cells were stained with calcium probe, Fluo-4, before excitation at 488 nm with a high pressure mercury lamp [301] (Lambda XL, Sutter Instrument Company, 28W, 25% intensity,). Emission fluorescence was collected directly, and the intensity of emission fluorescence was measured every 5 seconds. Change in fluorescence intensity was normalized to the fluorescence at t=0. The calcium signals were expressed as \( \Delta F/F_0 \), where \( \Delta F \) equals \( F_t - F_0 \) (\( F_t \): emission fluorescence of ROIs at specific time; \( F_0 \): emission fluorescence at t=0).

5.6. Preliminary results

5.6.1. Photon emission intensity measurement

Fluo-4 was used as a calcium probe to monitor cytosolic \( \text{Ca}^{2+} \) fluctuations, since it provides several advantages over other available calcium probes. First, compared to UV-excitable probes, using visible light-excitable probes (e.g. Fluo-4) can minimize the possibility of autofluorescence, as well as eliminate cytotoxicity arising from exposure to UV light. Compared to other visible light-excitable probes, such as rhodamine-based probe,

\[ \text{Mercury lamps do not generate continuous spectrum but mainly line sources. High-pressure mercury lamps generate light output over the UV-visible spectrum, compared to low-pressure mercury lamps which emit light mainly at 254 nm. High pressure mercury lamps provide light output 10 to 100 times brighter than incandescent lamps. For a typical 100-watt high-pressure mercury lamp, the intensity of output light at 470 nm to excite Fluo-4 is approximately 32.8 mW/cm}^2. \text{ Hence, the excitation power (25% power of a 28W lamp) of our system can be estimated to be around 2.3 mW/cm}^2. \]
Rhod-2, fluorescein-based Fluo-4 is negatively charged. Hence, it has a higher affinity for Ca\textsuperscript{2+}: the Kd of Fluo-4 is 345 nM, compared to that of Rhod-2 at 570 nM.

Intracellular calcium signals can be analyzed by imaging calcium probe-stained cells and measuring the fluorescence intensity specific for calcium probes. However, for cells cultured on responsive polymer (PNIPAM)-based actuator, the dynamic swelling/de-swelling behavior of PNIPAM gel along the Z-axis complicates the focusing and imaging process. Hence, instead of taking images and analyzing fluorescence intensity afterwards, we measured the intensity of the emission fluorescence directly. The intensity of captured photons was then used to quantify calcium signals.

Since there is a possibility that we may lose the focus due to gel swelling/de-swelling along the Z-axis, we investigated whether de-focusing will have a significant effect on the collected fluorescence intensity. Cells cultured on PS dishes, which are non-responsive substrates, were stained with calcium probes and used as samples.

Initially, samples were in focus and excited at 494 nm incident wavelength. The intensity of emission light was recorded. Then, the focus wheel of the microscope was adjusted to obtain 25, 50, 100, 200, and 400 μm of focus offset and the intensity of emission light was recorded at each focus offset. As shown in Figure 35, there is a correlation between the extent of defocus and the amount of intensity lost. The greater extent of defocus, the greater the observed loss of intensity. Although there is an approximately 22% decrease in fluorescence intensity if the focus offset was set to 400 μm, the decrease in fluorescence intensity is 0.5%, 3% and 4% at 25, 50, and 100 μm, respectively. Considering (a) cell size (approximately 40-50 um in the direction of the Z axis) and (b) amounts of
swelling/de-swelling along the Z-axis in our gel (less than 100 μm), 3\% loss of intensity in this range (max defocus 50 μm) should not affect our data interpretation.

The advantages of this approach, measuring intensity directly instead of taking fluorescence images and then estimating the intensity according to the brightness of the picture are:

(1) We can minimize the effect of defocus due to gel swelling/de-swelling. A defocused image may lead to significant loss of fluorescence intensity and biased data interpretation.

(2) We can minimize the possibility of variation in time needed for focusing. The responsive polymer gel is dynamically swelling/de-swelling along the Z-axis, so it may require more than the expected time to refocus the image. The time needed for refocusing may differ in each experiment, as well. Thus, the acquired signals may not be representative of real calcium fluctuations inside the cells.

I have demonstrated that, even though the gel will swell and de-swell along the Z-axis, the intensity of emission photons acquired will not be strongly influenced. Our approach, which records the intensity of emission light directly with the detector, may be a better approach for real-time calcium signaling investigation in a responsive-gel system.
Figure 35. Effect of de-focusing on the intensity of captured emission photons. Cells were cultured on non-responsive PS dishes and stained with fluo-4 calcium probe. Cells were identified and brought into focus before emission photon intensity was measured. Then, focus wheel was adjusted downward for 25, 50, 100, 200, or 400 μm to measure the emission light intensity. Differences in fluorescence intensity were calculated and plotted. (ROI=5)

5.6.2. Selection of excitation intensity to minimize photobleaching

5.6.2.1. Photobleaching

Photobleaching refers to the inability of an excited fluorescent dye to return to the ground state after repetitive excitation and emission cycles. When a fluorescent probe is
excited to the high energy excited state, it can undergo intersystem crossing and shift to the triplet state. Then, the probe has the probability to be knocked down to the dark state by oxygen or some other molecule. If the probe is knocked down to the dark state, it can then move back to the ground state, or it may remain in the dark state irreversibly. A molecule that remains in the dark state, while losing the capability to return to the ground state and being excited again, is thus photobleached.

5.6.2.2. Excitation intensity selection

To minimize photobleaching, different intensities (100% vs 25%) of excitation light from the light source (28-Watt electrodeless lamp, Lambda XL, Sutter Instrument Company) were used to find the minimal excitation for further experiments. Cells cultured on polystyrene (PS) dishes were stained with Fluo-4 calcium probes and excited at a wavelength of 494 nm. Then, a cell was selected as an individual region of interest (ROI) and the emitted photon intensities from 10 ROIs were measured every 5 seconds for 20 minutes. As shown in Figure 36, excitation intensity at 100% leads to a trend of continuous decrease in emitted fluorescence as time passes. During this 20 minutes process, the intensity of emitted photons decreased by 24.8%. On the other hand, if weaker excitation light (25%) was used, only an 18.9 % decrease of emission fluorescence was observed. More importantly, the signature calcium signaling fluctuation was observed if the excitation intensity was set at 25%. Too strong an excitation intensity may lead to photon-induced chemical damage to large numbers of fluorophores. The more fluorophores being damaged and permanently unable to be excited again, the fewer photons that can be emitted. As a result, excitation intensity of
100% causes a significant decline in acquired emission intensity as a function of time, along with a loss of the ability to display regular calcium fluctuation. In order to collect fluorescent outputs that are more representative of real calcium signals, excitation intensity was set at 25% of maximum for further studies.

Figure 36. Effect of excitation intensity on collected photon intensity. Different intensities (100% and 25%) of excitation light were used to excite fluo-4 calcium probes. Cells were selected as individual ROIs and the emitted photon intensity from 10 ROIs was measured every 5 seconds for 20 minutes at 30°C.
5.6.3. Effect of temperature

Mechanical stimulation exerted by PNIPAM gel is imparted by temperature change-triggered polymer swelling/de-swelling. PNIPAM gels swell when \( T < \text{LCST} \) (approximately 32°C) and de-swell when \( T > \text{LCST} \). To differentiate mechanical actuation-induced calcium signal fluctuations from calcium fluctuations due to temperature change, cells were cultured on PS dishes, which are non-responsive, and calcium signals were measured at different temperature. As shown in Figure 37, when cells were cultured at 30°C and 34°C, very similar calcium fluctuation patterns were observed, suggesting that temperature (at a range between 30°C and 34°C) may not cause large difference in regard to the calcium fluctuation pattern. This information is critical to enabling us to de-couple the calcium spikes related to mechanical actuation from fluctuations due to temperature change.

It is noteworthy that the magnitudes of calcium peaks are greater when temperature is fixed at 34°C (closer to physiological condition), compared to those when temperature is fixed at 30°C. Similar results were published in 2012 [302], showing that magnitudes of calcium signals at 37°C were greater than those of calcium signals at 21°C. This phenomenon may arise due to stronger, more active metabolism and calcium influx/outflux at a temperature closer to physiological condition.

On the other hand, for the cells that experienced temperature change between 30°C and 34°C, an increase (around 13%) in fluorescent signals was observed when temperature was raised from 30°C to 34°C for the first time (Figure 37). However, this clear increase was not observed in the second cycle of heating. This may due to lack of free \( \text{Ca}^{2+} \) in the
cytoplasm (Ca\textsuperscript{2+} efflux) after temperature has changed over time. Thus, small temperature variations used to induce mechanical actuation will not interfere with the actual Ca\textsuperscript{2+} signal (Ca\textsuperscript{2+} influx from outside the cells or Ca\textsuperscript{2+} released from internal storage) that results from mechanical stimulation.

In summary, based on the similar calcium fluctuation patterns observed from cells exposed to different temperatures, temperature may not be a critical factor that interferes with our observation of calcium fluctuations resulting from mechanical stimulation.

![Figure 37. Effect of temperature on calcium signaling. Cells were cultured on non-responsive polystyrene dishes and stained with fluo-4 calcium probes. Calcium signals were measured from](image)
cells exposed to different condition, which are (a) temperature fixed at 30°C, (b) temperature fixed at 34°C, and (c) temperature altered between 30°C and 34°C. In group (c), the cells were maintained at 30°C for 1min, heated and maintained at 34°C for 5.5 min, cooled and maintained at 30°C for 3.5 min, and then subjected to a repeat of the temperature alteration cycle. A cell was selected as an individual ROI and the emitted photon intensity from 10 ROIs was measured every 5 seconds.

5.7. Discussion

In summary, according to reports already published by other groups, intracellular calcium concentration is sensitive to mechanical stimulation. Stimulation as minute as approximately 10 piconewtons per cell can trigger calcium fluctuations of bovine aortic endothelial cells [248]. Previously, I have shown that cell shape (Figure 16) and size (Figure 17) can be changed by applying mechanical actuation to human fetal hepatocytes, though the results are not conclusive. The inconclusive results in cell size change may be the result of several factors. First, the contractile force exerted by cells through cytoskeleton molecules to resist externally applied force and maintain cell shape may lead to the unobvious cell size change. Second, the porous surfaces of hydrogel actuators may lead to cell images residing in different focal planes and thus hindering cell size characterization. The change in cell size is a dynamically evolving process, so the timing for cell imaging may be critical. Furthermore, cell motions (e.g. the extension of leading edges and retraction of trailing edges during cell migration) may compromise the effect of mechanical stimulation. As a result, a more sensitive approach to monitor cell behavior upon mechanical stimulation, measuring
cytosolic calcium concentration, was used. Table 4 is a summary of pros and cons of each method.

I have shown that by directly measuring the photon intensity of emission fluorescence, we can quantify the change in cytosolic Ca\(^{2+}\) concentration. Change in temperature, which is used to trigger gel actuation, may not influence cytosolic Ca\(^{2+}\) fluctuations significantly. These preliminary results suggest that measuring cytosolic Ca\(^{2+}\) concentration change will offer a promising approach to monitoring cell behavior changes triggered by our polymer actuator system. By using this platform, further detailed experiments can be conducted by researchers to study how calcium fluctuations change in response to mechanical actuation.
Table 4: Comparison of different approaches (cell size change vs calcium signaling change) that can be used to investigate if cells sense mechanical stimulation.

<table>
<thead>
<tr>
<th></th>
<th>Calcium signaling</th>
<th>Cell shape measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Why being considered?</strong></td>
<td>Mechanical stimulation may activate stretch-activated calcium channel (extracellular calcium) and/or turn on intracellular signaling to release calcium stored in endoplasmic reticulum (ER) (intracellular calcium)</td>
<td>Mechanically stretching and compressing the cell may cause cell deformation and change the cell shape.</td>
</tr>
<tr>
<td><strong>How to measure?</strong></td>
<td>Load the calcium probes into the cell. Apply mechanical stimulation. Detect and measure the change of fluorescence intensity.</td>
<td>Stain the cells with cell membrane dye. Apply mechanical stimulation and take images. Use software to measure cell size change.</td>
</tr>
<tr>
<td><strong>Sensitivity</strong></td>
<td>Superior. Very minute stimulation, for example vibration in surrounding environment, can lead to fluctuations in calcium signaling. Change in calcium signals can be detected in seconds or even milliseconds.</td>
<td>Inferior. Cytoskeleton molecules are connected to proteins on cell membrane to maintain and preserve cell shape. Cell will also exert contractile force to ensure cell shape does not change dramatically.</td>
</tr>
<tr>
<td><strong>Advantages</strong></td>
<td>1. Very high sensitivity, so even small mechanical stimulation can induce obvious calcium signaling change. 2. Calcium fluctuations can be decoded in a spatial and temporal manner. 3. Rapid calcium signaling changes can be observed seconds after mechanical stimulation. 4. Specific binding of probes and Ca²⁺. 5. Calcium probes do not emit fluorescence until (a) binding with calcium and (b) AM estser is removed by intracellular esterases. Hence, low background fluorescence can be expected. 6. Frequency, magnitude, and rate of rise of the calcium spikes can be indicators of mechano-sensing by cells.</td>
<td>Cell deformation can be direct evidence showing that cell sensed the mechanical stimulation.</td>
</tr>
</tbody>
</table>
| Concerns | 1. Inefficient loading of calcium probes into cells cultured on hydrogel system.  
2. Temperature change required to initiate gel actuation may be another factor to cause fluctuations in calcium signaling.  
3. Heterogeneity of calcium response from cell to cell. | 1. The deformation of cells may not be so obvious (5-10%) due to the contractile force exerted by cells.  
2. Longer time needed for noticeable shape change can be observed.  
3. Dye will be adsorbed on gel and lead to strong fluorescence background. |
Chapter 6. Fabrication of remotely controlled PNIPAM actuator

I have shown that our PNIPAM based polymer actuator is capable of serving as a platform to apply mechanical stimulation to cells for cell behavior study. In order to exert temporally and spatially controlled mechanical stimulation (such as desired frequency or location), CNTs are incorporated in our polymer actuator as the “nano switch” to sense remote signals and switch on the gel actuation motion.

6.1. Carbon nanotubes as optothermal-triggering materials: an overview

Increasing effort has been made to create hybrid materials that integrate the properties of each component within a composite system. Materials that respond to an optical stimulus attract significant interest, since a response can be triggered remotely with high temporal and spatial accuracy [303-306]. CNTs, whether they are MWNTs or SWNTs, have been shown to possess excellent NIR absorption [305, 307-309]. CNTs are capable of turning absorbed NIR into localized heat [305, 310, 311]. On the other hand, human tissues have slight absorption in the NIR region (700-1100 nm) [310, 312]. Hence, NIR can serve as a remote stimulus to specifically switch on the optothermal behavior of embedded CNTs for different purposes without damaging human tissues.

CNTs have been used as an optothermo-active species to trigger contents release from microcapsules [305]. NIR irradiation (785 nm, 400mW) of the CNTs, which are embedded in the microcapsules, leads to localized heating and rapid rupture of the microcapsules. Similarly, NIR laser (830 nm) stimulation has been used to heat embedded CNTs and so
achieve opening of microcapsules [313]. It has also been reported that NIR laser (808 nm) irradiation leads to selective heating and killing of tumor cells that uptake CNTs [310]. Not surprisingly, cells with no CNT uptake remain viable and proliferative. These results demonstrated that CNTs can serve as “nano-heaters”, which absorb NIR and convert the absorbed energy into heat.

6.1.1. CNTs trigger a volume phase transition of PNIPAM gels

PNIPAM is a thermal-responsive material that exhibits sharp and reversible phase transition behavior. When T<LCST, it forms hydrogen bonds with water and swells. In contrast, it de-swells and collapses when T>LCST. However, in the context of a practical device, it is inconvenient to have to adjust the temperature manually, and the slow heating/cooling process makes it difficult to generate controlled gel actuation. Optical stimulus is therefore desirable for applying controlled heating remotely with high temporal and spatial accuracy. By interacting with light from an NIR laser that is switched on and off, CNTs can function as localized heat-generators in PNIPAM gel to trigger a desired response. For example, it has been shown that PNIPAM gels incorporating CNTs displayed reversible swelling/de-swelling behavior upon remote NIR (1064 nm, 210mW) stimulation [307]. The sharp phase transition can be observed in seconds and the swelling/de-swelling transition process can be repeated for more than 1200 cycles. Other researchers also demonstrated that by using NIR laser irradiation (1064 nm, 1W, 5 min), the temperature of CNT-incorporating PNIPAM gel increased from 25°C to around 40°C [311]. A volume phase transition was observed in PNIPAM gels that incorporate CNTs. Meanwhile, the temperature of PNIPAM gels without CNTs remained at 25°C. Zhang et al.
also reported that programmable folding of the actuator can be achieved by temperature-triggered PNIPAM volume phase transition [178]. CNTs embedded inside the PNIPAM gel can be stimulated with NIR (785 nm, 30 mW), and opaque dots can be observed due to heating of the PNIPAM hydrogel. However, it is noteworthy that no direct evidence showed that folding of the actuator can be triggered by NIR irradiation. This is probably due to the small beam size of the NIR laser generating a limited responsive area.

6.1.2. Our ultimate goal: Controlled PNIPAM gel actuation by NIR stimulation

It is evident that CNTs have strong absorption in the NIR range and thus can be used to trigger a desired optothermal response. Therefore, NIR irradiation can be used to remotely heat the CNT “nano-heaters” embedded in our PNIPAM gel actuator. The localized temperature change due to NIR irradiation switches on the volume phase transition of the gel without having a direct impact on the adhered cells. Once the laser is turned off, the effect can be reversed immediately. As a result, the swelling/de-swelling behavior of the actuator can be controlled by manipulating the duration and power density of NIR irradiation. This system will shrink upon NIR irradiation and swell back to its original condition right after the irradiation is turned off. The magnitude and frequency of the mechanical stimulation “felt” by cells can therefore be tuned. Hence, desired cell behavior, such as frequency of calcium fluctuations, can be expected. Furthermore, by changing the NIR laser beam size, the responsive area may be tuned. Thus, mechanical stimulation may be applied from as small as \( \mu \text{m}^2 \) scale to whole device scale.
6.2. Materials and methods

6.2.1. Applying NIR stimulation to PA-PNIPAM-CNT composite gels

PA-PNIPAM-CNT gels were made and washed with PBS for 15 cycles (chapter 4, 4.5.1.). An optical fiber connected to the NIR laser source (LDC 210C, series 200, laser diode controller, Thorlabs) was attached to a tissue culture dish. PA-PNIPAM-CNT gel was placed in the tissue culture dish and immersed in PBS. Gels were swelled at RT for 5 seconds. Then, gels were stimulated with the NIR laser (980 nm, 200mW) for 40 seconds. The NIR laser was then turned off and the gel was swelled for 15 seconds. The image of the PA-PNIPAM-CNT gel was acquired by using confocal microscopy.

6.3. Results

6.3.1. NIR triggered gel actuation

To investigate if gel actuation and subsequent mechanical stimulation can be initiated remotely, the NIR laser (980 nm, 200 mW) was used to remotely “switch on” the device. CNTs have strong absorption in the NIR range and turn the absorbed light into heat. The localized heating effect can thus trigger PNIPAM gel matrix to undergo a volume phase transition and become de-swelled. Once the laser is turned off, heat dissipates and the PNIPAM gel swells. Figure 38 is a series of images showing that the actuation of our bilayer composite gel, PA-PNIPAM-CNT gel, can be remotely switched on and off by using the NIR laser. Gel was first immersed in PBS at RT for 5 seconds while the laser
was off (Figure 38a). Then, the NIR laser was turned on. As shown in Figure 38b, one second after turning on the laser, the gel shrunk and the edge of the gel regressed towards the side opposite to the laser source. The color change of the gel can be observed easily as well. Due to localized heating triggered by laser stimulation, the PNIPAM gel acquired a condensed, white/milky color. With increasing time of exposure to the laser, the shrinkage of the gel and the area that exhibited the color change increased, as shown in Figure 38c. After 40 seconds of laser stimulation, an enlarged area with changed color was observed and the edge of the gel regressed by approximately 200 μm (Figure 38d). Compared to Figure 38a, which is the image taken when the laser is not turned on, there is a strong difference regarding both (a) the position of the gel edge and (b) the color of the gel. The region illuminated with the NIR laser became white in color and exhibited de-swelling behavior. After 40 seconds of NIR stimulation, the laser was turned off. As shown in Figure 38e, termination of NIR stimulation abolished the local heating effect in the PNIPAM matrix. Fifteen seconds after the laser was turned off, the white color on the gel disappeared. This is an indication that the temperature in the PNIPAM matrix is now lower than its LCST. If temperature is below the LCST, PNIPAM gel becomes transparent, forms hydrogen bonds with water, and swells. This is evident in Figure 38e, as the edge of the PNIPAM gel swells almost halfway back to its original position before NIR stimulation. Here, we demonstrated for the first time, that NIR can be used to remotely trigger PA-PNIPAM-CNT gel swelling and de-swelling behavior. This phenomenon is due to CNTs embedded in gel matrix sensing the NIR and converting the light into heat. The localized heat thus promotes a volume phase transition of the gel, and the gel de-swells.
Figure 38. NIR triggered gel actuation. PA-PNIPAM-CNT gel was immersed in PBS at room temperature for 5 seconds before NIR stimulation (980 nm, 200 mW) for 40 seconds (the laser illumination was from the right). After that, the laser was turned off, allowing the gel to swell for 15 seconds. Images were taken every second during this entire one minute period. Prior to NIR stimulation, PA-PNIPAM-CNT gel was stained with DAPI for visualization. The scale bar in the middle indicates 200 μm.

6.4. Discussion and future direction

Incorporation of the optothermal triggering material, CNTs, in our polymer actuator provides several advantages relative to existing systems. First, it enables us to apply controlled stimulation with desired features, such as frequency, magnitude, size of responsive area, and location of the responsive area. Second, compared to adjusting temperature manually, which is inconvenient and slow, the remotely controlled platform provides an efficient way to exert the mechanical stimulation. Compared to available systems nowadays, which can only stimulate either single cells (e.g. by using optical tweezers) or the whole culture (e.g. by using deformable substrate systems), the responsive
area of our NIR responsive PA-PNIPAM-CNT gels can be tuned by adjusting the laser beam size. Thus, the number and location of cells subjected to mechanical stimulation can be controlled.

There are some unique features of our platform:

(1) By incorporating a cell-seeding polyacrylamide layer on top of the actuation-generating layer, the localized heat arising from NIR stimulated-CNTs may not have a direct effect on cells. The top cell seeding layer not only works as a flat surface for behavior monitoring, but can also function as an insulating layer to prevent over heating of the cells.

(2) Most importantly, incorporation of CNTs in the actuation-generating layer allows us to trigger the actuation remotely by stimulating the device with an NIR laser. Previously, a remote-controlled PNIPAM system has been developed to trigger drug release from PNIPAM-based microcapsules by using laser stimulation (785 nm, 400 mW) [305]. CNTs converted the absorbed NIR light into heat, and localized heating led to shrinkage of the PNIPAM microcapsules so that liquid drugs were released from the gel matrix. Our design is the first one in which a remote-controlled hydrogel device, using NIR as a trigger, can be used to exert mechanical stimulation on cells for cell behavior study.

In the long run, controlled mechanical stimulation can be applied by tuning the frequency, power, and spot size of the NIR laser. Thus, desired cell responses, such as calcium fluctuations with desired frequency or amplitude, can be achieved. At the same time, temperature mapping on the top cell-seeding layer should be performed to confirm that cells will not be overheated upon NIR stimulation. The actuation profile of the gel, after repeated
NIR stimulation, can be also investigated to ensure the durability and consistency of this platform.

My work mentioned above connects the common interests of engineers and biologists. By incorporating knowledge from materials science, tissue engineering and stem cell science, the stem cell differentiation process can be investigated from a different angle. The fabricated polymer-functionalized CNT thin films and remotely controlled nano hybrid hydrogel actuator can provide new opportunities to address some fundamental questions that cannot be solved by currently available approaches.
Chapter 7. Experimental design to investigate calcium signaling fluctuations upon mechanical stimulation

Mechanical actuation of PNIPAM gel can be triggered by imposing a temperature change. However, it is inconvenient to adjust the temperature manually, and the slow heating/cooling process makes it difficult to generate controlled gel actuation. Besides, a gradual temperature change may not create a significant change of Ca\(^{2+}\) signals. In order to (a) induce obvious calcium signal changes and (b) exert controlled mechanical stimulation (such as desired frequency), NIR stimulation can also be used to heat CNTs embedded in a PNIPAM matrix and trigger mechanical stimulation. On the other hand, it is noteworthy that calcium fluctuation patterns induced by NIR-triggered gel actuation may differ from that induced by gradual temperature change imposed by a heating stage.

To (a) investigate if gel actuation can be used to trigger cytosolic calcium fluctuations and (b) compare the differences between calcium signaling patterns induced by NIR-triggered gel actuation or gel actuation triggered by temperature change driven by a heating stage, two sets of experiments are designed.

1. Gel actuation triggered by temperature change imparted by a heating stage

2. NIR laser-driven gel actuation.
7.1. Gel actuation triggered by temperature change (30°C to 34°C) imparted by a heating stage

7.1.1. Background

1. Using the heating stage to raise the temperature above the LCST of PNIPAM (32°C) will cause de-swelling of the gel. Cooling by loss of heat to the environment will lead to swelling of the gel, due to the restoration of hydrogen bonds.

2. The change in temperature should not be too sudden, to prevent overheating by the heating chamber. Overheating may lead to a cytotoxic effect on the cells. However, a temperature change that is too gradual might not create a significant change of Ca\textsuperscript{2+} signal.

3. It was reported that there is no significant difference in resting cytosolic calcium concentration between cells exposed to 32°C and 24°C [314]. On the other hand, cells cultured at 37°C, which mimics physiological temperature, did show increased resting cytosolic calcium concentration.

4. However, it is better not to cool the temperature below 30°C, since another report indicates that decreasing temperature from above 30°C to below 30°C (28°C) induces calcium transients (Figure 39) [315], which may complicate our results. In that study, the temperature of perfusion saline solution was held at different temperature starting at 37°C. Calcium peaks could not be detected until the temperature was decreased to 28°C. Those researchers also found that the calcium signal profile consists of an initial peak with the largest amplitude, followed by peaks with decreased amplitude. After repeated temperature change cycles, the amplitude of the peaks decreased. By using (a) calcium-free solution and (b) depletion of internal Ca\textsuperscript{2+} storage, it was found that the temperature change-induced calcium transient is dependent on internal calcium storage.
Figure 39. Effect of temperature on calcium signals. Cooling the saline solution from 37°C to 28°C and below (upper panel) triggers Ca^{2+} fluctuations (lower panel) in mouse olfactory ensheathing glial cells. First calcium peaks (marked with red rectangular) can be observed when temperature is decreased to 28°C. X-axis represents time, while Y-axis represents change in temperature (upper panel) and fluorescence change (lower panel). (Adapted from Temperature-dependent calcium-induced calcium release via InsP(3) receptors in mouse olfactory ensheathing glial cells [315], Stavermann et al., 2012)

7.1.2. Materials, methods, and anticipated results

7.1.2.1. Effect of temperature change on Ca^{2+} signals

The effect of temperature change on Ca^{2+} signal level will be characterized using cells in the following conditions:
• Cells cultured on single layer PA gel (non-responsive, as a control); temperature fixed at 30°C.

• Cells cultured on single layer PA gel; temperature fixed at 34°C.

• Cells cultured on single layer PA gel; temperature changed between 30°C and 34°C.

7.1.2.2. Effect of mechanical stimulation on Ca²⁺ signals

The effect of mechanical stimulation on Ca²⁺ signal level will be characterized using cells in the following conditions:

• Cells cultured on PA-PNIPAM-CNT gel actuator; temperature changed between 30°C and 34°C.

• Cells cultured on single layer PA gel; temperature changed between 30°C and 34°C.

By comparing the results from these two groups, the effect of mechanical stimulation on calcium signaling can be determined.

7.1.2.3. Gel making procedure

PA gels (single layer)

PA gel precursor solution will be prepared by dissolving acrylamide (monomer) (1.3M), bisacrylamide (crosslinker; 0.019M), and TEMED (0.03M) in DI water. The solution will be mixed and purged with nitrogen for 30 minutes before mixing with purged ammonium persulfate (AP) (initiator; 0.02M) solution. The gel formation reaction will be performed at room temperature in 12 wells for 18 hours. The gels will be then washed with DI water (5 times) and PBS (5 times) in a beaker, with a stir bar continuously used.
After that, gels will be autoclaved and conjugated with collagen (chapter 4, 4.5.4.) before cell seeding.

PA-PNIPAM-CNT bilayer gels

Top layer PA gel precursor solution will be prepared by dissolving acrylamide (1.3M), bisacrylamide (0.019M), and TEMED (0.03M) in DI water. The solution will be mixed and purged with nitrogen for 30 minutes before mixing with purged ammonium persulfate (0.02M) solution. The gel formation reaction will be performed at room temperature in 12 wells for 5 minutes. Then, PNIPAM-CNT gel precursor solution (chapter 3, 3.6.5.) and AP solution will be added on top of the PA gels to make the PNIPAM-CNT gel layer. Gelation will be performed at room temperature for 18 hours, after which the PNIPAM-CNT gels had formed and bound with the PA gels, generating bilayer hybrid gels. The gels will then be washed for 10 cycles (5 cycles in DI water, 5 cycles in PBS, room temperature for 15 minutes and then 37°C for 15 minutes per cycle) before being autoclaved and conjugated with collagen (chapter 4, 4.5.4.) for cell seeding.

7.1.2.4. Staining the cells

Cells will be seeded on substrates at a density of 4 x 10^4 per cm². Twenty four hours later, cells will be washed with PBS for three times. Then, cells will be stained with 2X calcium probe solution¹³ for 1hr at 37°C before calcium signaling detection. Cytosolic

¹³ Compared to my old membrane staining protocols (chapter 3, 3.6.9.), the concentration of calcium probes is increased 2 folds now to achieve stronger staining of the cells on hydrogels. The calcium probes that we have are packaged in a bottle, which contains surfactants to improve solubility, with predetermined concentration (2X). If the fluorescence is still weak, we may need to consider (a) purchasing Fluo-4 probe powder or (b) staining twice.
calcium signals will be detected by measuring the fluorescence intensity of calcium probe, Fluo-4, inside the cells.

7.1.2.5. Calcium signaling measurement

The intensity of emission fluorescence will be recorded directly though the detector every 5 seconds. The excitation light source will be turned off between each measurement to minimize photobleaching. Emission fluorescence intensity (F) will be recorded and calculated as percent fluorescence change ($\Delta F/F_0 \times 100\% = (F - F_0)/F_0 \times 100\%$) relative to the basal fluorescence ($F_0$) at the initial time point. This normalization step can minimize possible errors resulting from different initial fluorescence intensities of the cells.

a. Effect of temperature on calcium signaling

A comparison of the change of calcium signal intensity at 30°C, 34°C, and T between 30°C and 34°C, will indicate the effect of temperature on calcium signals.

b. Effect of gel actuation on calcium fluctuations

PA-PNIPAM-CNT gels will be subjected to temperature change to investigate the effect of mechanical stimulation on calcium signaling. Temperature will be first maintained at 30°C for 5 min. Then, the temperature will be raised from 30°C to 34°C. The heating process will take around 3 min. Temperature will then be maintained at
34°C for 15 min before cooling. Temperature cooling from 34°C to 30°C will take around 3 min. After that, temperature will be maintained at 30°C for 15 min. Then, the temperature alteration cycle will be repeated 2 times. If the signal change due to mechanical stimulation is much greater, compared to that due to temperature change, the effect of mechanical stimulation on calcium signaling can be determined.

c. Measuring the amplitude of calcium peaks

The amplitude of calcium peaks can be acquired by measuring percentage change of ∆F (relative to F₀). On a plot in which the X axis represents time and the Y axis represents ∆F, the change of ∆F (e.g. from 3% to 70%) can be denoted as the amplitude of calcium peaks (67% of ∆F).

d. Measuring the frequency of calcium spikes

The frequency of calcium spikes can be acquired by calculating the number of spikes per unit time, denoting it as (number of events/min).

e. Measuring the duration of calcium peaks

The duration of calcium peaks can be acquired by calculating the time span of calcium peaks. For example, if a calcium peak arose at t=5 s and ended at t=35 s, the duration for this peak would be 30 seconds.
7.2. NIR laser stimulation triggers gel actuation and calcium fluctuations

Gel actuation triggered by temperature change is relatively slow and takes minutes to exhibit volume phase transition behavior. In order to exert controlled mechanical stimulation (such as higher frequency) to trigger calcium signaling with obvious fluctuations, an NIR laser can be used to stimulate the actuation behavior of PA-PNIPAM-CNT gel.

7.2.1. Materials and methods

7.2.1.1. Experimental groups (5 gels will be used in each group)

a. PA-PNIPAM-CNT gels stimulated with NIR

Expected results: NIR stimulation leads to gel de-swelling and triggers calcium fluctuations.

b. PA single layer gels stimulated with NIR

Expected results: No actuation generated and no mechanical stimulation-specific calcium peaks generated due to the shortage of optothermo-triggering CNTs and PNIPAM\(^\text{14}\).

c. PA single layer gels without NIR stimulation

Expected results: No actuation generated and no mechanical stimulation-specific calcium peaks generated, due to the shortage of NIR stimulation.

\(^{14}\) We may not be able to locate the NIR laser beam on a single layer PA gel, since it does not contain CNTs and it is relatively transparent to NIR stimulation. Hence, it will be difficult to identify the cells that are close to the NIR beam for calcium signaling measurement. But, we can stimulate the gel edge with the NIR laser, and characterize the response of the cells at the gel edge.
By comparing the data from groups a and b, the effect of NIR-driven mechanical stimulation on calcium signaling can be determined.

By comparing data from groups b and c, the effect of the NIR laser alone on calcium signaling can be determined.

7.2.1.2. NIR stimulation

An optical fiber connected to the NIR laser source (LDC 210C, series 200, laser diode controller, Thorlabs) will be attached to a tissue culture dish. PA-PNIPAM-CNT gel will be placed in the tissue culture dish and immersed in PBS. Gels will be exposed to RT for 30 seconds. Then, gels will be stimulated with the NIR laser (980 nm, 200mW as shown in chapter 6, 6.3.1.) for 2 minutes. After that, the NIR laser will be turned off and the gel will be swelled for 30 seconds. The intensity of emission fluorescence will be recorded directly though the detector every 5 seconds. The calcium signaling of cells sensed NIR-triggered actuation (swelling and de-swelling) can be compared to that of cells that (a) do not sense the actuation or (b) only sensed swelling from the gel.

7.2.1.3. Calcium signaling measurement

The measurement can be referred to the measurement part in 7.1.2.5.
Chapter 8. Conclusion and future directions

Stem cells are a promising cell source for the repair of damaged tissues. Despite their potential use in regenerative medicine, there are still some hurdles that hinder further application, such as a shortage of efficient approaches to generate terminally differentiated functional cells. Proper functional tissue development and regeneration require temporally and spatially controlled physical and chemical cues. Hence, one of the major focuses in this field is to provide stem cell-stimulating signals by using biocompatible materials that function as an artificial stem cell niche to direct stem cell fate.

8.1. Conclusion of my work

In this work, I focused on developing new platforms to apply (1) static physical inputs and (2) dynamic mechanical loadings on stem cells to promote functional differentiation.

For the developed PMAA-g-CNT thin films:

A. The PMAA-g-CNT thin films provide increased surface area due to nanofibrillar structure and lead to enhanced growth factor adsorption.

B. Increased neuron differentiation was observed in cells cultured on this novel substrate. Elevated expression of neuron specific marker β-Tubulin III, reduced expression of pluripotent marker Oct4, and polarized expression of more matured motor neuron marker synapsin I, were observed in cells differentiated on PMAA-g-CNT thin films.

C. The overall cell survival on PMAA-g-CNT surfaces is comparable to that on the standard PLO surfaces, indicating that no detrimental effect on cell growth was observed on PMAA-g-CNT surfaces.
D. Increased cell adhesion was also observed on PMAA-g-CNT thin films.

For the developed nanohybrid polymer actuators:

A. PNIPAM actuators exhibit faster swelling and de-swelling kinetics, compared to PNIPAM-CNT actuators. However, the responsiveness of PNIPAM actuators at the 51st cycle was diminished in comparison to the responsiveness at the 1st cycle. In contrast, similar actuation profile was observed in PNIPAM-CNT actuators at the 1st and 51st cycles.

B. Cells sense the mechanical stimulation from PNIPAM-CNT actuators by changing shape and size.

C. Cells can survive in the PNIPAM-CNT actuator after repeated actuation cycles (10 cycles per day for consecutive 5 days).

D. The mechanical stimulation can be exerted remotely in a temporally and spatially-controlled manner by NIR illumination.

8.2. Future directions

In the long run, by employing CNTs distinct features (their ability to promote cell adhesion, and the ease with which they can be tailored with different functional groups), our CNT-containing thin films can be applied to serve a variety of purposes. CNTs may be functionalized with bioactive moieties of different density or geometry creating a patterned substrate. The patterned substrate may be used to generate different types of cells with desired pattern and structure on a platform. This approach may pave the road for engineered artificial tissues. The CNT pattern can also be used to promote polarized protein expression and structure formation.
On the other hand, controlled mechanical inputs can be applied to cells to trigger desired intracellular signals, such as fluctuations of Ca$^{2+}$, to direct stem cell fate by using the developed PA-PNIPAM-CNT actuators. By tuning the NIR laser intensity, beam size, and stimulation interval, mechanical inputs with different magnitude, response area, and frequency can be generated by a single setup. By controlling the laser beam size and responsive area, the target exposed to mechanical stimulation can range from single cell to the whole culture. The capability to exert spatially and temporally controlled mechanical stimulation on stem cells by using our nanohybrid polymer actuator can broaden the application of stem cells in regenerative medicine.

In spite of growing appreciation, the correlations between physical cues and cell fate decision have not yet been fully elucidated. This is largely due to the shortage of platforms that are capable of generating diverse physical stimuli controllably on cells. The two novel platforms that I developed provide great opportunities to bridge the gap and can further unveil the mysteries of stem cells.
Chapter 9. References


Harrison BS, Atala A: **Carbon nanotube applications for tissue engineering.** *Biomaterials* 2007, 28(2):344-353.


Wood MD, Willits RK: **Applied electric field enhances DRG neurite growth: influence of stimulation media, surface coating and growth supplements.** *Journal of Neural Engineering* 2009, 6(4).


