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<th>Development of Simplified NIPAm-based Thermoresponsive Films for Cell Preservation, Expansion and Differentiation</th>
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<td><strong>Author(s)</strong></td>
<td>Fan, Xingliang</td>
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<tr>
<td><strong>Publication Date</strong></td>
<td>2013-12-16</td>
</tr>
<tr>
<td><strong>Item record</strong></td>
<td><a href="http://hdl.handle.net/10379/4261">http://hdl.handle.net/10379/4261</a></td>
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Development of Simplified NIPAm-based Thermoresponsive Films for Cell Preservation, Expansion and Differentiation

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Thesis presented for the Ph.D. degree
of the
National University of Ireland, Galway

National Centre for Biomedical Engineering Science
National University of Ireland, Galway

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Submitted September 2013

This candidate confirms that the work submitted is his own and that appropriate credit has been given to the work of others
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Acknowledgement

I would like to sincerely thank my supervisor for his guidance, expertise and unrelenting support throughout. Yury, it has been a pleasure from start to finish, thanks for your tolerance, encouragement and endless advice.

I would like to acknowledge the generous financial support of the Irish Research Council for Science, Engineering and Technology. My Grant No.: RS/2010/2807 has meant too much to me during past 3 years.

I would also like to thank all the people I have had the opportunity to work alongside during this time. Maria E Nash, professionally, thank you for your technical assistance and advice, personally, thank you for being such a supportive friend throughout. Rongbing Yang and Jennifer Alexander, my colleagues and housemates, for all your help and advice I sincerely thank you and more so for your support. Deirdre Healy and Olga Gladkovskaya, the Biomaterials group isn’t the same without you, ladies! Thanks also in no small way to all the students I had the privilege to mentor, Tomas Martin, Kevin-Caoimhghin Mears, Cathal Egan, David Coyle, Andrew Kemple and all involved in ‘Yury’s Land of Lifting’!

I would like to acknowledge the great support and guidance from Prof. William Carroll, may he rest in peace.

Alexander Gorelov, better known as Sasha, it has been a pleasure to have you for your invaluable advice professionally and your endless help.

Howard Fearnhead, my graduate research committee, thanks so much for your critical helps and encouragement. I would not have improved without you.

To all my collaborators, Frank Barry and Georgina Shaw, I wouldn’t have achieved so much in the area of hMSC without you. Mikhail Nosov introduced a brand new of immunological world to me. Dimitrios Zeugolis, Abhigyan Satyam and Pramod Kumar, thanks million for your support and encouragement, it is you made me cheer up
when I was lost in the tissue engineering research. Sanbing Shen, Min Liu, Martin Madill, Katya McDonagh, Areechun Sotthibundhu, it has been a great opportunity to work in your lab, thanks also for your precious advice.

To all staff and students in NCBES and REMEDI past and present, thank you for making this a great place to do research and a great place to have lots of joy. David Connolly, thanks you for guiding me with the microscopies and giving me the opportunities to be the M.Sc. practical demonstrator. Siobhán Gaughan, thanks for your support throughout the last few years. Kerry Thompson, Eadaoin Timmins and Shirley Hanley, thanks for the supports with Time Lapse Microscopy, AFM, FTIR, SEM and Flow Cytometry. Robert Giblin, thanks for your support with the orders, both for the lab and for my personal.

Most of all, thank you to Daddy and Mammy, who have always been there to support me from day one. I am aware that you have felt the stresses and successes with me throughout. I dedicate this thesis to you both.
List of abbreviations

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<tr>
<td>AFM</td>
<td>atomic force microscope</td>
</tr>
<tr>
<td>AFP</td>
<td>α-fetoprotein</td>
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<td>ASM</td>
<td>α isoform of smooth muscle actin</td>
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<td>BCA</td>
<td>bicinechonic acid</td>
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<td>BSA</td>
<td>Albumin from bovine serum</td>
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<td>cell adhesion molecules</td>
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<td>complete chondrogenic medium</td>
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<td>DMEM</td>
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<tr>
<td>ECM</td>
<td>extra cellular matrix</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EtOH</td>
<td>anhydrous ethonal</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FC</td>
<td>Ficoll™70 &amp; Ficoll™400</td>
</tr>
<tr>
<td>FGF-2</td>
<td>Fibroblast Growth Factor-basic</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared Spectroscopy</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward Scatter</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks’ balanced salt solution</td>
</tr>
<tr>
<td>HCK</td>
<td>human corneal keratocytes</td>
</tr>
<tr>
<td>HMGB1</td>
<td>high mobility group box 1</td>
</tr>
<tr>
<td>hMSC</td>
<td>human mesenchymal stem cell</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HS</td>
<td>human serum</td>
</tr>
<tr>
<td>ICC</td>
<td>Immunocytochemistry</td>
</tr>
<tr>
<td>ICM</td>
<td>incomplete chondrogenic medium</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin 1β</td>
</tr>
<tr>
<td>iPS cell</td>
<td>induced pluripotent stem cell</td>
</tr>
<tr>
<td>ISCT</td>
<td>International Society for Cellular Therapy</td>
</tr>
<tr>
<td>LCST</td>
<td>lower critical solution temperature</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>macrophage inflammatory protein 1β</td>
</tr>
<tr>
<td>MIX</td>
<td>3-Isobutyl-1-Methyl-Xanthine</td>
</tr>
<tr>
<td>MMC</td>
<td>macromolecule crowding</td>
</tr>
<tr>
<td>MSC</td>
<td>mesenchymal stem cell</td>
</tr>
<tr>
<td>NaN₃</td>
<td>Sodium azide</td>
</tr>
<tr>
<td>NCBES</td>
<td>National Centre for Biomedical Engineering Science</td>
</tr>
<tr>
<td>NFB</td>
<td>Network of Excellence for Functional Biomaterials</td>
</tr>
<tr>
<td>NIPAm</td>
<td>N-isopropylacrylamide</td>
</tr>
</tbody>
</table>
NIPAm-co-NtBAm: N-isopropylacrylamide-co-N-\textit{t}ert-buty lacrylamide
NtBAm: N-\textit{t}ert-buty lacrylamide
NUI Galway: National University of Ireland, Galway
PAMPs: pathogen-associated molecular patterns
PBS: phosphate buffered saline solution
PDMS: polydimethylsiloxane
PE: phycoerythrin
PFA: Paraformaldehyde
PGA: polyglycolic acid
PLLA: poly(L-lactic acid)
PMN: polymorphonuclear leukocyte
pNIPAm: poly (N-isopropylacrylamide)
PRR: pattern recognition receptors
PVDF: polyvinyl difluoride
RAW264.7: macrophage-like transformed murine cell line RAW264.7
REMDI: Regenerative Medicine Institute
RMS: root-mean-square
s-GAG: sulphated glycosaminoglycan
SDS: Sodium dodecyl sulfate
SEM: Scanning Electron Microscope
SSC: Side Scatter
TBST: Tris-Buffered Saline and Tween 20
TCP: tissue culture plastic
TE: Tris-EDTA
UCST: upper critical solution temperature
WI-38: primary human lung fibroblasts
WS-1: primary human skin fibroblasts
Abstract

Traditional cell recovery methodologies cleave cell-to-cell junctions and thus the recovery of an intact cell sheet for use in tissue engineering is rendered impossible. Additionally, these traditional cell detachment techniques can be damaging to cell surface receptors, which in turn can impair subsequent cell function. Thermoresponsive polymer film mediated cell growth and recovery, has become a popular way to recover undamaged cells, with cell to cell junctions and basally deposited ECM maintained. These cell sheets can be then used for tissue engineering purposes or tissue damage repair. Thermoresponsive polymer has a lower critical solution temperature (LCST) in aqueous solution, which phenomenon has been exploited in temperature controlled cell harvesting. It has been shown that variety of thermoresponsive surfaces are generally conducive to reasonable cell growth. Okano et al. have grafted pNIPAm onto tissue culture plastic using electron beam polymerisation to yield an ultra-thin layer of pNIPAm. Maria E Nash has demonstrated that thermoresponsive films deposited using spin coating method were able to yield cell culture delivery substrates. This issue has been focused on further to simplify the preparation techniques for fabricating thermoresponsive films with a view to cell preservation. To this end, thermoresponsive platforms were deposited using the solvent cast method to yield thin, uniform, reproducible films. Solvent casting is a basic, cheap and effective method for fabricating films in the micrometre range of thickness. It was first reported as a method to deposit a thermoresponsive film for cell culture in 1990. For comparison purposes films were prepared using solvent cast method and the spin coating method.

Two types of NIPAm-based thermoresponsive polymers were used in this research; the first employed commercially sourced pNIPAm, the second a NIPAm-co-NtBAm copolymer. The advantages of using a commercially sourced polymer system paired with the operationally simple solvent cast/spin coating technique for cell sheet regeneration are that films prepared in this manner can be produced with minimal
training and expense and the use of a commercially sourced product avoids the need for complex polymerisation processes. The NIPAm-co-NtBAm copolymer was selected for similar applications, it showed better cell compatibility and had a LCST lower than room temperature, which makes the biomaterial much easier and flexible for routine applications.

The deposited films were characterised using a variety of analytical techniques before biological assessment. Studies have shown that there is a correlation between the thickness of the deposited pNIPAm films and successful cell adhesion and proliferation; therefore it was imperative that this parameter could be assessed. Successful cell adhesion onto a biomaterial surface is dependent on a number of physiochemical characteristics such as surface wettability, roughness and composition, therefore where a discernible difference in cell growth was observed between films of different thicknesses or deposited by different means; comparative assessments of such characteristics were made. Investigations into films prepared from commercially sourced pNIPAm show that it is the thickness determining factor for successful cell adhesion, thinner films supported more cell adhesion. Human mesenchymal stem cell and macrophage-like transformed murine cell line RAW264.7 grew on thinner solvent cast films better than that on their thicker counterparts. Films prepared from the NIPAm based thermoresponsive polymer via spin coating and solvent cast successfully hosted a wide variety of cells and cell lines to confluence and cell detachment was achieved through temperature modulation. Optimisation of the NIPAm based thermoresponsive films for cell adhesion, proliferation and differentiation allowed for the refinement of crucial parameters to thermoresponsive modifications.
Chapter 1. BACKGROUND AND INTRODUCTION

1.1 Smart polymers

1.1.1 Definition of smart polymer

Basic components in living organisms such as proteins, nucleic acids and carbohydrates are considered as natural occurring smart polymers. These polymers comprise of connecting monomer units linked by chemical bonds. The individual monomer reactions and interactions are triggered to have the same reaction with thousands of monomer units to the macro-level, from the cellular level upward to functioning living organisms such as DNA self-replication, energy storage and hydrophobic and hydrophilic reversible transitions (Wolf 1985). Synthetic polymers, which are designed to mimic these biopolymers, have been developed into a variety of functional forms to meet desirable industrial and scientific applications. Galaev and Mattision defined smart polymers as polymers which ‘undergo strong conformational changes when only small changes in the environment occur’ (Galaev and Mattisson 1999). Such materials can be sensitive to a number of environmental changes such as temperature, the presence of water, pH, the presence/intensity of light, etc. The polymer response can take various forms, such as: colour alteration, becoming transparent, conductive, permeable to water or changing shape (shape memory polymers) (Lomadze and Schneider 2005; Okano 1993; Zrinyi 2000). Usually, slight changes in the environment are sufficient to induce greater change in the polymer’s properties. It is important to note that not only are these polymers able to significantly change conformationally in reaction to a small alteration in an ambient parameter but that this change is also reversible upon reversal of the stimulant trigger (Yamada et al. 1990).

What makes smart polymers unique and interesting for biomedical and general scientific applications is their non-linear response, i.e. a significant change in structure and properties can be induced by a very small stimulus. Once that change occurs, there
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is no further change, meaning a predictable all-or-nothing response occurs, with complete uniformity throughout the polymer. The strength of smart polymer’s response to stimuli is able to create a considerable force for driving biological processes. Such property changes can be exploited for various highly specialised applications in biological systems.

Smart polymers can be classified by their stimuli sources and some of the more common ones are:

1) Temperature sensitive smart polymers
2) pH sensitive smart polymers
3) Polymers with dual stimuli-responsiveness
4) Light sensitive smart polymers
5) Phase sensitive smart polymers, etc.

There are many advantages of smart polymers, such as their biocompatible, increase patient compliance, no integrated electronics, biodegradability, blood contactable, modification for cell adhesion ligands, and the ability to alter release profiles of the incorporated agents, etc. (Al-Tahami and Singh 2007; Chaterji et al. 2007; Hoffman 2000; Hoffman et al. 2002).

There are also some shortcomings of smart polymers for example they can be hard to handle, usually they are mechanically weak, difficult to load with drugs or cells, difficult to crosslinking in vitro as a prefabricated matrix, difficult for sterilization (Mahajan and Aggarwal 2011).
1.1.2 Smart polymer applications

Smart polymers are used for a variety of ‘everyday’ applications such as in: hydrogels (nappies), plasters (band aids) that lose their stickiness when taken off. Smart polymers are also utilised in many forms for biotechnology and bioengineering, as they can be dissolved in solution, adsorbed or grafted on aqueous-solid interfaces or cross-linked in the form of hydrogels. Polymers as linear free chains in solution are used in bioseparation; covalently cross-linked, reversible gels are used in microfluidics; polymers which are chain adsorbed or surface-grafted are used as smart surfaces for tissue engineering (Kumar et al. 2007a). Smart polymers may be physically mixed with or chemically conjugated to biomolecules to yield polymer-biomolecule systems which respond to biological, physical or chemical stimuli. These polymeric biomaterials have potential for a wide variety of applications in biotechnology and bioengineering. When an enzyme molecule is bound covalently to a smart polymer, the polymer can be used to create reversibly soluble biocatalysts by forming a separate phase with stimuli. pH sensitive smart polymers consist of the polymers for which the transition between the soluble and insoluble state is created by decreasing net charge of the polymer molecule. The net charge can be decreased by changing pH to neutralize the charges on the macromolecule and hence to reduce the hydrophilicity of the macromolecule. These macromolecules can be modified as drug delivery systems (Mahajan and Aggarwal 2011). Examples of smart polymer biomedical applications are given in Table 1-1.
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Table 1-1 Summary of smart polymer biomedical applications.

<table>
<thead>
<tr>
<th>Smart polymer application</th>
<th>Example</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioseparation</td>
<td>poly(acrylic acid) (PAAc), poly(ethylene glycol) (PEG)-dextran</td>
<td>(Teotia et al. 2001)</td>
</tr>
<tr>
<td>Tissue engineering</td>
<td>poly(N-isopropylacrylamide) and its copolymers</td>
<td>(Chan and Mooney 2008; Kim et al. 2002)</td>
</tr>
<tr>
<td>Protein Folding</td>
<td>poly(propylene oxide)-phenyl-poly(ethylene glycol)</td>
<td>(Kuboi et al. 2000)</td>
</tr>
<tr>
<td>Gene Therapy</td>
<td>poly(azobenzene), poly[α-(4-aminobutyl)-l-glycolic acid], poly(ethylacrylic acid)</td>
<td>(Nagasaki et al. 2000; Pack et al. 2005)</td>
</tr>
<tr>
<td>Biocatalyst</td>
<td>α-chymotrypsin in acrylamide copolymers, polysaccharide chitosan, poly(N-isopropylacrylamide)</td>
<td>(Ivanov et al. 2003; Vazquez-Duhalt et al. 2001; Willner et al. 1993)</td>
</tr>
<tr>
<td>Drug delivery</td>
<td>poly(lactic acid), polyesters, poly(N-isopropylacrylamide-co-methacrylic acid)</td>
<td>(Moselhy et al. 2000; Zhang and Wu 2002)</td>
</tr>
<tr>
<td>Molecular Gates and Switches</td>
<td>Acrylamide-co-acrylate</td>
<td>(Shimoboji et al. 2002)</td>
</tr>
</tbody>
</table>
1.2 Thermoresponsive polymers

1.2.1 Basic terminology

Thermoresponsive polymers are polymers that exhibit a drastic and discontinuous change in their physical properties with temperature (Hoffman 1995). Thermoresponsive polymers can be divided into two classes: the first presents a lower critical solution temperature (LCST) while the second presents an upper critical solution temperature (UCST). LCST is the critical temperature below which the components of a mixture are miscible for all compositions. UCST, on the contrary, is the critical temperature above which the components of a mixture are miscible in all proportions. Certain polymers’ LCST or UCST in solvent are polymer specific, it is determined by polymer composition, polymer chain length and polymer molecular weight, etc. But the polymers which generate the most scientific interest and those which will be focused on here, are those which exhibit LCST transition in solution.

![Diagram showing water soluble and water insoluble states of a thermoresponsive polymer](image)

Figure 1-1 As the temperature is elevated from below the thermoresponsive polymer LCST to above, the polymer goes from its extended relaxed coil state to a collapsed globular state and precipitates out of its aqueous solution or assumes a gelatinous form.
LCST polymers are amphiphilic in nature, possessing both hydrophilic and hydrophobic parts. The hydrophilic segments form hydrogen bonds with water molecules thereby solubilising the polymer at the lower temperatures (T<LCST), which means the molecular chains are fully stretched out. But the hydrogen bonds become weaker and weaker as the temperature is raised through the LCST; finally breaking down as the ambient temperature exceeds the LCST, leading to the formation of coiled aggregates (Figure 1-1). The LCST of a thermoresponsive polymer is governed by the polymer’s molecular weight and its distribution; the nature of amphiphilic groups and the content of hydrophilic monomers in block or graft co-polymers. (Luo et al. 2003). The LCST can be explained thermodynamically by considering the 1st and 2nd laws of thermodynamics:

\[ \Delta G = \Delta H - T\Delta S \]

Where \( \Delta G \): Gibbs free energy, \( \Delta H \): enthalpy, T: temperature and \( \Delta S \): entropy.

It is noteworthy that LCST is an entropically driven effect while UCST is an enthalpically driven effect (Ward and Georgiou 2011). For a process to be favourable the Gibbs free energy term \( \Delta G \) should be negative. Consider liquids such as oil and water, mixing would be characterised by a large unfavourable enthalpic contribution and thus demixing is favourable. Then consider a simple solution of salt and water, mixing is favourable and is characterised by an increasing system disorder and thus a large and positive entropic contribution to the Gibbs free energy. Now consider a polymer solvent system which possesses a UCST. At low temperatures the enthalpic contribution may dominate the entropic term but as the temperature rise the entropic contribution increases until at the UCST it outweighs the unfavourable \( \Delta H \). Thus the higher temperature enhances solubility.
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However, polymers which possess a LCST exhibit highly directional interactions such as hydrogen bonding in aqueous solution. This hydrogen bonding leads to a large dominant negative $\Delta H$, thus mixing is favoured at low temperature. In this state, phase separation at the LCST is mostly due to entropy. Specifically, the main driving force is the entropy of the water, that when the polymer is not in solution the water is less ordered and has higher entropy. This is also called the “hydrophobic effect” (Klouda and Mikos 2008; Lutz 2008; Southall et al. 2002).

Poly(N-isopropylacrylamide) (pNIPAm) is the most commonly studied thermoresponsive polymer with a LCST of approximately 32°C, close to physiological temperature (37°C) (Shimizu et al. 2010; Twaites et al. 2005). It is possible to manipulate the LCST of N-isopropylacrylamide (NIPAm) based thermoresponsive systems by copolymerizing the NIPAm monomer together with hydrophilic or hydrophobic monomers rendering the overall hydrophilicity of the polymer higher or lower respectively (Doorty et al. 2003; Feil et al. 1993; Hacker et al. 2008). Some polymers with thermoresponsive properties include poly(N,N-diethylacrylamide) (PDEAM) with a LCST of 33°C which is near that of pNIPAm (Idziak et al. 1999) and poly(N-(2-methoxy-1,3-dioxan-5-yl) methacrylamide) (PNMM) and poly(N-(2-ethoxy-1,3-dioxan-5-yl) methacrylamide) (PNEM) (L11) which were determined to have transition temperatures of 22 and 52°C, respectively. Poly(N-acryloyl-N’-propylpiperazine) (PNANPP) exhibited a transition temperature of around 37°C in aqueous solution, but the measured value was highly sensitive to pH changes, etc (Roy et al. 2013). A table detailing a list of popular thermoresponsive polymers and their corresponding LCSTs is given below in Table 1-2.
Table 1-2 LCSTs of thermoresponsive homopolymers

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

1.2.2 pNIPAm and NIPAm-co-NtBAm

The thermoresponsive polymers used in this work are pNIPAm and NIPAm-co-N-tert-butylacrylamide (NIPAm-co-NtBAm). pNIPAm is by far the most extensively studied stimuli responsive polymer. Conformationally, it consists of a hydrocarbon back bone, hydrophilic groups in the form of the adjacent amide group and hydrophobic moieties in the form of isopropyl groups (Della Volpe et al. 1998). There is only one subgroup difference between NIPAm monomer and N-tert-butylacrylamide (NtBAm) monomer, but inclusion of the NtBAm monomer results in a more hydrophobic polymer system...
the LCST of which is dependent on the relative molar contribution of the copolymer monomers. For example Rochev et al. report on a family of NIPAm-co-NtBAm copolymers with LCSTs over the range of 10 to 32°C, depending on the NtBAm content (Figure 1-2) (Rochev et al. 2004).

Figure 1-2 Chemical structure of NIPAm, NtBAm monomers and LCST of NIPAm-co-NtBAm copolymers as a function of NtBAm contents (Rochev et al. 2004).

### 1.2.3 Application of thermoresponsive polymer

Interest in thermoresponsive polymers has steadily grown over the past couple of decades and a great deal of work has been dedicated to applying the polymers with a view to biomedical applications. Their ability to change conformationally in response to temperature has been utilised in areas such as bioseparation, drug delivery, tissue engineering, hydrogels, films and chromatography, etc. (Table 1-3). There are many advances in the field of thermoresponsive polymers, but numerous challenges and opportunities remain for impacting the field of smart materials. It is not only the transition temperature which has to be taken into account, but also the effect of the materials on the body, which is critical for biomedical applications (Roy et al. 2013).
<table>
<thead>
<tr>
<th>Thermoresponsive polymer application</th>
<th>Example</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioseparation</td>
<td>pNIPAm-protein conjugates, poly(ethylene oxide-co-propylene oxide), poly(N-vinyl caprolactam-co-vinyl imidazole), poly (N-vinylisobutryamide)</td>
<td>(J. P. Chen and Hoffman 1990; Galaev and Mattiasson 1999)</td>
</tr>
<tr>
<td>Thermoreponsive surfaces</td>
<td>pNIPAm, N-isopropylacrylamide-based polyelectrolyte, Poly(N-vinyl caprolactam), poly(N-isopropylacrylamide-co-N-hydroxyethylacrylamide)</td>
<td>(T. Liao et al. 2010a; Mattiasson et al. 1996; Okano et al. 1995)</td>
</tr>
<tr>
<td>Drug delivery</td>
<td>PLGA-block-P(EGMEMA-co-PGMA), poly(N-isopropylacrylamide-b-butylmethacrylate), poly(N-isopropylacrylamide-co-poly(methyl methacrylate)</td>
<td>(Abulateefeh et al. 2009; Chung et al. 1999; Rapoport 2007)</td>
</tr>
<tr>
<td>Tissue engineering</td>
<td>pNIPAm, NIPAm-co-acrylic acid (AAc), NIPAm-co-4-(N-cinnamoylcarbamide)methylstyrene, pNIPAm crosslinked with methylenebis(acrylamide)</td>
<td>(da Silva et al. 2007; E.L. Lee and von Recum 2010)</td>
</tr>
<tr>
<td>Hydrogels</td>
<td>pNIPAm, NIPAm-co-acrylic, NIPAm-co-propylacrylic acid, poly(N-isopropylacrylamide-co-N,N-dimethylacrylamide)</td>
<td>(Castellanos et al. 2007; Klouda and Mikos 2008)</td>
</tr>
<tr>
<td>Chromatography</td>
<td>pNIPAm, NIPAm-co-2-(dimethyl-amino) ethyl methacrylate, NIPAm-co-butyl methacrylate</td>
<td>(Ayano and Kanazawa 2006; Kanazawa 2007; Kanazawa et al. 2008)</td>
</tr>
</tbody>
</table>
Chapter 1

1.3 Cell adhesion and differentiation

1.3.1 Cell adhesion

Cell adhesion is the binding of a cell to a surface, such as an extracellular matrix (ECM) or another cell. Several different families of cell adhesion molecules (CAMs) mediate cell-cell and cell-matrix interactions. The families of adhesion molecules identified to date include selectins, integrins, cadherins, and immunoglobulin (Ig) superfamily members (Gumbiner 1996). Members of these adhesion receptor families do not only play critical roles in cell adhesion, but also in differentiation, migration, inflammation processes and embryonic formation. The mechanisms regulating adhesive interactions are complex and integral membrane proteins are built of multiple domains. Figure 1-3 briefly demonstrates the mechanism of each cell-cell adhesion CAMs. Cadherin and the Ig superfamily of CAMs mediate homophilic cell adhesion. For cadherin, calcium binding to sites (orange) between the five domains in the extracellular segment is necessary for cell adhesion; the N-terminal domain (blue) causes cadherin to dimerise and to bind cadherin dimers from the opposite membrane. The Ig superfamily contains multiple domains (green) similar in structure to immunoglobulins and frequently contain type III fibronectin repeating units (purple). In a heterophilic interaction, the lectin domain of selectins binds carbohydrate chains in mucin-like CAMs on adjacent cells in the presence of Ca$^{2+}$. The lectin domain is separated from the membrane by a series of repeated domains. The major cell-matrix adhesion molecule, integrin, is a heterodimer of α and β subunits. They bind to the cell-binding domain of fibronectin, laminin, collagen (type I, II and IV) or other matrix molecules (Figure 1-4) (Harvey Lodish et al. 2000; Hynes 1999; Lobert et al. 2010). The detailed classification and major examples for CAMs are shown in Table 1-4.
Table 1-4 Cell adhesion molecules classification and examples.

<table>
<thead>
<tr>
<th>Membrane proteins: cell adhesion molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium-independent</td>
</tr>
<tr>
<td>Ig SF CAM</td>
</tr>
<tr>
<td>N-CAM (Myelin protein zero), ICAM (1, 5), VCAM-1, PE-CAM, L1-CAM, Nectin (PVRL1, PVRL2, PVRL3)</td>
</tr>
<tr>
<td>Integins</td>
</tr>
<tr>
<td>LFA-1 (CD11a+CD18), Integrin alphaXbeta2 (CD11c+CD18), Macrophage-1 antigen (CD11b+CD18), VLA-4 (CD49d+CD29), Glycoprotein IIb/IIIa (ITGA2B+ITGB3)</td>
</tr>
<tr>
<td>Calcium-dependent</td>
</tr>
<tr>
<td>Cadherins</td>
</tr>
<tr>
<td>Classical</td>
</tr>
<tr>
<td>CDH1, CDH2, CDH3</td>
</tr>
<tr>
<td>Desmosomal</td>
</tr>
<tr>
<td>Desmoglein (DSG1, DSG2, DSG3, DSG4), Desmocollin (DSC1, DSC2, DSC3)</td>
</tr>
<tr>
<td>Protocadherin</td>
</tr>
<tr>
<td>PCDH1</td>
</tr>
<tr>
<td>Unconventional/ungrouped</td>
</tr>
<tr>
<td>T-cadherin, CDH4, CDH5, CDH6, CDH8, CDH11, CDH12, CDH15, CDH16, CDH17, CDH9, CDH10</td>
</tr>
<tr>
<td>Selectins</td>
</tr>
<tr>
<td>E-selectin, L-selectin, P-selectin</td>
</tr>
<tr>
<td>Other</td>
</tr>
<tr>
<td>Lymphocyte homing receptor: CD44, L-selectin, integrin (VLA-4, LFA-1)</td>
</tr>
<tr>
<td>Carcinoembryonic antigen CD22, CD24, CD44, CD146, CD164</td>
</tr>
</tbody>
</table>

Adhesion molecules also enable cells to contact and specifically interact with each other, which is a requirement of all multicellular organisms. Specialised cell-cell interactions allow communication between adjacent cells by soluble chemical signals binding to cell surface or intracellular receptors and are crucial for cell development.
and functional activity. For instance, cell migration is a coordinated process that involves rapid changes in the dynamics of actin filaments, together with the formation and disassembly of cell adhesion sites, it requires endocytosis and recycling of integrins (Figure 1-4). Integrins that bind to the ECM proteins transduce external stimuli into intracellular biochemical signals for cell migration (Lobert et al. 2010; Mitra et al. 2005).

Figure 1-3 Major families of cell-cell adhesion molecules (CAMs) (Harvey Lodish et al. 2000).
Integrins are major cell-matrix adhesion molecules, which also participate in cell migration which requires endocytosis and the recycling of integrins (Lobert et al. 2010).

In general cell culture work, cell adhesion on to a surface can be classified as a two-step mechanistic process: first, physicochemical interactions combination complexes such as van der Waals forces, gravity between the cell membrane and substrate control the initial attachment of cells. It is an adsorption mechanism which can be termed as a ‘passive adhesion’ mechanism, this step mainly is facilitated by the initial contact of the cell and the substrate. ‘Active adhesion’ is the next step and it requires the cellular metabolic process. When cells are attached onto the surface, external stimuli triggered the CAMs to bind to an appropriate substrate so as to make an interface between the cell membrane and the underlying substrate stable. This is achieved by both biological and physicochemical mechanisms (Baier et al. 1985; Kataoka K 1989; Okano et al. 1995).
1.3.2 Cellular differentiation

Cell differentiation is a process in which a generic cell develops into a specific type of cell in response to triggers from the body or the cell itself. Cell differentiation plays a critical role in the function of many organisms, particularly complex organisms, throughout their lives. The most typical cell differentiation is a single celled zygote developing into a multicellular adult organism that can contain hundreds of different types of cells. In mammals, zygote and subsequent blastomeres are totipotent as they can differentiate into any kind of cells. In plants, many differentiated cells can become totipotent themselves or with simple laboratory techniques. Cells that can differentiate into several different cell types, but not all, are considered to be pluripotent. Such cells are called embryonic stem cells in animals and meristematic cells in higher plants. Pluripotent stem cells undergo further specialisation into multipotent cells that then give rise to functional cells (Diez Villanueva et al. 2012). Stem cells are cells found in most multi-cellular organisms. They have the ability to differentiate into other cells and the ability to self-renew. Unlike the embryonic stem cell that can become any type of cell in the body, adult stem cells are thought to be limited to differentiating into different cell types of their tissue of origin. Mesenchymal stem cells are typical adult stem cells; they are harvested and cultured from normal human bone marrow. They are able to differentiate into osteogenic, chondrogenic and adipogenic lineages (Figure 1-5A). Induced pluripotent stem cells are another type of stem cell artificially derived from non-pluripotent cells – typically an adult somatic cell. They are similar to natural pluripotent stem cells in many aspects, such as the expression of certain stem cell genes and proteins, morphology, doubling time, potency and differentiability, etc. (Figure 1-5B)
Figure 1-5 A. Mesenchymal stem cell can be cultured to generate differentiated cells. B. Induced pluripotent stem cell pathways (Bonfield and Caplan 2010).

Differentiation dramatically changes a cell’s size, shape, membrane potential, metabolic activity and responsiveness to signals. Cell differentiation, with a few exceptions such as mutation, does not change the cell’s genome, the changes are largely due to highly controlled modifications in gene expression. Thus, different cells can have very different physical characteristics despite having the same genome. Cell differentiation has become a critical area of general and critical cellular process research, in the context of adult and embryonic stem cells as well as induced pluripotent stem cells (Kishore et al. 2010; Totey et al. 2009).
1.3.3 Factors which influence cell adhesion and differentiation on biomaterial surfaces

Biomaterial development has been focusing on the surface modification of biomaterials for decades in order to promote a greater understanding and control of the material characteristics for regulating biocompatibility. The ideal biomaterial surfaces should provide a framework and initial support for cell adhesion, proliferation, differentiation and ECM formation (Agrawal and Ray 2001). Successful cell adhesion and differentiation onto a biomaterial is dependent on a number of factors such as surface topography and chemistry (wettability, stiffness, roughness, chemical composition); microstructure (porosity, pore size, pore shape, interconnectivity and specific surface area) and mechanical character (Engler et al. 2006; O'Brien et al. 2005).

Figure 1-6 Illustration of the multitude of different factors affecting cells, which together determines the cell fate (Kolind et al. 2012).

When cells adhere to a surface, a sequence of physicochemical reactions happens between the cells and the surface. It has been well established that cellular and biological entities exhibit dramatically different behaviour when interacting with hydrophilic and hydrophobic surfaces (Figure 1-6) (da Silva et al. 2007). Neither ultra-
hydrophilic nor ultra-hydrophobic surfaces are ideal for optimum cell adhesion and proliferation. Many literature studies report different cell behaviour depending on surface hydrophobicities, which may mediate protein absorption. Surface wettability modified the sort and the quantity of adsorbed cell adhesion molecules. Hydrophobic surfaces tend to adsorb more proteins, while hydrophilic surfaces tend to resist protein adsorption (Xu and Siedlecki 2007). It was found that moderately hydrophilic surfaces were most conducive to successful cell adhesion. Maximum adhesion on a surface may vary from cell line to cell line due to their independent characteristics (Goddard and Hotchkiss 2007; J. H. Lee et al. 1998; Tamada and Ikada 1993).

Substrate topography and roughness or texture also influences cell adhesion, proliferation and differentiation. The literature has reported that cells grown on microrough surfaces, were stimulated towards differentiation; as shown by their gene expression in comparison with cells growing on smooth surfaces (Hatano et al. 1999; Lim et al. 2005). Purposeful patterns can be induced on surfaces through etching, sandblasting and micromachining etc. to induce guided or structured cell growth (von Recum and van Kooten 1995; Yim et al. 2005). Depending on the scale of the irregularities of the material surface, surface roughness can be divided into macroroughness (100μm – milimeters), microroughness (100nm – 100μm), and nanoroughness (less than 100nm), each with its specific influence (Vagaska et al. 2010). The effect that substrate roughness and topography has on cells varies between cell lines. For larger cells, such as osteoblasts and neurons, macroscopic descriptions of the surface roughness could be reasonable (Donoso et al. 2007). Cells such as fibroblasts and epithelial cells prefer smooth surfaces (Boyan et al. 1996). Bartolo et al. investigated neuronal cell behavior on surfaces with nanoscale (6.26 nm) to microscale (200 nm) roughness and report that the axonal length increased and the neurites became highly branched on surfaces with roughness 6.26-49.38nm. Therefore, the nanoscale rough membranes seemed to be more supportive of neurite outgrowth modulating the development process of the neurons (Bartolo et al. 2008). A significant amount of
Chapter 1

research has been dedicated to the effect that biomaterial topographical roughness has on a wide-variety of cell lines, the careful selectivity of surface for cells could be used as an advantage during the development of biomedical devices.

Cells are capable of sensing and adapting to substrate mechanical strength or flexibility. Several studies have reported that cell attachment, proliferation and differentiation are all modulated by the substrate rigidity (Engler et al. 2006; Khatiwala et al. 2007). On the other hand, research has found that cells may actively alter the stiffness of their microenvironment or that of other cells (Marquez et al. 2007). Studies show that cells prefer stiffer surfaces as cells tend to migrate towards the stiffer sector when seeded on a surface with varying levels of stiffness (Lo et al. 2000; Yeung et al. 2005).

1.4 Biomaterials & Stem cells

Stem cells hold great promise as a tool in regenerative medicine, cell therapies represent the potentially and most fruitful applications for stem cells. Upon differentiation into a suitable phenotype, stem cells can be introduced at a damaged site in a tissue in order to facilitate its regeneration (Higuchi et al. 2013). However, it is still challenging to maintain, expand, and differentiate stem cells on the scale and with the uniformity necessary for clinical applications. By researching how biomaterials’ physical, chemical, and mechanical properties influence cell phenotype, it is possible to manipulate these properties to guide stem cell behavior in overcoming these obstacles. A variety of biomaterials are being developed that support cellular attachment, proliferation and most importantly lineage specific differentiation (Dawson et al. 2008; Singh and Elisseeff 2010).

Chemical and biological modifications to biomaterials can directly influence stem cell behavior by altering substrate properties, surface interactions, scaffold degradation rate, microenvironment architecture. Cellular responses to biomaterials may be controlled by altering the interactions of material with serum components. Numerous studies have demonstrated how modified biomaterials and scaffold surfaces introduce specific
biological responses in stem cells (Burdick and Vunjak-Novakovic 2009; Dawson et al. 2008; Lutolf and Hubbell 2005). It is speculated that mechanical stimuli activate bone marrow-derived MSCs cell surface receptors and focal adhesion sites, which in turn triggers intracellular signalling cascades. This leads to specific gene activation that modulates ECM secretion (Altman et al. 2002; Engler et al. 2006). MSC differentiation is influenced both by their physical microenvironment e.g. by mechanical cyclic stresses applied directly to the cells as well as by the inherent biomaterial properties (Simmons et al. 2003). Plasma grafting was also used to alter the surface chemistry, surface energy and surface topology of poly(L-lactic acid) (PLLA) films, by increasing their ability to support cell retention (Wan et al. 2003). Monomers such as vinyl acetate, acrylic acid and acrylamide were grafted onto PLLA films by means of photo-induced grafting. Such modifications with functional groups (carboxyl, hydroxyl and amide groups) were shown to improve the ability of biomaterials to support chondrocyte growth (Chu et al. 2002). Chastain et al. used two different materials, poly-dl-lactic-co-glycolic acid (PLGA) and polycaprolactone (PCL), to modulate the preferential adsorption of ECM proteins from serum and demonstrated that depending upon the adsorbed protein the material showed differential effect on osteogenic differentiation of hMSCs (Phadke et al. 2010).

As biomaterial research advances, biomaterials provide a sophisticated microenvironment as a support and signalling media for stem cells. Different biomaterials can be blended together to achieve additional properties and benefits. However as new materials as well as innovations in research continues, there are still many different biomaterials which exist that have not yet been adapted for use with stem cell culture, which could be explored in future stem cells applications (Phadke et al. 2010; Singh and Elisseeff 2010; Willerth and Sakiyama-Elbert 2008).
1.5 Biomaterials & Immune responses

Biocompatibility is concerned with the interactions that occur between biomaterials and host tissues (Remes and Williams 1992). Immune response is key for long-term survival and function of biomaterials, which can have a profound impact on the host immune response. With this in mind the concept emerged to design biomaterials that are able to trigger desired immunological outcomes and thus support the healing (Franz et al. 2011). Therefore, it is important to understand the mechanism of the host inflammatory and wound healing response to implanted materials. The host responses toward biomaterials include injury, blood–material interactions, provisional matrix formation, acute inflammation, chronic inflammation, granulation tissue development, foreign body reaction, and fibrosis/fibrous capsule development (Figure 1-7) (Anderson 2001; Anderson et al. 2008). The events particularly involved in the foreign body reaction to biomaterials include protein adsorption, monocyte/macrophage adhesion, macrophage fusion to form foreign body giant cells, consequences of the foreign body response on biomaterials, and cross-talk between macrophages/foreign body giant cells and inflammatory/wound healing cells (Anderson et al. 2008).
Figure 1-7 Sequence of events involved in inflammatory and wound healing responses leading to foreign body giant cell formation. This shows the importance of Th2 lymphocytes in the transient chronic inflammatory phase with the production of IL-4 and IL-13, which can induce monocyte/macrophage fusion to form foreign body giant cells (PMN, polymorphonuclear leukocytes) (Anderson 2001).

Proteins from blood and interstitial fluids adsorb onto the biomaterial surface very quickly after the first contact. The layer of adhered protein determines the complex interactions of coagulation, complement and platelets that occur on biomaterial surfaces and they provide adhesion receptors for monocyte, lymphocyte and polymorphonuclear leukocyte (PMN) recognition. Apart from that, there are other receptor-ligand interactions activating immunocompetent cells called alarmins, which are the endogenous equivalent of pathogen-associated molecular patterns (PAMPs) and include heat shock proteins, HMGB1 (high mobility group box 1), ATP and uric acid. They act as potential activators of leukocytes following biomaterial implantation.
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(Bianchi 2007; Franz et al. 2011). Inflammatory cells (predominantly PMN) migrate from the blood toward the implant site immediately post protein deposition. Circulating PMNs are rapidly recruited to infection sites by host- and pathogen-derived mediators, acting as a first line of defence against invading pathogens (Franz et al. 2011). PMN normally disappear from the adhesion site within two days (Anderson et al. 2008). Chronic inflammation follows as the biomaterial persists with macrophages representing the driving force in perpetuating immune responses (Hamilton 2003). Macrophages act as both inflammatory mediators and wound heal regulators in the foreign body reaction. Macrophages attached to the biomaterial can foster the invasion of additional inflammatory cells by secreting chemokines like IL-8, MCP-1, MIP-1β for further dissemination of chemoattractants (Jones et al. 2007), they also phagocytosis wound debris, release enzymes important for tissue reorganisation, cytokines and growth factors inducing migration of fibroblasts. (Broughton et al. 2006; Franz et al. 2011). Macrophages play a key role in forming foreign body giant cells (FBGCs) on biomaterial surfaces as well as modulating cellular events to biomaterials result in their rejection or degradation (Figure 1-8). Therefore, it is necessary to modulate immune responses to biomaterils by surface modificaition, incorporation of bioactive molecules and designing ‘immunomodulating’ biomaterials based on artificial ECM, etc.
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Figure 1-8 Immune response toward biomaterials. A) Adsorption of blood proteins and activation of the coagulation cascade, complement and platelets result in the priming and activation of PMNs, monocytes and resident macrophages. B) Alarmins released from damaged tissue additionally prime the immune cells for enhanced function via PRR (pattern recognition receptors) engagement. C) The acute inflammatory response is dominated by the action of PMNs. PMNs secrete proteolytic enzymes and ROS (reactive oxygen species), corroding the biomaterial surface. IL-8 released from PMNs enhances PMN influx and priming. In the transition from acute to chronic inflammation, PMNs stop secreting IL-8 in favour of cytokines promoting immigration and activation of monocytes and macrophages. D) Macrophages are the driving force of chronic inflammation. Constant release of inflammatory mediators like TNFa, IL-6, and MCP-1 results in permanent activation of macrophages. Fusion-inducing stimuli like IL-4 and IL-13 promote the fusion of macrophages to FBGC, which form a highly degradative environment on the biomaterial surface. Furthermore, FBGC promote ECM remodeling and fibroblast activation resulting in excessive fibrosis and biomaterial encapsulation. E) Macrophage-derived cytokines and PRR engagement activate DCs (dendritic cells) on the biomaterial surface. Depending on the nature of the stimulus, DCs mature to either immunogenic or tolerogenic subtypes, amplifying or suppressing the inflammatory response (Franz et al. 2011).

1.6 Biomaterial & Tissue engineering

Tissue engineering is a biomedical technology and methodology to assist and accelerate the regeneration and repairing of defective and damaged tissues based on the natural healing potentials of patients themselves. For this therapeutic strategy, it is indispensable to provide cells with a local environment that enhances and regulates their proliferation and differentiation for cell-based tissue regeneration. Biomaterial technology plays an important role in the creation of this cell environment (Tabata 2009). Biomaterials can provide physical supports for engineered tissues and powerful
topographical and chemical cues to guide cells. Introducing nanoscale cues such as nanotopography or nanoparticles as therapeutic agents provide an exciting approach to modulate cell behaviour. Cell scaffold and biosignalling molecule delivery technologies with biomaterials have been utilized to create cell environments suitable for tissue regeneration (C. K. Kuo et al. 2006; Saltzman and Olbricht 2002; Tabata 2008, 2009). There are many biomaterials used in tissue engineering in attempts to regenerate different tissues and organs in the body. A number of key considerations are important for designing and determining the suitability of a biomaterial scaffold in tissue engineering regardless of the tissue type: 1) biocompatibility, 2) biodegradability, 3) mechanical properties, 4) architecture, 5) manufacturing technology and 6) choice of biomaterial for manufacturing (O'Brien 2011).

Typically, three individual groups of biomaterials, ceramics, synthetic polymers and natural polymers, are used for tissue engineering. Ceramics are widely used in dental and orthopaedic surgery for their high mechanical stiffness, low elasticity and hard brittle surface. For instance, hydroxyapatite and tri-calcium phosphate are widely used for bone regeneration applications (Hench 1998; Saavedra et al. 2001; Wang 2003). Synthetic polymers have shown great success in tissue engineering as they can be fabricated with tailored architecture, and their degradation characteristics controlled by varying the polymer itself or the composition of the individual polymer (O'Brien 2011; Rowlands et al. 2007). The shortcomings for them are the risk of rejection and cytotoxicity during degradation (Liu et al. 2006). Most common synthetic polymers used in tissue engineering include polystyrene, PLLA, polyglycolic acid (PGA) and PLGA. Natural polymers are excellent in promoting cell adhesion and proliferation as they are biological active. Unfortunately they generally possess poor inherent mechanical properties, which make natural polymers impossible for load-bearing orthopedic applications. Collagen, various proteoglycans and chitosan are often used in the production of scaffolds for tissue engineering (O'Brien 2011).
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Biomaterials are available with a variety of different chemical, mechanical and biological properties. Tissue engineering along with integration of mechanical and chemical engineering can be ideal for body injury healing through promoted angiogenesis or the infiltration and recruitment of key cells at the injured site (Patel and Fisher 2008; Tabata 2009).

1.7 Objectives

This study has been focused on further to simplify the preparation techniques for fabricating thermoresponsive films with a view to cell preservation. Two different polymer deposition techniques which are technologically facile, accessible and economical were used to deposit bare thermoresponsive films on which cells can be cultured and subsequently harvested. The details of the thermoresponsive film composition, method of fabrication and cell types used are listed in Table 1-5. The general divisions of this body of research were shown in Figure 1-9.

Table 1-5 Summary of thermoresponsive films as well as cell types used in the study.

<table>
<thead>
<tr>
<th>Film Type</th>
<th>Fabrication method</th>
<th>Thickness</th>
<th>Cell type used</th>
</tr>
</thead>
<tbody>
<tr>
<td>pNIPAm</td>
<td>Spin coating</td>
<td>100 nm</td>
<td>3T3, RAW264.7^</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 µm</td>
<td>RAW264.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 µm</td>
<td>hMSC^, HCK^, RAW264.7</td>
</tr>
<tr>
<td></td>
<td>Solvent casting</td>
<td>2 µm</td>
<td>hMSC, RAW264.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 µm</td>
<td>hMSC, RAW264.7</td>
</tr>
<tr>
<td>NIPAm-co-NtBAm</td>
<td>Spin coating</td>
<td>100 nm</td>
<td>WS-1^, WI-38^, RAW264.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 µm</td>
<td>RAW264.7</td>
</tr>
<tr>
<td></td>
<td>Solvent casting</td>
<td>1 µm</td>
<td>iPS cell^, RAW264.7, HCK</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 µm</td>
<td>RAW264.7, HCK</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 µm</td>
<td>RAW264.7</td>
</tr>
</tbody>
</table>

^RAW264.7: macrophage-like transformed murine cell line RAW264.7; hMSC: human mesenchymal stem cell; HCK: human corneal keratocytes; WS-1: primary human skin fibroblasts; WI-38: human lung fibroblasts; iPS cell: induced pluripotent stem cell.
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The use of commercially sourced pNIPAm allows for the avoidance of time-consuming and expertise driven polymerisation processes. The synthesised NIPAm-co-NtBAm significantly increased the NIPAm based thermoresponsive polymer biocompatibility for cell recovery. Depositing the films via the spin coating and solvent casting technique is routine, cheap and accessible to most laboratory personnel without the need for prior knowledge of chemical and engineering processes. The development of thermoresponsive platforms through both spin coating and solvent casting technique would therefore allow a tissue engineering lab to produce their own thermoresponsive dishes.

Figure 1-9 General sub-sections and cell types used in this body of research.
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The objectives of this project were to:

- **Stem cells**
  Simplify human mesenchymal stem cell detachment by non-enzymatic thermoresponsive technique.
  Grow human mesenchymal stem cell and preserve its immunophenotype on thermoresponsive films.
  Preserve and differentiate induced pluripotent stem cells with modification of thermoresponsive films.

- **Immunological assessment**
  Define the macrophage cell activation level and receptor profile based on different compositions and thicknesses of polymer films.
  Setup a practical acceptable and simple thermoresponsive substrate for macrophage routine lab work.

- **Tissue engineering**
  Optimise thermoresponsive substrates for different human primary cell lines for non-invasive cell harvesting techniques.
  Produce ECM-rich cell-assembled constructs from thermoresponsive films.
Chapter 2

Chapter 2. Materials and methods

The materials and methods used during this research project may vary in sections; therefore this chapter introduces the general materials and methods used throughout. A more succinct specific methodology will be summarised in each chapter.

2.1 Materials

Poly (N-isopropylacrylamide) (Mw 19,000-30,000, LCST 32°C) was purchased from Sigma-Aldrich and was used as received. NIPAm-co-NtBAm was synthesised as previously described, the LCST of NIPAm-co-NtBAm was measured in previous studies using microcalorimetry and was assessed to be 16°C (Kavanagh et al. 2005; M. E. Nash et al. 2013b; Rochev et al. 2001). The copolymer (65% NIPAm and 35% NtBAm) was synthesised by free radical polymerisation, using 2,2’-Azobis(2-methylpropionitrile) (0.5mol%) as an initiator in benzene (10% w/w) under argon. After polymerisation at 60°C for 24h, the mixture was precipitated from acetone to hexane three times, the product was dried at room temperature in vacuum (Rochev et al. 2004).

Albumin from bovine serum (BSA), Ammonium persulphate, anhydrous ethanol (EtOH, 200 proof, 99.5%), collagen, Dimethyl sulfoxide (DMSO), Glycine, HCl, H₂SO₄, NaOH, Lipopolysaccharides (LPS), NaCl, Paraformaldehyde (PFA), poly-l-lysine, Sodium dodecyl sulfate (SDS), Sodium azide (NaN₃), Tris-Base, Tween-20, Triton-X 100, were purchased from Sigma-Aldrich and used as received.

Swiss Albino 3T3 cells were provided by the National Centre for Biomedical Engineering Science (NCBES), NUI Galway. Human mesenchymal stem cells (one donor), Induced Pluripotent Stem (iPS) cells and RAW264.7 mouse leukaemia monocyte macrophage cell line were kindly provided by the Regenerative Medicine Institute (REMEDII), NUI Galway. WS-1 primary human skin fibroblasts cell, WI-38
human lung fibroblast and human corneal keratocytes cell were kindly provided by the Network of Excellence for Functional Biomaterials (NFB), NUI Galway.

Dulbecco’s modified Eagles medium (DMEM), Hanks’ balanced salt solution (HBSS), penicillin streptomycin, foetal bovine serum (FBS), phosphate buffered saline solution (PBS), Trypan blue stain, trypsin-EDTA were purchased from Sigma-Aldrich and used as received. PicoGreen® dsDNA assay, CellTrace CFSE cell proliferation Kit from Invitrogen, alamarBlue® assay from Biosource, Mouse IL-1 beta ELISA Ready-SET-Go!® from eBioscience, Pierce™ BCA Protein Assay Kit from Thermo Scientific, Amersham ECL Prime Western Blotting Detection Reagent from GE Healthcare Life Sciences, Thermanox™ plastic 25mm discs, NUNC™ 35mm dishes from NUNC™, all other plastic consumables from Sarstedt.

2.2 Methods

2.2.1 Film preparation

1) Solution Preparation

1. The appropriate amount of commercial pNIPAm or NIPAm-co-NtBAm was weighed by Adventurer Pro AV114 balance.

2. The polymer was then put into a 15ml tube and then the appropriate amount of EtOH was added to the tube, depending on the method of film deposition or the desired concentration, e.g. 20mg polymer into 1ml EtOH. (2% pNIPAm solution was prepared by 20mg/ml and 4% pNIPAm was prepared by 40mg/ml)

The EtOH was housed in a capped bottle with a soft centre. A syringe was used to extract the alcohol from the bottle and it was quickly dispensed into the vial. The vial was then immediately closed to minimize exposure to moisture in the air. The reason for such a more succinct measure is due to an unusual property of pNIPAm; while pNIPAm is soluble in ethanol and water separately, it is not soluble in a mixture of both. This phenomenon is known as co-nonsolvency.
3. The cap was closed and then sealed with Parafilm™.

4. Finally the tube was put on a rotator for over-night at 400rpm to ensure that the polymer is completely dissolved in the solvent.

2) Film fabrication

A number of different coating techniques are used to deposit polymer coatings such as spin coating, solvent casting, dip coating, spray coating and physical adsorption and each technique offers its own advantages and disadvantages. A brief overview of spin coating and the solvent casting technique used in this body of research is given below.

Two types of underlying substrate were used: tissue culture plastic (TCP) (35mm) and Thermanox® disc (25mm).

Spin coating

The typical process of spin coating involves depositing a small amount of a fluid polymer solution onto the centre of a substrate, and followed by acceleration to a high speed spinning the substrate. Static dispense and dynamic dispense are the two methods of deposition commonly used. Centripetal acceleration will cause the solution to spread out over the substrate, and finally off of the edge of the substrate leaving a thin film of polymer on the surface (Figure 2-1). Finally, the film thickness as well as other properties will depend on the nature of the solution and parameters of spin coating process (viscosity, drying rate, polymer concentration, surface tension, spin time and spin speed, etc.). Many factors contribute to how the properties of coated films are defined, such as final rotational speed, acceleration, and fume exhaust (Giraldi et al. 2006; Lawrence and Zhou 1991).
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Figure 2-1 Schematic illustrating the basic stages of the dynamic spin coating method.

Repeatability is one of the most important factors in spin coating (Charpin et al. 2007). Drastic variations in the coated film could result from subtle variations in the parameters that define the spin process (Zhao and Marshall 2008). Generally speaking, higher spinning speed fabricate thinner final films. Minor variations of ±50 rpm during the high spin speed process could cause a thickness change of up to 10%. Film thickness is not only largely dependent on the force applied to shear the fluid polymer solution towards the edge of the substrate, but is also sensitive to the drying rate which affects the viscosity of the solution (Fraysse and Homsy 1994).

The thickness of the spin coated films was based on previous works (M. E. Nash et al. 2011b).

1. The Laurell Technologies WS-400B-6NPP/LITE spin coater was set up.

2. 150 μl of polymer solution was dispensed onto a substrate which was rotating slowly at 150 rpm for 9 sec.

3. The programmed speed was quickly accelerated to 5000rpm and spinning lasted for 30 sec. As the rapid rotation proceeds, the volatile EtOH solvent evaporates and a thin film of the polymer remains.

4. For all depositions, concentration of 2% polymer in EtOH was used unless otherwise stated.
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Solvent casting

Solvent casting was first reported as a method to deposit a thermoresponsive film for cell culture in 1990. 0.5% w/v pNIPAm solution was initially prepared by dissolving the polymer in water, then the solution was cast onto a hydrophilic plate so as to manufacture 1μm pNIPAm film (Takezawa et al. 1990). After that, anhydrous ethanol was used to dissolve pNIPAm for the solvent casting, thus making the drying process much quicker due to solvent volatility (Rochev et al. 2004).

Solvent casting is a basic, cheap and effective method for fabricating films in the micrometre range of thickness. Briefly, a solution of the polymer is prepared in a volatile solvent, which is then deposited and spread evenly and carefully over the substrate. However, not all types of solution can be used due to the limitations associated with the drying process. During the drying process, the surface dries initially, and then proceeds towards the inside of solution (Osborne 2001). It should be noted that during the drying procedure if there is an inadequate number of polymer molecules for casting, (for instance the polymer concentration is too low or the volume of the solution is too small), the forces experienced by the polymer molecules, may cause the surface to crack which will cause the film to retain what is termed as a “mud-cracked” surface. Such an effect will make the surface rougher, and as a result nonhomogeneous films fabricated (B. Zhou 2001). The thickness of the solvent cast film can be estimated from total mass of polymer spread over the defined area of the Petri dish assuming a dry polymer density 1g cm\(^{-3}\) (M. E. Nash et al. 2013a). The thickness of the solvent casting film in this research can be calculated by:

\[
H = \frac{(C \times V \times \rho)}{S} \leftrightarrow V = \frac{(H \times S)}{(C \times \rho)}
\]

Where H is the thickness of the final film; C and V are concentration and volume of polymer solution, respectively; \(\rho\) is polymer density; S is the surface area of the substrate upon which the film was coated.
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The correct amount of pNIPAm solution was aliquoted onto the substrate and spread evenly and quickly to fabricate films of a pre-defined thickness. The thickness of the films was determined by the volume of solution dispensed and the concentration of the polymer solution. Larger solution volume will require longer drying time, therefore the solution deposited onto the substrate was kept between 50 to 200μl.

3) Drying process

Solvent casting and spin coating are both wet coating technologies; therefore a drying regime was developed.

After deposition of the film, either spin coating or solvent casting, the coated substrate was transferred to a desiccator overnight which was saturated with an ethanol environment. This is to make sure that the films dry slowly thus retaining a smooth film topography.

Then the substrate was transferred to a 40°C, 600mBar vacuum incubator for at least 4 hours. This is to remove any residual solvent.

4) UV sterilization

All films were sterilized under mild UV condition before cell seeding, the films were exposed to the UV in a laminar flow hood for 2h. Films were freshly sterilized before experimentation, any unused ones were sealed in dishes with Parafilm™ until needed.

2.2.2 Physical characterisation

1) Fourier Transform Infrared (FTIR) Spectroscopy

FTIR analysis was carried out on a Shimadzu FTIR-8300 Fourier transform infrared spectrophotometer equipped with a golden gate diamond ATR accessory. Polymer samples were prepared on TCP substrates. Prior to sample analysis a background TCP scan was taken to account for any environmental interference.
FTIR is a technique which is used to obtain an infrared spectrum of absorption, emission, photoconductivity or Raman scattering of a solid, liquid or gas. An FTIR spectrometer simultaneously collects spectral data in a wide spectral range. This confers a significant advantage over a dispersive spectrometer which measures intensity over a narrow range of wavelengths at a time. FTIR technique has made dispersive infrared spectrometers all but obsolete (except sometimes in the near infrared) and it has opened up new applications for infrared spectroscopy.

FTIR signals can be used to distinguish between different molecular compositions due to the inherent characteristic vibration and stretching frequencies of different bonds. Certain frequencies are absorbed by the sample and others are not when a broadband spectrum beam of light passes through a sample (Codling 1997). The light that reaches the detector is then analyzed and the specific frequencies that were absorbed are identified. It is then possible to compare these frequencies to a “peak library” to identify the various chemical functional groups of the sample materials (Markovich and Pidgeon 1991).

2) Film thickness assessment by Atomic Force Microscopy

The Atomic Force Microscope (AFM) (Model accessories used: Dimension 3100 AFM (Digital Instruments, Santa Barbara, CA, USA) with Veeco 1-10 Ohm-cm phosphorus (n) doped Si tips) is a powerful tool used to measure the properties of a sample surface. Such properties include roughness, height and depth. It yields topographic images and allows structure and detail to be seen with high resolution (1-10nm) without the need for rigorous sample preparation.

Film thickness measurements were performed using the simple scratch test method. Dried polymer films prepared as described above were scratched with sharp blade to reveal the underlying substrate. AFM analysis was used to assess to z height difference between the underlayer and the polymer surface. AFM images were obtained in tapping mode in air and a matrix of 512 x 512 data points along the x-y plane were analyzed in
a single scan. Four 100 μm x 100 μm scans were recorded at a scan rate of 1 Hz on each ablated area to ensure statistical accuracy.

3) Film roughness assessment by Atomic Force Microscopy

AFM was also used to assess the roughness of the deposited pNIPAm coatings using 10μm x 10μm scans. The roughness of the films was reported as root-mean-square (rms) roughness values, where rms denotes the standard deviation of the Z-values along the reference line.

4) Hitachi Scanning Electron Microscope

The Hitachi S-4700 Scanning Electron Microscope (SEM) was used to analyze the cell sheets which detached from thermo-responsive films. The SEM is an extremely powerful tool for surface analysis, allowing high depth-of-field and high magnification imaging. The scanning electron microscope SEM allows for the examination and analysis of microstructural characteristics of solid objects. Topographical features, morphology, compositional differences, and the presence and location of defects can be examined in a wide range of sample types.

For the SEM analysis conducted in this research, the cells sheets were stuck to SEM stubs with double-sided carbon tap, the samples were then gold coated using an Emitech™ K550X gold sputter coater. The coater was equipped with a piezo-electric controlled film thickness monitoring option set to 10nm before analysis using a Hitachi S-4700 SEM. The SEM was operated in low magnification mode for analysis with an accelerating voltge of 15kV.

5) Contact angle measurement

Drop shape analysis is a convenient way to measure contact angles and thereby determine surface energy. The principal assumptions are:

- The drop is symmetric about a central vertical axis: this means it is irrelevant from which direction the drop is viewed.
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- The drop is not in motion in the sense that viscosity or inertia is playing a role in determining its shape: this means that interfacial tension and gravity are the only forces shaping the drop.

A home-built goniometer was used to take advancing contact angle measurements in the Materials Science laboratory, in the School of Chemistry in University College Dublin (Figure 2-2). The relative hydrophilicity/hydrophobicity of the polymer films was measured by advancing contact angle above the thermoresponsive polymers’ LCST.

![Image of goniometer]

Figure 2-2 Home-built goniometer contact angle system used for advancing contact angle measurements in the Materials Science laboratory, in the School of Chemistry in University College Dublin.
2.2.3 General cell culture technique

1) Cell recovery from cryo-preserved state

Normally, cells are stored in liquid nitrogen for long-term cryo-preservation. To thaw, the vials were removed from the liquid nitrogen and heated in a 37°C water bath until almost thawed, after which they were transferred to a T75 tissue culture flask. Immediately, a total of 10ml of media pre-heated to 37°C was added drop by drop, and the flask was placed into an incubator (37 °C, 5% CO₂). The following day, the medium was changed in order to remove remnants of toxic DMSO.

2) Cell cryo-preservation

It is essential to freeze cells in liquid nitrogen in order to maintain them for future research.

1. Once there were enough cells, they could be detached by trypsinisation or scraping and then spun down to a pellet by centrifugation.

2. The pellet was re-suspended in a freezing medium (10%-20% DMSO in FBS).

3. The cells were aliquoted into cryogenic vials and placed in the -80°C freezer overnight.

4. Subsequently, vials were transferred to liquid nitrogen storage, maintenance temperature -196°C.

3) Subculture of adherent cell line

The base medium for general adherent cell lines is Dubecco’s Minimum Essential Medium with high glucose. To make complete growth medium, 10% FBS and 1% penicillin streptomycin antibiotics have to be added to the base medium. The routine cell culture procedure of the cells used in this body of research:

1. To split the cells, the medium in the flask was removed and the flask was rinsed with HBSS to remove any residue.
2. A 1% trypsin solution was then added to the flask after the solution was removed. The flask was removed to a 37°C incubator until the cells had detached which was verified microscopically.

3. 5 ml fresh culture medium was then added to neutralise the trypsin.

4. The cell suspension was collected and spun down at 1500 rpm for 5 min after which the cell pellet was isolated by aspirating the supernatant.

5. Fresh medium was used to resuspend the cells.

6. For subculturing, appropriate aliquots of the cell suspension were added to new culture flasks and the cultures were incubated at 37°C and 5% CO₂.

7. For experimentation, the concentration of the cells was counted using a haemocytometer and an appropriate number of cells were seeded. Normally, cells will be subcultured when they reached 95% confluent. Incubation conditions unless otherwise mentioned were a humidified atmosphere of 5% CO₂ at 37°C.

4) Cell seeding on thermoresponsive films

It is imperative that the ambient temperature is maintained above the LCST’s of the polymer films. The sterilized films were first placed on a thermoplate set to 40°C and allowed to equilibrate for 15 min. The cells were harvested and counted as described above and then diluted to a suitable concentration. The diluted solution was then kept in a water bath set to 37°C. After the solution was fully warmed, the desired number of cells was then seeded onto the films in triplicate as well as onto controls. The seeding density was 40,000 cell/cm² for all cell lines with the exception of stem cells which were seeded at a density of 20,000 cell/cm². The film samples were transferred to an incubator immediately after seeding.
5) Quantitative assessment of cell growth

AlamarBlue® Assay

AlamarBlue® is a reagent which was used to test the cell proliferation and cytotoxicity. Resazurin is the active agent in AlamarBlue® and is nontoxic to cells (Goegan et al. 1995). Viable cells actively take up resazurin, which in its oxidized form, shows almost no fluorescence (blue colour), but after permeating into living cells metabolic assimilation, reduces resazurin to resorufin which emits strong fluorescence (pink colour). This metabolic reduction is facilitated through oxidoreductases and the mitochondrial electron transport chain (Nakayama 1997). The cell viability can therefore be measured directly with a calorimetric reader. The relative level of the fluorescent signal (minus a blank signal collected from 10 % AlamarBlue® dye only) equates to the metabolic activity of the cells seeded on the samples and can be compared to the signal collected from controls. The AlamarBlue® test was employed in the experiments over the more traditionally used MTT assay due to its comparatively higher sensitivity and lower toxicity (Hamid et al. 2004). Briefly, the procedure used to make the AlamarBlue® assay is outlined below.

1. Cells were seeded on thermoresponsive films and controls as described in 2.2.3 and incubated for the desired period of time.

2. Candidate cells were washed with HBSS in advanced to make sure there was no other colour in the plate from media etc., which could contribute to a false result. A 10% AlamarBlue® solution prepared in HBSS was added into cells and then incubated at 37°C for 1 to 4 hours in a cell culture incubator.

3. 100µl of the co-incubated solution and 10% AlamarBlue® alone were added into a 96-well-plate in triplicate.

4. Wallac Victor Fluorescent Plate Reader was used to read the fluorescence using a fluorescence excitation wavelength of 550nm, read fluorescence emission at
595nm. The results were then analyzed and a chart was generated.

**Quant-iT™ PicoGreen® dsDNA Assay**

When using thermoresponsive dishes, it is imperative that physiological isothermal conditions are maintained until the ‘freeze-thaw step’ to prevent premature cell detachment. Therefore, all samples were removed from the incubator directly to a pre-heated (40°C) thermoplate.

1. Candidate cells were washed with HBSS to make sure there was no other colour in the plate from medium *etc.* which could contribute to a false result. A pre-defined amount of distilled water was added into each sample (the volume of which depended on the size of the wells being used for the experiment).

2. The cells were then repetitively frozen and thawed. This was carried out by freezing the cells for 30 minutes at –80 °C and subsequent thawing for 30 minutes at room temperature. This was repeated 3 times in order to ensure complete cleavage of the cell membranes.

3. During the freeze and thaw process, the following assay solutions were prepared:
   - 1X TE buffer (10mM Tris-HCl, 1mM EDTA, pH 7.5); 20X TE buffer diluted in distilled water
   - 2mg/ml DNA stock; 588μl distilled water + 12μl 100mg/ml DNA standard (from kit)
   - 50mg/ml DNA stock; 585μl distilled water + 15μl 2mg/ml DNA stock.
   - The PicoGreen dye was diluted 200 times (from kit) and covered in aluminum foil. (The PicoGreen was maintained in a dark atmosphere and was not added to the test plate until just before the assay was to be read due to being photosensitive).

4. A standard curve was prepared in a 96 well plate according to the table below,
each DNA concentration was triplicated in the plate to ensure statistical accuracy (Table 2-1).

Table 2-1 Standard curve for concentrations PicoGreen® Assays

<table>
<thead>
<tr>
<th>DNA concentration (ng/ml)</th>
<th>DNA stock = 50 ng/ml</th>
<th>DNA stock = 2μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA (μl)</td>
<td>0 20 40 100</td>
<td>5 10 50 100</td>
</tr>
<tr>
<td>Buffer(μl)</td>
<td>100 80 60 0</td>
<td>95 90 50 0</td>
</tr>
</tbody>
</table>

5. After the final freeze-thaw cycle, 100μl of cell lysate was transferred from each experimental well into a well of a 96-well-plate, away from the standard curve wells to ensure experimental clarity.

6. 100μl of diluted PicoGreen was then added into each well in the 96-well-plate.

7. The plate was allowed to sit at room temperature for 2-5 minutes in the dark, and then fluorescence was measured using the Wallac Victor Fluorescent Plate Reader at an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

6) Temperature induced cell detachment and imaging

Temperature induced cell detachment

As the cells became confluent on the samples and the controls, they were removed from the incubator and transferred to a thermostate set to 4°C. The culture media was removed using disposable transfer pipettes and then 2ml of cooled medium was added to initiate cell detachment.

Phase contrast and fluorescence Microscopy

Microscopy images of all cell types grown on each kind of polymer films and controls were captured using the Olympus IX71 inverted microscope, equipped with a Hamamatsu ORCA-ER digital camera, X-Cite Series 120 UV light box and controller.
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Images were captured and analysed using cell^p™ software for non-fluorescence images and Volocity® 6.2 for fluorescence images.

**Time lapse microscopy**

An Olympus IX81-ZDC time lapse microscope with Andor IQ™ software was used to image RAW264.7 cell detachment and migration upon initiation of cold treatment. The software was programmed to take images at regular intervals (20s) which were later compiled to make a time-lapse video of cell behaviour which clearly mapped the spatiotemporal changes which occurred with time.

**Confocal microscopy**

An Olympus IX81 motorised inverted microscope fitted with a piezo controlled z-stage, high-resolution EMCCD camera (Andor iXonEM+), Yokogawa CSU-X1 Spinning Disk Unit and controller was used to take fluorescence images at 405nm, 488nm and 561nm. The iPS cell immunochemistry staining was captured and analysed using Andor IQ™ software.

7) **Flow cytometry**

Flow cytometry is a laser based, biophysical technology employed in cell counting, cell sorting and biomarker detection, etc.

1. For flow cytometry analysis, cells were detached from the thermoresponsive films as described, followed by gentle and repetitive pipetting to yield singular cells. Cells were detached from controls via conventional trypsinization and scraper.

2. The cells were placed centrifuged and resuspended in Flow Cytometry Staining Buffer to a final cell concentration $1 \times 10^6$ cells/ml.

3. BD FACSCanto™ Flow Cytometer was used to obtain the results, cells were gated according to their physical characteristics by Side Scatter (SSC) versus
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Forward Scatter (FSC). Flowjo™ software was used to analyze the results.

8) CFDA-SE cell proliferation assay

Before opening the carboxyfluorescein diacetate succinimidyl ester (CFDA-SE), the product was allowed to warm to room temperature. 10mM CFDA-SE stock solution was prepared immediately prior to use by dissolving the content with high-quality DMSO. The stock solution was then diluted in PBS to the working concentration (5μM). It is important to note that any solution of this reagent should be used promptly.

1. When the cells had reached the desired density, the medium in the dishes was removed and pre-warmed PBS containing the probe was added.

2. After 10 min incubation, the loading solution was replaced with fresh, pre-warmed medium and the cultures were incubated for further 30min at 37°C. During this time, CFDA-SE underwent acetate hydrolysis.

3. A control was analyzed by flow cytometry immediately after incubation to define the starting density.

4. The samples and the rest of the controls were incubated for another 48/72h in order to assess cell proliferation.

9) Enzyme-linked immunosorbent assay (ELISA)

Mouse IL-1 beta ELISA was used to assess the RAW264.7 inflammatory activation level of cells grown on sample substrates and controls. Interleukin-1 beta (IL-1β), produced by activated macrophages as a proprotein, plays an important role in the body’s inflammatory response, while it is also involved in cell proliferation, differentiation and apoptosis (Maruyama et al. 2005). The experimental procedure followed was the standard protocol provided by eBioscience®.

1. Corning Costar 9018 ELISA plate was coated with 100μl/well of capture antibody in Coating Buffer. The plate was sealed and incubated overnight at 4°C.
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2. The wells were aspirated and washed 5 times with >250 μl/well Wash Buffer*. Allowing time for soaking (~ 1 minute) during each wash step increases the effectiveness of the washes. The plate was blotted on absorbent paper to remove any residual buffer.

3. The wells were blocked with 200μl/well of 1X Assay Diluent, and then incubated at room temperature for 1 hour.

4. Aspirate/wash was repeated for a total of 5 washes.

5. 1X Assay Diluent was used to dilute standards; 100μl/well of standard was added to the appropriate wells. A 2-fold serial dilution of the top standards was performed to make the standard curve. 100μl/well of samples was added to the appropriate wells. The plate was sealed and incubated at room temperature for 2h (or overnight at 4°C for maximal sensitivity).

6. Aspirate/wash cycles were repeated for a total of 5 washes.

7. 100μl/well of detection antibody diluted in 1X Assay Diluent was added. The plate was sealed and incubated at room temperature for 1h.

8. Aspirate/wash cycles were repeated for a total of 5 washes.

9. 100μl/well of Avidin-HRP (horseradish peroxidase) diluted in 1X Assay Diluent was added. The plate was sealed and incubated at room temperature for 30min.

10. The aspirate and wash step was repeated as in step 2. In this wash step, the wells were soaked in Wash Buffer for 1 to 2 min prior to aspiration.

11. This was repeated for a total of 7 washes.

12. 100μl/well of Substrate Solution was added to each well. The plate was then incubated at room temperature for 15min.

13. 50μl of Stop Solution (1M H₂SO₄) was added to each well.
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14. The plate was read by a plate reader at 450nm and 570nm. The values of 570nm were subtracted from those of 450nm and analyzed.

10) **Protein isolation & quantification from cell**

Cells were harvested as previously described in Section 2.2.3. The detached cells were collected and spun down at 400g for 5min. The supernatant was aspirated to retain the pellet. Protein lysis buffer was used to resuspend the cells and then they were left on ice for 30min. Samples were centrifuged for 20min at 900g at 4°C and the supernatant containing the protein was aspirated.

The protein concentration was determined using Pierce™ BCA Protein Assay Kit according to manufacturer’s instructions. The assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. Briefly, protein standards ranging from 0-2mg/ml were used to prepare a standard curve. Protein samples were diluted 1/3 in distilled H2O and measured in duplicate. 5µl of each sample & standard were added to 200µl of the working solution in a flat bottom 96-well-plate. Lysis buffer was used as a blank control. The samples and standards were incubated for 30min at 37°C and the absorbency was then read at 550nm. The samples were then stored at -80°C until further use.

11) **Western blotting**

Western blotting identifies which specific antibodies proteins have been isolated from cells. The proteins are separated from one another according to their size by gel electrophoresis.

1. For each sample, 20µg of protein was mixed with 4X buffer and heated for 10 min at 95°C. Samples, including a molecular weight marker, were then loaded onto a SDS/polyacrylamide gel and ran for 1.5h at 100 volts.

2. Next the transfer step was set up. Sponges and filter paper (6X pieces) were
soaked in transfer buffer. The PVDF membrane was soaked in methanol for 2-3min prior to use and then rinsed in TBST (Tris-Buffered Saline and Tween 20) to equilibrate the membrane.

3. The protein samples on the gel were subsequently transferred onto the PVDF membrane for 1h 20min at 100V 0.35A on ice.

4. The membrane was separated from the gel following the run and washed (X3) in TBST for 5min at a time.

5. Non-specific protein interactions were inhibited by incubating the PVDF membrane in block buffer containing 5% low fat milk in TBST for at least one hour at room temperature while being rocked gently.

6. Antibodies were titrated and membranes were then probed for the protein of interest by incubation with the antibodies diluted in blocking buffer. The membrane containing the protein samples were incubated with the primary antibodies in blocking buffer overnight at 4°C.

7. The following day the membrane was washed (X3) in TBST for 5min at a time.

8. Next the membrane was incubated with the appropriate secondary antibodies in blocking buffer for 1h at room temperature.

9. Proteins were detected via enhanced chemiluminescence ECL kit. Antibody binding can be visualized using the Fluorchem Imager at varying exposure times, typically 1 min, 5 min and 10 min.

10. Following detection, the membrane can be stripped of bound primary and secondary antibodies via the incubation of the membrane with 20ml Restore Western Blot Stripping Buffer for 30 min at 55°C.

11. The use of restoring buffer enables each membrane to be stripped several times without affecting the membrane bound protein. The membrane can be
Chapter 2

subsequently washed three times with TBST, blocked for 1h in blocking buffer and re-probed using a different antibody.

12. Equal loading of protein samples was verified by the detection of β-actin.

12) **Immunocytochemistry**

Immunocytochemistry (ICC) is a common laboratory technique that uses antibodies that target specific peptides or protein antigens in the cell via specific epitopes. The bound antibodies can then be detected using fluorescence detections. ICC allows researchers to evaluate whether or not cells in a particular sample express the antigen in question.

1. Cells were rinsed briefly in PBS and then covered to a depth of 2-3mm with 4% paraformaldehyde. The cells were allowed to fix for 15 min at room temperature.

2. The cells were then rinsed 3 times in PBS for 5 min each.

3. A sufficient amount of 0.2% of Triton-X was added to each sample and allowed to incubate for 5 min and then it was removed.

4. The cells were then rinsed in PBS for 5 min.

5. 5% goat serum was used to inhibit the non-specific protein interactions for 1h at room temperature.

6. The primary antibody was applied after aspirating the blocking buffer, the cells with the primary antibody were then incubated overnight at 4°C

7. The following day the cells were washed (X3) in PBS for 5 min at a time. To decrease background staining, the cells were rinsed in high salt PBS for 2 min between then second and third PBS rinses.

8. The fluorochrome-conjugated secondary antibody was then added to the cells for 1-2h of incubation at room temperature in the dark.
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9. The cells were then rinsed in PBS/high salt PBS as above.

10. The cells were then covered with PBS for examination under confocal microscopy.
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Chapter 3. Stem cell adhesion, proliferation and differentiation on thermoresponsive films

Sections of this chapter were written based on the published communication:

3.1 Introduction

As previously outlined, the generation of cells for the repair of damaged tissues using stem cell therapy holds much promise due to the pluripotent potential of stem cells. It is preferable to eliminate the use of any animal derived products, such as trypsin which is routinely used in cell culture, for clinical and therapeutic purposes. Thermoresponsive platforms offer an alternative route for stem cell regeneration by hosting and releasing stem cells without the use of animal derived products. This is becoming increasingly desirable as the regulatory biomedical landscape suggests a collective movement away from the use of animal derived products and the evolution of new techniques that avoids their use.

The underlying potential of MSCs in stem cell therapy is their innate ability to differentiate. In order to ultimately realise this potential it is essential, to be able to culture MSCs for large scale applications in an undifferentiated state in a reproducible and reliable fashion so that they can be differentiated when desired. There are a number of factors capable of influencing or inducing stem cell differentiation including; cell-material interactions, material-surface properties such as chemical composition and energy and cell growth factors (Curran et al. 2006; Re'em et al. 2012). There has been extensive research on the use of biomaterials with a view to preserving and differentiating not only MSCs, but also embryonic stem cells and hematopoietic stem cells, researchers have examined biomaterials in the form of 2-D and 3-D scaffolds, microparticles, hydrogels and coatings etc (Bratt-Leal et al. 2011; Dawson et al. 2008; Singh and Elisseeff 2010). Modifications to the cell substrate used to guide hMSC differentiation in vitro indicate that -NH₂ and -SH modified surfaces promote osteogenesis and -OH and -COOH modified surfaces promote and maintain chondrogenesis (Curran et al. 2006). Thermoresponsive polymers were also used for hMSC proliferation and multi-differentiation potentials, for example pNIPAm-grafted polydimethylsiloxane (PDMS) and NIPAm-based polyelectolyte multilayer films,
hMSC cells reserved their potential for differentiation and showed better viability on NIPAm based polymer films than on the plate surfaces coated with gelatine (T. Q. Liao et al. 2010b; Shi et al. 2010). It has been shown using rat bone marrow MSCs and human adipose tissue MSCs that when these cells are detached from thermoresponsive polymer coatings the ECM proteins are preserved which has been shown to facilitate the maintenance of important cell membrane proteins (L. Yang et al. 2012).

However, the film fabrication procedures are complicated and how the cells behave post detachment has not been reported. In previous studies commercially sourced polymer was used to produce pNIPAm solutions for fabrication of ultra-thin pNIPAm based films for cell and cell sheet regeneration via the spin coating method. A number of different cell lines including hMSCs were successfully grown under standard physiological conditions and were subsequently gently detached upon temperature reduction (M. E. Nash et al. 2011a). No relationship between the thickness of the film produced and successful cell attachment and proliferation within the thickness-scale employed (> 30 nm to < 2000 nm) was observed in Nash et al.’s study. However, studies have shown that cells attach and therefore proliferate poorly on thick or bulk pNIPAm coatings and many studies report a thickness limitation above which there is a dramatic reduction in the number of cells attaching to the polymer surface (Kumar et al. 2007b; Matsuda et al. 2007; M.E. Nash et al. 2012; Rayatpisheh et al. 2012; H. Takahashi et al. 2010). In studies where pNIPAm was covalently grafted for this purpose, this thickness limitation is in the order of tens of nanometers with the exception of coatings formed via plasma polymerization (Canavan et al. 2005; Kikuchi A. 2005; Kumar et al. 2007b; Kumashiro et al. 2010). Hence, while some studies show no relationship between the thickness of pNIPAm based films and optimum cell growth, it is still a parameter that should be carefully investigated and controlled when designing substrates for regenerative cell harvesting. Finally, the grafting techniques used to produce thermoresponsive coatings are technologically difficult and
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economically expensive and many studies seek to investigate less complex and cheaper alternatives.

The aim of the present study was to develop a simple method to produce pNIPAm coatings with a view to cell regeneration, particularly non-differentiated stem cell regeneration. It seeks to offer a significant simplification on methods employed elsewhere by investigating the possible thickness limitation of films produced using the simple solvent casting method. Previous investigations using cell adhesion promoters as an over layer on top of thick solvent cast pNIPAm films to improve cell adhesion as well as the aforementioned spin coating approach (Moran et al. 2006; M. E. Nash et al. 2011a). Solvent casting is a very simple, routine and inexpensive technique used to produce polymer films mainly in the micrometer range. Moreover, solvent casting is a common method used in cell culture protocols for example when depositing cell adhesion promoting layers such as fibronectin or poly-l-lysine and therefore is a method familiar to most cell culture operators making it a widely accessible approach for film production compared to methods used elsewhere (Baldwin et al. 1996; Foldberg et al. 2011). To further simplify the method; commercially sourced pNIPAm was used thus avoiding the expense, equipment and expertise needed for polymerization or grafting procedures which makes the method accessible to all laboratories where non-invasive cell or cell sheet detachment is desirable. While using non-grafting techniques to produce films may lack the elegance of the grafting techniques due to polymer dissolution upon temperature reduction, studies have shown that pNIPAm is not cytotoxic to cells (Malonne et al. 2005; Takezawa et al. 1990).
3.2 Methodology

3.2.1 Materials

hMSCs were kindly provided by the Regenerative Medicine Institute (REMedI) group in the National Centre for Biomedical Engineering Science (NCBES), NUI Galway. For human mesenchymal stem cell culture, Minimum Essential Medium (1X), GlutaMAX™ I were purchased from Biosciences LTD, Fibroblast Growth Factor-basic (FGF-2) from PeproTech and non-heat inactivated foetal bovine serum from Hyclone was used to supplement the growth media. Dexamethasone, Insulin, Indomethacin, 3-Isobutyl-1-Methyl-Xanthine (MIX) were purchased from Sigma Aldrich. Phycoerythrin (PE) labeled CD19, CD34, CD45, CD73, CD90 and CD105 (CD: cluster of differentiation) cell surface markers were from Biosciences and used as received. All other plastic consumables were from Sarstedt.

3.2.2 hMSC stem cell expansion, preservation and differentiation procedure

1µm, 2µm and 4µm solvent cast pNIPAm films were prepared on 35mm TCP for cell seeding. The prepared films were characterized before cell seeding. FTIR was used to analyze the deposited films. The film thickness and roughness measurements were performed by AFM. Advancing contact angle measurements were performed using the advancing drop method on a home-built goniometer as described.

1) Cell culture and imaging techniques

hMSCs were maintained in Minimum Essential Medium, supplemented with 10% non-heat inactivated foetal bovine serum, 1% penicillin streptomycin and 1ng/ml of FGF-2. For experimentation, hMSC cells were seeded in triplicate on solvent cast pNIPAm film at a density of 20,000cells/cm² for 5 days with 95% air and 5 % CO₂ at 37 °C. In all cases the samples were placed on a thermoplate to maintain a working temperature above the polymer’s LCST. Cell proliferation and cell detachment was microscopically observed using an Olympus BX51 with Image Pro-Plus analysis system phase contrast.
microscope. AlamarBlue\textsuperscript{®} assay was used to test the cell metabolic activity; total dsDNA content was also quantified using Quant-iT\textsuperscript{TM} PicoGreen\textsuperscript{®} dsDNA Assay.

2) Temperature detached hMSCs surface antigen characteristics

For flow cytometry analysis, cells were detached from the thermoresponsive films as described in 2.2.3 followed by gentle and repetitive pipetting to yield single cells. Cells were detached from TCP controls via conventional trypsinization. The cells were stained with CD19, CD34, CD45, CD73, CD90, CD105, all PE-labelled, as per the manufacturer’s instructions and were analyzed by flow cytometry using BD FACS Canto from Bioscience, cell analysis was standardized for side/forward scatter and fluorescence. The results were analyzed by Flowjo\textsuperscript{™} software. Additionally, half of the cells that were detached from the thermoresponsive surfaces were reseeded on TCP and incubated for a further 3 days after which the same marker expressions were checked to define the post-detachment effects. All FACS experiments were performed in triplicate.

3) Post thermo-detached differentiation assessment

To test the hMSC differentiation potential on thermoresponsive films, cells were repetitively seeded and detached on and from thermoresponsive films 3 times. Cells were detached from the films by reducing the temperature and gently seeded onto the next film. Then specific differentiation media were used to trigger the hMSCs differentiation for adipogenesis, chondrogenesis and osteogenesis.

hMSC adipogenic differentiation was measured by Oil Red O staining, chondrogenic differentiation was assessed by measuring sulphated glycosaminoglycan (s-GAG) and normalizing between pellets by measuring the DNA content (s-GAG per µg DNA), osteogenic differentiation was stained by Alizarin Red and measured by Stanbio Calcium (CPC) LiquiColour\textsuperscript{®} Test Kit.
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3.3 Results

FTIR analysis confirmed film deposition. As seen in Figure 3-1, FTIR spectra displayed peaks at 1500 cm\(^{-1}\), 3300 cm\(^{-1}\), 3000 cm\(^{-1}\), 1450 cm\(^{-1}\), 1180 cm\(^{-1}\), 1640 cm\(^{-1}\) corresponding to N-H bending peaks, a N-H stretching vibration, a C-H stretching vibration, a C-H asymmetric stretching vibration, a C-H symmetric stretching peak and a C=O stretching peak respectively which correspond to the fingerprint peaks of pNIPAm. The 2µm thick film’s spectrums were the same as the 4µm’s, only the 4µm is shown for clarity.

Figure 3-1 FTIR spectrum of pNIPAm powder and solvent cast films. The assignment of peaks confirms successful pNIPAm film deposition. (pNIPAm powder: grey; 1µm thick solvent cast pNIPAm film: purple; 4µm thick solvent cast pNIPAm film: dark green)
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The relative thickness & roughness of the films was assessed via AFM analysis; the results of which can be seen in Table 3-1 & Table 3-2 and all of the films were assessed to be relatively smooth.

Table 3-1 Predicted solvent cast film thickness versus thickness measured by AFM analysis.

<table>
<thead>
<tr>
<th>Predicted Film Thickness (µm)</th>
<th>Measured Film Thickness (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.07 ± 0.07</td>
</tr>
<tr>
<td>2</td>
<td>2.04 ±0.03</td>
</tr>
<tr>
<td>4</td>
<td>4.02 ±0.04</td>
</tr>
</tbody>
</table>

Table 3-2 Solvent cast film RMS roughness as measured by AFM analysis. The roughness of the films was reported as root-mean-square (RMS) roughness values, where RMS denotes the standard deviation of the Z-values along the reference line.

<table>
<thead>
<tr>
<th>Film Thickness (µm)</th>
<th>RMS Roughness (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16.01 ± 0.90</td>
</tr>
<tr>
<td>2</td>
<td>24.01 ± 4.88</td>
</tr>
<tr>
<td>4</td>
<td>28.85 ± 1.55</td>
</tr>
</tbody>
</table>

The relative hydrophilicity/hydrophobicity of the polymer films was measured by advancing contact angle above the polymer’s transition temperature. Distinct stick/slip behaviour was clearly evident as the water droplet interacts with the film surface. This type of behaviour is described in detail by Gilcreest et al.. The stick angle is generally reported as close to the real contact angle of the films in cases where stick/slip behaviour is observed, which in this case was an average of 68 ± 2 degrees for 1 µm films, 72 ± 1 degrees for 2 µm and 57 ± 1 degrees for 4 µm films from 500 s to 1800 s (allowing for the initial calibration of the advancing drop) (Gilcreest et al. 2004).
3.3.1 hMSCs attachment and detachment by temperature control

hMSCs were seeded on the cast pNIPAm films and observed microscopically for cell proliferation after 120 hours incubation. hMSCs proliferation on 1 µm films was slightly less than on conventional TCP controls and the numbers of cells adhering and proliferating reduced as the thickness of the cast films increased with a modest reduction in cell numbers observed on 2µm films and a marked reduction in cell numbers on thicker 4 µm films. This was confirmed by metabolic activity alamarBlue® assay results with the activity of cells on the 4 µm films only 20% compared to controls, the PicoGreen dsDNA quantification assay displaying a similar trend with only 20% of dsDNA compared to TCP controls (Figure 3-2). Metabolic activity alamarBlue® assay results suggest that not only did fewer cells adhere on the thicker substrates, but additionally their metabolic activity was reduced in comparison. These results indicate that while 1 µm thick films were capable of hosting hMSCs to confluence the proliferation is slower than on the TCP controls i.e. 74% dsDNA compared with TCP controls. The reason why the films become increasingly cell repulsive with increasing film thickness is unclear though it is probably associated with the increased mobility of the polymer chains with increasing film thickness leading to increased hydration which is non-conducive to protein adsorption which mediates cell attachment, this increased hydrophilicity for the thicker 4 µm thick films was analytically reflected in the advancing contact angle measurements.

hMSCs were completely detached under cold treatment and cell to cell contacts were maintained and full cell sheets were recovered within less than 30 minutes (Figure 3-3).
Figure 3-2 A. AlamarBlue® assay test results comparing hMSC metabolic activity on TCP controls and solvent cast films of differing thicknesses. B. PicoGreen total dsDNA assay results comparing hMSC dsDNA quantities on TCP controls and solvent cast films of differing thickness. Assays were performed on the samples after 120h incubation with an initial seeding cell density of 20,000 cells/cm². Error bars refer to standard deviation where 3 separate samples of each type of substrate were used and assays were performed in triplicate.
Figure 3-3 hMSC cell sheet detaching from a 1µm cast pNIPAm film. Cell to cell junctions are maintained as the cell sheet lifts off. Cells began detaching after 5 minutes with complete detachment achieved after 30 minutes. This montage follows the progression of cell detachment upon exposure to cold treatment; (A) 5 min, (B) 6 min, (C) 7 min, (D) 9 min, (E) 11 min, (F) 13 min, (G) 15 min, (H) 18 min. Scale bar: 500µm.
3.3.2 hMSCs surface antigen characteristics after temperature detachment

As defined by the International Society for Cellular Therapy (ISCT); MSC cells must be plastic-adherent in standard cell culture conditions using tissue culture flasks. Secondly, >95% of the MSC cell surface must express CD105, CD73 and CD90, as measured by flow cytometry. Additionally, these cells must lack the expression (<2% positive) of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA class II. Third, the cells must be able to differentiate into osteoblasts, adipocytes and chondroblasts under standard in vitro differentiating conditions (Dominici et al. 2006). With this in mind, the ‘positive markers’ CD73, CD90 and CD105 and ‘negative markers’ of CD19, CD34, and CD45 for hMSCs were tested using FACS, as the presence or lack of expression respectively would indicate that the polymer films did not induce hMSC differentiation. Furthermore, half of the detached cells were reseeded and incubated on TCP for 3 days after which marker expression was again checked to define the post-detachment affects. hMSC were detached from controls via conventional trypsinization, by temperature control from thermoresponsive films and again by trypsinization after reseeding and incubation for 3 days prior to testing for the membrane markers. In terms of the ‘positive’ markers the expression of all 3 was >95% which indicates that no hMSC differentiation had occurred in the cases of cells grown and detached from pNIPAm or the controls and moreover no post detachment affects occurred after reseeding and incubation. In all cases the expression of all 3 ‘negative’ cell membrane surface markers was <2% positive, Table 3-3. As these markers are rarely expressed on MSC cells these results indicate that no hMSC differentiation had occurred in the cases of cells grown and detached from pNIPAm or the controls, furthermore no post detachment affects occurred after reseeding and incubation.
To assess if this method of expansion and propagation is suitable for maintaining hMSCs in an undifferentiated state over serial passages, cells were seeded and detached as previously described from 1µm & 2µm thermoresponsive surfaces and TCP controls, but this time the cells were reseeded and detached through 3 passages. After the final passage the validatory pluripotency markers, as described by the ISCT, were assessed by FACS Canto as previously described. It was observed that cells grown and detached on 1µm thick solvent cast films were able to successfully attach and proliferate on subsequent cycles again using 1 µm thick films. The multifold expansion of hMSCs on the 1µm thick films yielded cells which maintained all the ‘positive markers’ and lacked the so-called ‘negative markers’ tested for. Cells grown and detached from 2µm thick pNIPAm films were not able to maintain C73 or CD90 as the ‘positive markers’, also the ‘negative markers’ significantly increased on the cell surface (Table 3-4). Therefore, only the cells adhered on 1µm thick solvent cast pNIPAm film maintained their multipotentiality in accordance with the prescribed ISCT guidelines.
### Table 3-4 hMSCs cell surface antigen expression as assessed by FACS of cells passaged and detached 3 times for solvent cast pNIPAm surfaces.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>1micro CA pNIPAm</th>
<th>2micro CA pNIPAm</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD73 (+)</td>
<td>96.0%±1.5%</td>
<td>95.5%±1.2%</td>
<td>63.4%±1.8% (-)</td>
</tr>
<tr>
<td>CD90 (+)</td>
<td>95.6%±0.9%</td>
<td>95.2%±1.1%</td>
<td>50.5%±2.3% (-)</td>
</tr>
<tr>
<td>CD105 (+)</td>
<td>98.0%±1.2%</td>
<td>98.8%±1.0%</td>
<td>95.4%±0.5%</td>
</tr>
<tr>
<td>CD45 (-)</td>
<td>0.9%±0.1%</td>
<td>0.7%±0.2%</td>
<td>20.8%±2.0% (+)</td>
</tr>
<tr>
<td>CD34 (-)</td>
<td>1.1%±0.4%</td>
<td>1.5%±0.4%</td>
<td>5.5%±3.4% (+)</td>
</tr>
<tr>
<td>CD19 (-)</td>
<td>0.8%±0.1%</td>
<td>1.0%±0.2%</td>
<td>12.7%±2.7% (+)</td>
</tr>
</tbody>
</table>

+ Positive expression  
- Negative expression or extremely low expression

### 3.3.3 Differentiation of hMSCs post detachment from thermoresponsive films

To define hMSCs it is necessary to be able to differentiate into osteoblasts, adipocytes and chondroblasts under standard in vitro differentiating conditions. Therefore, after the hMSCs were reseeded and detached through 3 passages, the cells were harvested and treated by one of three differentiation mediums to assess the cell differentiation potential.

1) hMSCs osteogenic differentiation after temperature detachment

The harvested hMSCs were seeded with a density of \(2 \times 10^4\) cells/cm\(^2\) into 24-well-plate as follows. 8 wells were seeded with cells detached from thermoresponsive surfaces via temperature control, (4 of these to be used as control wells for temperature detached cells, 4 for testing for induced differentiation in temperature detached cells), and another 8 wells were seeded with cells trypsinised, conventionally from TCP, (4 of
these were used as control wells for conventionally detached cells, 4 for testing for induced differentiation in conventionally detached cells). Cells were seeded at a density of 4x10^4 cells/well and incubated as normal. After 48 hours cells should have adhered to the plastic of the plate and appear confluent, the medium was changed using 1 ml complete hMSC medium in the control wells and 1 ml of osteogenic medium in the test wells. Medium was changed twice a week for 10 to 17 days, depending on the condition of the monolayer. Cells needed to be harvested before they intended to peel. 1 well from each group was stained by Alizarin red. The remaining 3 wells of each were used for calcium quantitation.

As can be seen in Figure 3-4, in both the film and trypsin detached test groups, a significant amount of Alizarin red stain can be observed which is indicative of calcium deposits associated with osteogenic differentiation. In contrast, in both control groups there was barely any staining observed. These results indicate that the hMSC cells were able to differentiate into osteoblasts after 3 repetitive thermal-mediated cell attachment-detachment cycles using thermoresponsive films. Calcium quantification confirmed hMSCs osteogenic differentiation with both test groups exhibiting 7 times more calcium than the control groups. The cells detached from thermoresponsive films produced slightly more calcium in both the control and the test groups compared to their trypsin detached counterparts (Figure 3-5).
Figure 3-4 hMSCs stained with Alizarin red used as an indicator of calcium deposition after 3 cycles of temperature mediated cell growth and detachment. (A. Control group post cyclic detachment from thermoresponsive substrates; B. Osteogenic differentiation post cyclic detachment from thermoresponsive substrates; C. Control group post conventional detachment; D. Osteogenic differentiation post conventional detachment. Scale bar: 500µm)
Chapter 3

Figure 3-5 Statistics results of calcium deposition from osteogenic differentiated hMSCs after 3 cycles of repetitive temperature detachment. Control: control hMSCs detached from thermoresponsive film; Control_T: control hMSCs detached by trypsin; Osteo: osteogenic differentiated hMSCs detached from thermoresponsive film; Osteo_T: osteogenic differentiated hMSCs detached by trypsin. (n=3)
Chapter 3

2) hMSCs adipogenic differentiation after temperature detachment

The harvested hMSCs were seeded at a density of $2 \times 10^4$ cells/cm$^2$ into a 24-well-plate. 8 wells were seeded with cells detached from thermoresponsive surfaces via temperature control, (4 of these to be used as control wells for temperature detached cells, and 4 for testing for induced differentiation in temperature detached cells), and another 8 wells were seeded with cells trypsinized conventionally from TCP, (4 of these were used as control wells for conventionally detached cells, and others 4 for testing for induced differentiation in conventionally detached cells). After the cells adhered to the plastic of the plate and appeared confluent, 1ml/well of the adipogenic induction medium was added to the test wells and left for 3 days, control wells receive normal growth medium. After 3 days, the medium in the test wells was changed and 1ml/well of maintenance medium was added and left under normal incubation conditions for 1 day. The induction and maintenance cycle was repeated 3 times before staining the cells with Oil Red O.

The lipid staining was not observed in either control groups whereas clear Oil Red O lipid staining was observed within both test groups indicating adipogenic differentiation (Figure 3-6). After imaging the Oil Red O was extracted by pipetting 99% Isopropanol over the surface of the well several times. The extractions were then measured on a plate reader at 520nm. Significantly higher readings were found in the test groups, most notably in the case of cells repetitively regenerated from thermoresponsive surfaces. No significant difference was detected between the control groups (Figure 3-7). The statistical results also confirmed that the hMSCs adipogenic differentiation potential was maintained after consequential thermoresponsive film cultivations.
Figure 3-6 hMSCs adipogenic differentiation after 3 cycles of repetitive temperature detachment. (A. control of thermoresponsive film detached cells; B. adipogenic differentiation after thermoresponsive film detached cells; C. control of trypsin detached cells; D. adipogenic differentiation after trypsin detached cells. Scale bar: 200µm)
Figure 3-7 Statistical results of the extracted Oil Red O stained lipid from adipogenic differentiated hMSCs after 3 cycles of repetitive temperature detachment. Ctrl_F: control hMSCs detached from thermoresponsive film; Ctrl_T: control hMSCs detached by trypsin; Adipo_F: adipogenic differentiated hMSCs detached from thermoresponsive film; Adipo_T: adipogenic differentiated hMSCs detached by trypsin. (n=3)
3) hMSCs chondrogenic differentiation after temperature detachment

The harvested hMSCs were seeded with a density of $1 \times 10^5$ cells/well into U-bottom 96-well-plate using hMSC growth medium. After 48h, hMSC spheroids spontaneously formed. 8 wells were seeded with cells detached from thermoresponsive surfaces via temperature control, (4 of these to be used as control wells for temperature detached cells, and 4 for testing for induced differentiation in temperature detached cells), and another 8 wells were seeded with cells trypsinized conventionally from TCP, (4 of these were be used as control wells for conventionally detached cells, and 4 for testing for induced differentiation in conventionally detached cells). The control groups were treated with incomplete chondrogenic medium (ICM) and the test groups were treated with complete chondrogenic medium (CCM). The medium was changed every other day for 21 days. The spheroids were then harvested for s-GAG measurement and the results were normalized between spheroid DNA content and expressed levels of s-GAG. The results indicate that there was no s-GAG expressed in either hMSC control groups. The thermally detached chondrogenic differentiated hMSCs had more s-GAG per µg DNA than that of the trypsin detached hMSCs. The statistical results showed that hMSCs which were repetitively cultured and detached on and from thermoresponsive surfaces were capable of induced differentiation into chondroblasts, even more so than their trypsinized counterparts (Figure 3-8).
Statistical analysis of hMSCs chondrogenesis differentiation after 3 cycles of repetitive temperature detachment. The level of chondrogenesis is assessed by measuring s-GAG and normalizing between pellets by measuring the DNA content and expressed levels of s-GAG per µg DNA. Control: control hMSCs detached from thermoresponsive films; Control_T: control hMSCs detached by trypsin; Chondro: chondrogenic differentiated hMSCs detached from thermoresponsive films; Chondro_T: chondrogenic differentiated hMSCs detached by trypsin. (n=3)


3.4 Conclusion

The most commonly used cell medium supplement FBS isolated from cow fetal blood, is highly variable, consisting of ill-defined bio-components leading to batch-to-batch inconsistencies, similar to the disassociation enzyme trypsin. Due to being isolated from animal sources its inclusion therefore introduces contamination risks such as inadvertent exposure to adventitious pathogens, which, from a clinical application viewpoint, is a risk that should be minimized or eradicated. Therefore, hMSCs were also seeded on 1µm pNIPAm films without supplementary serum. Cells attached to the surfaces successfully but the proliferation was severely impeded in the absence of serum compared to proliferation in the presence of serum, and the cells were easily detached upon temperature reduction in a matter of a few minutes. It is necessary to expand the usage of thermoresponsive systems for multiple passage expansion in reproducible cell culture conditions with a view to establishing a method which would eliminate the use of any animal derived products (serum, trypsin). But a chemically defined medium capable of supporting hMSC adhesion and proliferation has yet to satisfactorily developed and used in routine cell culture.

Another important concern is that the unimmobilized polymer dissolves upon temperature reduction and is released into the cell culture medium. The toxicity of pNIPAm has been investigated in mice with no detectable toxicity after 28 days using a relatively high concentration of 2000mg/kg, also no pNIPAm cytotoxicity was detected under cell culture conditions studied by Malonne et al. and Takezawa et al. (Malonne et al. 2005; Takezawa et al. 1990). While in this study the dissolved polymer did not seem to have any deleterious effect on the cells collected, it is best to remove as much polymer as possible via media exchange or/and centrifugation before reseeding or further experimentation.

Solvent cast films prepared from commercially sourced pNIPAm offer a convenient, simple and cheap alternative to producing thermoresponsive surfaces with a view to
cell and cell sheet regeneration. This study found that the optimal thickness of such a film is 1µm. As the thickness of the solvent cast films increased the surfaces became less bioadhesive and the numbers of cells, which attached and consequently proliferated declined. hMSC cell sheet detachment was achieved through simple temperature control. hMSC immunophenotypic surface profile FACS analysis indicated that thermoresponsive surfaces do not induce hMSC differentiation and therefore this protocol offers a gentle and non-destructive approach for cell detachment therapies where the collection of undifferentiated MSCs is desirable. Most importantly, after repetitive attachment and detachment from thermoresponsive films, the hMSCs were able to differentiate into osteoblasts, adipocytes and chondroblasts, all differentiation formations are better than that from trypsin detached hMSCs. Finally, this simple method of surface preparation allows for the preparation of substrates of differing geometries and sizes offering a greater flexibility in experimental design.
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Chapter 4. Macrophages behaviour on different NIPAm based thermoresponsive substrates

Sections of this chapter have been published:


Some parts were modified for thesis writing.
Chapter 4

4.1 Introduction

Thermoresponsive materials and surfaces are powerful tools for creating tissue-like constructs that imitate native tissue geometry and mimic its spatial cellular organization (da Silva et al. 2007). A lot of attention is focussed on the rational design of biomaterials for cell culture and for further utilization in tissue reconstructions (Hatakeyama H. 2006). Interest in thermoresponsive polymers has steadily grown over the decades, and a great deal of work has been dedicated to developing temperature sensitive macromolecules that can be crafted into new smart materials (Roy et al. 2013). A recently emerging area is the development of thermoresponsive polymers to cultivate and harvest cells without cross contamination from animal sources. For instance, previous studies using solvent cast films prepared from commercially sourced pNIPA with an optimal film thickness of 1 µm offer a convenient, simple and cheap alternative for producing thermoresponsive surfaces with a view to cell and cell sheet regeneration. hMSCs proliferation was similar in all cases and the pluripotent nature of the hMSCs was preserved through multi-fold passaging on the film (M. E. Nash et al. 2013a). Another example is the use of thermoresponsive nanocomposite gels to proliferate and harvest hMSCs, which should be effectively cultured in vitro for tissue engineering and regenerative medicine (Kotobuki et al. 2013). There are some thermoresponsive products for cell culture available on the commercial market. An example is Nunc UpCell Surface, which supports non-enzymatic harvesting of adherent cells for preservation of cell viability and surface proteins; however, the optimal thermoresponsive coating is still under development.

Some research has been conducted on cell-thermoresponsive material interactions, where cells exhibited different adhesion and proliferation behaviour depending on the kind of thermoresponsive polymers due to their individual cell adhesion behaviours (H. Takahashi et al. 2012). One important and yet outstanding question to be answered is: do thermoresponsive materials change the adhered cell’s phenotype? In order to answer
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this question all parameters related to thermoresponsive films should undergo comparative analysis. It is not only chemical composition which can affect cell behaviour, but also film thickness with poor cell adhesion on thicker films (Moran et al. 2007a). Complete deciphering of the cell-material communication code is still a long way off (Kobayashi J. 2010; M. E. Nash et al. 2011b; Ventre et al. 2012; Yamada et al. 1990). With this in mind, this study is to compare cell behaviour on thermoresponsive films deposited using two film deposition methods (spin coating and solvent casting). Furthermore, two types of thermoresponsive polymers were comparatively assessed in terms of influence on cell behaviour; pNIPAm and a more hydrophobic copolymer analogue NIPAm-co-NtBAm. The NIPAm-co-NtBAm copolymer was selected because it showed better cell compatibility and it has a lower critical solution temperature (LCST), lower than room temperature, which makes this biomaterial much easier for routine lab usage (Rochev et al. 2004).

For biological evaluation, the macrophage-like transformed murine cell line RAW264.7 was selected as it is a widely used standard model for modern immune activation analysis. Macrophages orchestrate the immune response to an implanted biomaterial and play a key role in determining the biocompatible outcomes (Anderson and McNally 2011; Gordon and Taylor 2005; Lamers et al. 2012; Sharma et al. 2010). It is also well known that RAW264.7 cells are difficult to remove from certain growth substrates, so thermoresponsive materials are a useful utility. Macrophage adhesion to plasma processed surfaces is distinct from that exhibited by fibroblasts as they tend to establish stronger cell-surface contacts (Godek et al. 2006). Incubating cells with too high a trypsin concentration for too long a time period may damage cell membranes and kill the cells. Alternatives have been utilized such as scraping. However, scraping can dramatically reduce the total viability of any cell type and it is not certain if the dead cells might also release intracellular contents that might affect the viable cells (Batista et al. 2010).
Based on these considerations, the main goals for this study are 1) to define the macrophage cell activation level and receptor profile based on different compositions and thicknesses of polymer films and 2) to set up a practically acceptable and simple thermoresponsive substrate for routine lab work. RAW264.7 cells were seeded onto both pNIPAm and NIPAm-co-NtBAm thin films ranging from 100nm to 4 µm in thickness, which were fabricated either by spin coating and solvent casting. The in-vitro response of RAW264.7 cells to these ultrathin and thin films with regards to adhesion, morphology changes, cytokine secretion and cell surface molecule expression were studied.

4.2 Methodology

4.2.1 Materials

The macrophage-like transformed murine cell line RAW264.7 was kindly provided by the Regenerative Medicine Institute (REMedi) in NUI Galway. CellTrace™ CFSE Cell Proliferation Kit was purchased from invitrogen™, Mouse IL-β ELISA Ready-SET-Go assay was purchased from eBioscience®. Anti-mouse CD80, Anti-mouse MHCII, Armenian Hamster IgG Isotype Ctrl, κ Isotype Ctrl were from Biolegend™. Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich.

4.2.2 Film fabrication and film characterization

The following films were fabricated for this study: solvent cast pNIPAm (1µm, 2µm, 4µm); spin coated pNIPAm (100nm, 1µm); solvent cast NIPAm-co-NtBAm (1µm, 2µm, 4µm); spin coated NIPAm-co-NtBAm (100nm, 1µm).

FTIR was used to ascertain the films’ chemical composition using a Shimadzu FTIR-8300 spectrometer in absorbance mode. AFM was used to measure film roughness and thickness. AFM images were obtained in tapping mode in air using a Dimension 3100 AFM (Digital Instruments, Santa Barbara, CA, USA) and Veeco 1-10 Ohm-cm phosphorus (n) doped Si tips. A matrix of 512×512 data points along the x-y plane.
were analyzed in a single scan. The roughness assessment of the deposited films was taken by 10µm×10µm scanning. The roughness of the films was reported as root-mean-square (rms) roughness values, where rms denotes the standard deviation of the Z-values along the reference line. For thickness measurement, a sharp blade was used to scratch the surface of the fully dried polymer film fabricated as described above, from the underlying substrate. 100µm×100µm scanning images were recorded at a scan rate of 1 Hz in the area around the scratch. Contact angle measurements were made in order to access the hydrophobicity of the films. Advancing contact angle measurements were taken on a home-built goniometer as previously described (Gilcreest et al. 2004).

4.2.3 Cell attachment and detachment

RAW264.7 cells were maintained in DMEM, supplemented with 10% FBS and 1% penicillin-streptomycin antibiotics. Cells from passage 10 to passage 25 were used for experiments and assays where appropriate.

Briefly, RAW264.7 cells were seeded in triplicate at a density of 40,000 cell/cm² on the pNIPAm and NIPAm-co-NtBAm films. Blank TCP was used as negative control, while LPS (200ng/ml) was used as positive control. Cells were incubated for 48h in a humidified atmosphere of 95% air and 5% CO₂ at 37 ºC thus maintaining a working temperature above the polymers LCST. Care was taken to make sure that the sample temperature was maintained above the LCST during any operation to prevent premature cell detachment.

Cell adhesion on the thermoresponsive films was microscopically observed using an Olympus BX51 phase contrast microscope with Image Pro-Plus software. Total DNA content of the cells attached on each kind of film and controls was quantified using the Quan-iT PicoGreen dsDNA assay kit. Cell detachment was observed using a Time Lapse Olympus IX81 motorised inverted microscope with a temperature/CO₂ humidified incubation chamber for live cell experiments (Anatomy Department in NUI Galway). For initial cell detachment, the original warm medium was removed followed
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by the addition of cold medium (20ºC for pNIPAm films, 4ºC for NIPAm-co-NtBAm films) and the temperature was maintained at these sub-LCST temperatures for the duration of observations. The samples were then placed under the time lapse microscope immediately for imaging with 20 sec per photo.

4.2.4 Cell proliferation and flow cytometry analysis

For cell proliferation assessment, Carboxyfluorescein succinimidyl ester (CFSE) staining was performed 24h after cell seeding. This ensures that the cells on all substrates had enough time to adhere and proliferate. The seeding density for CFSE staining was reduced to 20,000 cells/cm² in order to prevent cell over growth. The cells were incubated for a further 48h after staining. Flow cytometry was performed using BD FACSCanto to test the cell proliferation, cells were gated according to their physical characteristics by Side Scatter (SSC) versus Forward Scatter (FSC). For flow cytometry analysis, cells were detached from the thermoresponsive films as described, followed by gentle and repetitive pipetting to yield singular cells. Cells were detached from TCP controls via conventional trypinization and scraping. At least 10,000 events were detected for each experiment; the geometric mean values of FITC signal strength summation were then calculated for each sample.

4.2.5 Interleukin 1β assessment

Interleukin 1β (IL-1β) release was assessed by the ELISA assay. The cell culture medium was harvested for testing after 48 hours of incubation and the standard IL-1β was diluted 2 fold with a starting point of 1,000pg/ml. The experimental procedure followed was the standard protocol provided by eBioscience®.

4.2.6 Immunofluorescence staining

Cell surface proteins were also stained and examined by flow cytometry. The cells were stained with CD80 (APC labelled) and MHC II (FITC labelled) antibodies from Bioscience as per the manufacturer’s instructions and were analyzed by the flow
cytometry using BD FACSCanto, cell analysis was standardized for side/forward scatter and fluorescence. At least 10,000 events were detected for each experiment; the results were analyzed by FlowJo™ software. All experiments were performed in triplicate.

4.3 Results and discussion

4.3.1 Physical characterisation

FTIR confirmed the deposition of the thermoresponsive films. The FTIR spectrum results are shown in Figure 4-1, Figure 4-2 and Figure 4-3. Typical fingerprints of pNIPAm and NIPAm-co-NtBAm were detected. From the FTIR spectra, a C-N bending peak can be observed at around 1150 cm\(^{-1}\), a C-H symmetric stretching peak at around 1180 cm\(^{-1}\), and a C-H asymmetric stretching vibration at about 1450 cm\(^{-1}\). A N-H bending peak can be observed at approximately 1500 cm\(^{-1}\). A C=O stretching peak is observed at approximately 1640 cm\(^{-1}\). The characteristic C-H stretching vibrates at around 3000 cm\(^{-1}\). A N-H stretching vibration can be observed at approximately 3300 cm\(^{-1}\). The FTIR spectra of the pNIPAm film is very similar to that of the NIPAm-co-NtBAm film, as there is only one sub group difference between the NIPAm monomer and NtBAm monomer. The 1µm thick spin coated and solvent cast film spectrums were similar to each other, therefore, only the 1µm solvent cast film’s spectrum is shown to ensure clarity. The 2µm thick film’s spectrums were the same as the 4µm’s and powdered samples (pure pNIPAm powder/crystals of NIPAm-co-NtBAm), only the 4µm is shown for clarity.
Figure 4-1 FTIR spectrum of pNIPAm powder and films. The assignment of peaks confirms successful pNIPAm film deposition. (pNIPAm powder: dark green; 100nm spin coated pNIPAm film: green; 1µm thick solvent cast pNIPAm film: grey; 4µm thick solvent cast pNIPAm film: purple)

Figure 4-2 FTIR spectrum of NIPAm-co-NtBAm powder and films. The assignment of peaks confirms successful NIPAm-co-NtBAm film deposition. (NIPAm-co-NtBAm crystals: green; 100nm spin coated NIPAm-co-NtBAm film: blue; 1µm thick solvent cast NIPAm-co-NtBAm film: grey; 4µm thick solvent cast NIPAm-co-NtBAm film: brown)
Figure 4-3 FTIR spectrum of pNIPAm and NIPAm-co-NtBAm powders. pNIPAm: green; NIPAm-co-NtBAm: blue.

The thicknesses of all the fabricated films were obtained using AFM by measuring the z height distance between the TCP substrate which was exposed by a surgical blade and the polymer layer which remained on the substrate. The results showed good agreement with the expected theoretical thickness values (Table 4-1). The film roughness was also obtained using the AFM, and the results are shown in Table 4-2.
Table 4-1 Theoretically predicted film thickness versus thickness measured by AFM analysis.

<table>
<thead>
<tr>
<th>Film Type</th>
<th>Expected Thickness</th>
<th>Measured Film thickness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spin coating</td>
<td></td>
</tr>
<tr>
<td>pNIPAm</td>
<td>100 nm</td>
<td>101.1 nm±0.5</td>
</tr>
<tr>
<td></td>
<td>1 µm</td>
<td>1.05 µm±0.13</td>
</tr>
<tr>
<td></td>
<td>1 µm</td>
<td>1.00 µm±0.07</td>
</tr>
<tr>
<td></td>
<td>Solvent casting</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 µm</td>
<td>2.04 µm±0.03</td>
</tr>
<tr>
<td></td>
<td>4 µm</td>
<td>4.02 µm±0.04</td>
</tr>
<tr>
<td>NIPAm-co-NtBAm</td>
<td>100 nm</td>
<td>109.1 nm±0.9</td>
</tr>
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<td>1 µm</td>
<td>1.10 µm±0.18</td>
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<tr>
<td></td>
<td>1 µm</td>
<td>1.03 µm±0.05</td>
</tr>
<tr>
<td></td>
<td>Solvent casting</td>
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<td></td>
<td>2 µm</td>
<td>2.05 µm±0.04</td>
</tr>
<tr>
<td></td>
<td>4 µm</td>
<td>4.03 µm±0.03</td>
</tr>
</tbody>
</table>

Table 4-2 Film RMS roughness as measured by AFM analysis, where RMS refers to the standard deviation of the Z-values along the reference line.

<table>
<thead>
<tr>
<th>Film Type</th>
<th>Film Thickness</th>
<th>RMS Roughness (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spin coating</td>
<td></td>
</tr>
<tr>
<td>pNIPAm</td>
<td>100 nm</td>
<td>13.5±0.4</td>
</tr>
<tr>
<td></td>
<td>1 µm</td>
<td>12.8±0.7</td>
</tr>
<tr>
<td></td>
<td>1 µm</td>
<td>16.0±0.9</td>
</tr>
<tr>
<td></td>
<td>Solvent casting</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 µm</td>
<td>24.0±4.9</td>
</tr>
<tr>
<td></td>
<td>4 µm</td>
<td>28.8±1.5</td>
</tr>
<tr>
<td>NIPAm-co-NtBAm</td>
<td>100 nm</td>
<td>12.1±0.8</td>
</tr>
<tr>
<td></td>
<td>1 µm</td>
<td>16.4±1.1</td>
</tr>
<tr>
<td></td>
<td>1 µm</td>
<td>13.4±2.5</td>
</tr>
<tr>
<td></td>
<td>Solvent casting</td>
<td></td>
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<tr>
<td></td>
<td>2 µm</td>
<td>15.0±1.7</td>
</tr>
<tr>
<td></td>
<td>4 µm</td>
<td>18.2±1.4</td>
</tr>
</tbody>
</table>
The relative hydrophilicity/hydrophobicity of the polymer films was measured by advancing contact angle above the thermoresponsive polymers’ LCST. Contact angle results indicated that the 1µm solvent cast pNIPAm films were slightly more hydrophobic than their spin coated analogues (60º to 50º, respectively). NIPAm-co-NtBAm films were significantly more hydrophobic than pNIPAm films (~80º) (Table 4-3).

In the previous study of cell adhesion and proliferation we found that copolymerization of NIPAm with more hydrophobic monomer NtBAm produces polymers which are able to support cell adhesion and proliferation better than the NIPAm homopolymer (Rochev et al. 2001; Rochev et al. 2004). The same tendency is observed in the present work, as it will be shown later.

Table 4-3 Advancing contact angle results obtained by advancing drop method on a home-built contact angle measurement system.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Stick angle(º)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (TCP)</td>
<td>64.7±0.5</td>
</tr>
<tr>
<td>pNIPAm</td>
<td></td>
</tr>
<tr>
<td>Spin coating</td>
<td></td>
</tr>
<tr>
<td>100nm</td>
<td>51.2±1.0</td>
</tr>
<tr>
<td>1µm</td>
<td>55.3±1.6</td>
</tr>
<tr>
<td>Solvent casting</td>
<td></td>
</tr>
<tr>
<td>1µm</td>
<td>67.6±2.0</td>
</tr>
<tr>
<td>2µm</td>
<td>71.6±1.1</td>
</tr>
<tr>
<td>4µm</td>
<td>57.3±1.4</td>
</tr>
<tr>
<td>NIPAm-co-NtBAm</td>
<td></td>
</tr>
<tr>
<td>Spin coating</td>
<td></td>
</tr>
<tr>
<td>100nm</td>
<td>80.2±0.9</td>
</tr>
<tr>
<td>1µm</td>
<td>80.4±0.6</td>
</tr>
<tr>
<td>Solvent casting</td>
<td></td>
</tr>
<tr>
<td>1µm</td>
<td>81.3±0.8</td>
</tr>
<tr>
<td>2µm</td>
<td>82.4±1.8</td>
</tr>
<tr>
<td>4µm</td>
<td>84.6±1.2</td>
</tr>
</tbody>
</table>
4.3.2 Cell adhesion on thermoresponsive films

RAW264.7 cells were seeded on the prepared pNIPAm and NIPAm-co-NtBAm films and observed microscopically for cell adhesion after 48h of incubation. Cell adhesion was also checked both with and without serum on the TCP controls. Under normal cell culture conditions, it takes approximately 6 hours of incubation for RAW264.7 cells to adhere to a substrate, but the adhesion process is also affected by the type of substrate. The cells attached on all NIPAm-co-NtBAm copolymer films fabricated by both spin coating and solvent casting in 6 hours. However, only solvent cast pNIPAm films are able to support the cell attachment. The attached cells elongated and spread on the substrate surface similar to TCP control. On the spin coated pNIPAm films, there were few RAW264.7 cells attached onto the film surface with the majority still floating in the medium, and the adhered cells also took 2-3 hours more than usual to attach (Figure 4-4). As the thickness of the solvent cast pNIPAm films increased, fewer cells were found on the thermoresponsive film, while the adhesion time also increased. The PicoGreen results quantitatively confirmed the microscopy results. As the solvent cast pNIPAm film thickness increased, less DNA content was detected, which confirmed that fewer cells adhered on the thicker solvent cast pNIPAm films (Figure 4-5). The RAW264.7 cells showed the same tendency as hMSCs on solvent cast pNIPAm films which was described in Chapter 3.
Figure 4-4 RAW264.7 cell adhesion on the different types of thermoresponsive films. Cells attached successfully on the solvent cast pNIPAm films as well as on both the spin coated and solvent cast NIPAm-co-NtBAm films, where the cell morphology is the same as the positive control. Cells seeded without serum showed unhealthy sign; the majority of cells seeded on the spin coated pNIPAm film were rounded up and clamped together. (A. Bare TCP without serum; B. RAW264.7 in full growth medium on TCP; C. RAW264.7 seeded on a 100nm thick spin coated pNIPAm film; D.
RAW264.7 adhesion on a 1µm thick spin coated pNIPAm film; E. RAW264.7 adhesion on a 1µm thick solvent cast pNIPAm film; F. RAW264.7 adhesion on a 2µm thick solvent cast pNIPAm film; G. RAW264.7 adhesion on a 4µm thick solvent cast pNIPAm film; H. RAW264.7 adhesion on a 100nm thick spin coated NIPAm-co-NtBAm film; I. RAW264.7 proliferation on a 1µm thick spin coated NIPAm-co-NtBAm film; J. RAW264.7 adhesion on a 1µm thick solvent casted NIPAm-co-NtBAm film; K. RAW264.7 adhesion on a 1µm thick solvent casted NIPAm-co-NtBAm film; L. RAW264.7 adhesion on a 1µm thick solvent casted NIPAm-co-NtBAm film. Scale bar: 200µm)

Figure 4-5 The total DNA content of RAW264.7 cells attached on each kind of film and control was quantified using the Quan-iT PicoGreen dsDNA assay after 48h incubation. A. PicoGreen results confirmed what was observed microscopically with only 20% DNA content left on the spin coated pNIPAm films compared with ‘gold standard’ TCP controls assumed to be 100% ideal cell culture surfaces. Cell attachment on the solvent casted pNIPAm films decreased as the film thickness increased with a deduction rate of around 10%. B. DNA content on both the spin coated and solvent casted NIPAm-co-NtBAm copolymer was similar to that on the TCP controls. *: significantly lower DNA content.
4.3.3 RAW264.7 detachment by temperature control

RAW264.7 cells were seeded on the prepared pNIPAm and NIPAm-co-NtBAm films and they were observed microscopically for cell detachment by simply reducing the temperature below the polymers’ LCST. RAW264.7 cells started detaching immediately after the temperature dropped below the LCST. The cell morphology of the adhered cells changed dramatically within 5 min of the environmental temperature being dropped. The average attached cell length shrank from over 80 µm to less than 30µm in 10min after the temperature dropped, but the rounded up cells remained stuck to the underlayer TCP substrate and they migrated in order to reattach under room temperature (Figure 4-6). Generally, completely cell detachment was achieved with in 30 mins of cold treatment.

![Figure 4-6](image)

Figure 4-6 Macrophage cell detachment from a 1 µm solvent cast pNIPAm film as viewed under a time lapse microscope. Cell morphology started to change within 1min
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after temperature reduction. The average attached cell length shrank from over 80 µm to less than 30µm due to the dessolving of thermoresponsive film. Cells tended to stick and migrate to reattach to the underlayer TCP substrate afterwards with quick morphological movement, the cell length increased as the cell reattached to the TCP substrate. (Magnification: 400X)

4.3.4 Cell proliferation on thermoresponsive films

Table 4-4 RAW264.7 proliferation on different thermoresponsive films. The signal strength was obtained as $\sum$ (FITC-A): Geom. Mean values.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Average Signal strength</th>
<th>$G_0$</th>
<th>Doubling time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>$G_0$ 48h</td>
<td>45771±113</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6386±143</td>
<td>2.84±0.04</td>
<td>16.9±0.3</td>
</tr>
<tr>
<td>pNIPAm</td>
<td>Spin coating 100nm (48h)</td>
<td>12674±845</td>
<td>1.85±0.45</td>
</tr>
<tr>
<td></td>
<td>Spin coating 1µm (48h)</td>
<td>10150±210</td>
<td>2.17±0.03</td>
</tr>
<tr>
<td></td>
<td>Solvent casting 1µm (48h)</td>
<td>5239±199</td>
<td>3.12±0.06</td>
</tr>
<tr>
<td>NIPAm-co-NtBAm</td>
<td>Spin coating 100nm (48h)</td>
<td>6944±134</td>
<td>2.72±0.03</td>
</tr>
<tr>
<td></td>
<td>Spin coating 1µm (48h)</td>
<td>6224±248</td>
<td>2.87±0.06</td>
</tr>
<tr>
<td></td>
<td>Solvent casting 1µm (48h)</td>
<td>5221±173</td>
<td>3.13±0.05</td>
</tr>
</tbody>
</table>

$G_0$: control cells which were analyzed immediately to define the starting density
$G_0$: the samples and control proliferation times after 48 hours incubation

CFSE stain enters the cytoplasm of cells and is converted to a fluorescent ester by removal of its acetate groups by intracellular esterases. CFSE is then retained within cells and covalently couples via its succinimidyl group, to intracellular molecules (Parish 1999). The covalently coupled fluorescent CFSE will stay inside cells for extremely long periods and cannot be transferred to adjacent cells. With the
proliferation of individual cells, the fluorescent molecules will be divided evenly to each subsequent generation, and the signal will become weaker and weaker as the cells passage down. Cells were treated with CFSE 24h after cell seeding and incubated for a further 48h before flow cytometry assessment. The statistics in Table 4-4 shows that cells on the spin coated pNIPAm films proliferate less than the control cells. Cells on the solvent cast polymers proliferated similar to the control in both cases, but the proliferation on the spin coated pNIPAm was dramatically reduced compared to spin coated NIPAm-co-NtBAm films and the controls. Cell proliferation on spin coated NIPAm-co-NtBAm is comparable to the controls. Cells on the NIPAm-co-NtBAm films and solvent cast pNIPAm films have uniform and similar peak readings as the controls, while cells seeded on the spin coated pNIPAm films have higher values showing that cell proliferation was prohibited by the under layer substrate (Figure 4-7).

Figure 4-7 Proliferation rate of RAW264.7 cells on different substrate films as measured by CFSE staining. Cells adhered to the spin coated pNIPAm films had higher fluorescence value, which means that the pNIPAm film fabricated by spin coating
prohibited RAW264.7 cell proliferation. However, cell proliferation on both the solvent cast pNIPAm and NIPAm-co-NtBAm films were similar or better than that of the control.  

(A. RAW264.7 CFSE staining measured on pNIPAm films; B. RAW264.7 CFSE staining measured on NIPAm-co-NtBAm films)

**4.3.5 Cellular immunological response to thermoresponsive films**

Interleukin-1 beta, is produced by activated macrophages as a proprotein, and it plays an important role in the body’s inflammatory response; it is also involved in cell proliferation, differentiation and apoptosis (Maruyama et al. 2005). RAW264.7 cells were seeded and kept for 48h. IL-1β ELISA Ready-SET-Go assay was used to assess the cytokine release. LPS (200ng/ml) was used to treat RAW264.7 as a positive control. Figure 4-8 shows the IL-1β cytokine release produced by RAW264.7 cells on each kind of sample. The RAW264.7 cells IL-1β release on spin coated pNIPAm films was significantly higher than that on any other type of films and negative control, the thicker the spin coated pNIPAm films, the more cytokines were detected. The cells adherence on the 100nm spin coated pNIPAm was not stable with very high standard deviation. Cells on the NIPAm-co-NtBAm films and solvent cast pNIPAm films showed low levels of IL-1β, but the overall cytokine release on the pNIPAm type of films was higher than that on the copolymer films. There was no significant difference between the release from spin coated and solvent cast NIPAm-co-NtBAm films.
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Figure 4-8 IL-1β production in response to seeding and incubation on different thermoresponsive films. RAW 264.7 cells (4×10^5 cells/ml) were seeded onto the indicated films for 48h. Blank tissue culture plastic dishes were taken as the negative controls. LPS (200ng/ml) was the positive control. Cells seeded on all types of films maintained silent status, cells attached on pNIPAm films secreted a higher level of IL-1β than that on the copolymer films. *: positive reaction.

By examining the cell surface molecules CD80 and MHC class II, we can determine how the cells incubated in the presence of thermoresponsive films, trypsinized or scraped can affect macrophage surface molecules. In general cell culture experiments, RAW cells are always detached with the help of a scraper due to the difficulty of detachment using trypsin-EDTA. CD80 is a protein found on activated monocytes that provides a co-stimulatory signal, which is necessary for T cell activation and survival.
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(Peach et al. 1995). The MHC II molecule is found on antigen-presenting cells and is also required for T cell activation.

It was necessary to analyse RAW264.7 cell surface protein expression under the same conditions for the different modes of detachment and the different polymer surfaces and controls to assess if the underlayer surfaces and detachment methods used induced negative effects on the detached cells. We assessed CD80 and MHC II expression of cells detached from thermoresponsive films, cells detached by trypsin and scraper using immunofluorescence and flow cytometry. To this end, APC-conjugated CD80 and FITC-conjugated MHC II monoclonal antibody were used to stain the cells. CD80 expressions on the cells detached by scraping present wide, multiple peaks, which indicate that this type of detachment could damage the cells. Cells detached from the NIPAm-co-NtBAm films expressed lower levels of CD80 compared to those detached from pNIPAm films. Spin coated films expressed more CD80 molecules than solvent cast films. Only 60% of attached cells were removed by trypsin after a 20 min of treatment, and the expression of CD80 on trypsinized cells was not detectable (Figure 4-9). The level of MHC II expression stays low in the presence of all types of polymers compared to control LPS stimulation. The co-incubation of RAW264.7 cells with all polymer films does not affect MHC expression (Figure 4-10). Overall, the activation of attached RAW264.7 cells on pNIPAm films was higher than that of cells detached from the TCP controls. The activation of cell surface MHC II molecule expression and IL-1β release on NIPAm-co-NtBAm films was relatively low. From the results, it was found that IL-1β release was negatively correlated to RAW264.7 cell proliferation. A decrease in proliferation and a higher level of IL-1 serve as the evidence of the final differentiation of RAW to macrophage, which means that pNIPAm leads to RAW264.7 cell differentiation.
Figure 4-9 Surface expression of CD80 on RAW264.7 cells. CD80 surface levels were assessed by direct immunofluorescence and FACS analysis directly after the cells were detached by trypsinization, scraping or temperature in the case of cells detached from thermoresponsive film. Lower levels of CD80 were found on the cell surfaces which were detached from the copolymer films, expression was not detectable on the cell surfaces of cells which were detached by trypsinization.
Figure 4-10 Surface expression of MHC II on RAW264.7 cells. MHC II surface levels were assessed by direct immunofluorescence and FACS analysis immediately after the cells were detached by trypsinization, scraping or temperature in the case of cells detached from thermoresponsive films. The expression was maintained at low level in all cases except the LPS control, and it was observed to be detachment method dependent.
Spin coating and solvent cast film fabrication techniques allowed for the preparation of smooth, thin and ultrathin thermoresponsive film coatings on the TCP substrates. Immunological responses to films were then conducted on the RAW264.7 cells which were seeded on the pNIPAm and NIPAm-co-NtBAm films. This study also initiates the development of non-invasive monocyte/macrophage cell detachment techniques and could have further tissue engineering applications. It also improves the current strategies to limit macrophage adhesion, fusion and fibrous capsule formation in the foreign body response which have focused on modulating material surface properties (S. Chen et al. 2010).

RAW264.7 cell adhesion and elongation was clearly observed on solvent cast pNIPAm (1, 2, 4µm) and NIPAm-co-NtBAm films (spin coating 100nm, 1µm; solvent cast 1, 2, 4µm), independent of the film hydrophobicity differences. However, the spin coated pNIPAm films (100nm, 1µm) failed to support RAW264.7 cell adhesion which indicates that the fabrication method affects macrophage adhesion. Our previous study has shown that other cell lines could attach to the spin coated films of pNIPAm confirming that attachment is cell line dependent (M. E. Nash et al. 2011b). Solvent cast films were able to support RAW264.7 cell adhesion and proliferation better than spin coated films, especially on thinner pNIPAm films. The cell proliferation curve measured by flow cytometry, showed a positive correlation between cell adhesion and proliferation on each kind of thermoresponsive film. RAW264.7 cells exhibited not only better cell adhesion, but also better cell proliferation and less IL-1β production on NIPAm-co-NtBAm copolymer films compared to that on pNIPAm films. This showed that the chemical composition of the films can impact monocyte/macrophage activation behaviour.

The results from the functional response assays of RAW264.7 cells in terms of their IL-1β secretion and cell surface molecule expression showed that RAW264.7 cells do respond to thermoresponsive substrates. IL-1β is a sensitive indicator of immune
stimulation that can help monitor the levels of cellular activation induced by different biomaterials (Ding et al. 2007). CD80 and MHC class II expression detection can be used to monitor the progress of an inflammatory response to an implanted material (Petillo et al. 1994). Higher IL-1β release was found on pNIPAm films compared to the TCP and other sample types. Cellular surface protein expressions can be modified by changing thermoresponsive film composition and fabrication method, which will make sure there is no further multiple-step activation to the thermoresponsive substrate. Detachment from the thermoresponsive polymer films also preserved the cellular surface molecules comparable to trypsin treatment. This gives the thermoresponsive polymer, especially NIPAm-co-NtBAm, a most promising advantage for macrophage culture and implant coating.
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4.4 Conclusion

In this study the consistent evaluation of polymer films with variable compositions and fabrication methods confirmed that NIPAm based polymers and copolymers provide a promising non-destructive alternative for RAW264.7 cell detachment. Film induced monocyte/macrophage activation can be controlled by modifying the fabrication method or polymer composition. It is widely known that cell functions such as adhesion, strength, spreading, intracellular signalling and differentiation potential are affected by the cellular response to the substrates (Kulangara and Leong 2009; Vogel and Sheetz 2006; Yim and Leong 2005). Improving the understanding of macrophage cell response to thermoresponsive films will provide new opportunities for tissue cultivation as well as for biocompatible coatings.

The chemical composition, fabrication method and thickness of thermoresponsive films have a major impact on monocytes/macrophages activation behaviour. Cell response to the films fabricated by spin coating and solvent casting showed a distinctive pattern with less activated on solvent cast films. While the cell adhered to pNIPAm, especially the spin coated one, exhibited activation effects. Variations in the preparation method and the chemical composition of the thermoresponsive film can significantly affect cell behaviour on thermoresponsive substrates. NIPAm based copolymer offers a gentle alternative for cell detachment. Successful cell attachment and detachment was achieved by simply controlling the environmental temperature, offering an alternative should trypsin not be capable. This study demonstrates that by modifying biomaterial thermoresponsive films, an easily produced thin film can be used for daily non-invasive macrophage culture as well as further thermoresponsive biomaterial applications.
Chapter 5

Chapter 5. Cell sheet regeneration from thermoresponsive films

Sections of this chapter were written based on manuscript submitted to *Advanced Materials*:

A. Satyam, P. Kumar, X. Fan, Y. Rochev, L. Joshi, H.P. Selgas, D. Lyden, B. Thomas, B. Rodriguez, M. Raghunath, A. Pandit and D. Zeugolis (2013) 'Macromolecular polydispersity modulates extracellular matrix synthesis and deposition in vitro - A paradigm shift in regenerative medicine'.
5.1 Introduction

Advancements in molecular and cell biology have led to the development of cell-based therapies to treat injured or degenerated tissues (Adler and Maddox 2007; Chidgey et al. 2008; Dove 2002; Lindvall et al. 2004). The rationale of this concept is that functional regeneration can be achieved best using the innate capacity of cells to create their own tissue-specific matrix avoiding the shortfalls of artificial devices. Direct cell injections offer little control over the local retention and distribution of the injected cells which leads to a scattered therapeutic efficiency, although this mode of administration has demonstrated very promising clinical outcomes (Matsuda et al. 2007; Speck et al. 1984; Tateishi-Yuyama et al. 2002; Thomas 1978; van Ramshorst et al. 2011). This deficiency has led to the development of living substitutes for skin and blood vessel composed of cells seeded on a collagen scaffold (Bell et al. 1979; Nanchahal et al. 1989). The scaffold delays tissue regeneration and function despite the possible efficacious results in preclinical models and clinical trials (Anderson et al. 2008; Broekhuizen et al. 2009; Nilsson et al. 2007; Tang and Eaton 1995; Wick et al. 2010). Therefore it is desirable to develop scaffold-free cell-sheet tissue engineering by cell self-assembly, in other words fabricate intact cell sheets with complete cell-cell connections and endogenously produced ECM (Peck et al. 2011; J. Yang et al. 2006). There have been a lot of positive outcomes in the preclinical and clinical area of tissue engineering such as skin (Green et al. 1979; Oconnor et al. 1981), blood vessels (Konig et al. 2009; L’Heureux et al. 2006; L’Heureux et al. 2007), cornea (Griffith et al. 1999; Nishida et al. 2004), heart (Masuda et al. 2008), lung (Nandkumar et al. 2002), liver (Ohashi et al. 2007) and bone (Pirraco et al. 2011) replacement, but only Epicel® (Genzyme, USA) for skin and LifeLine™ for blood vessel (Cytograft, USA) have been commercialised so far. The main limitation so far is the long period of time required for inducing and activating cell-based tissue regeneration. For instance, it requires 14-35 days for corneal epithelium (Nishida et al. 2004), 70 days for lung cell-sheet
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(Nandkumar et al. 2002), 196 days for blood vessel (L'Heureux et al. 2006) of ex vivo culture, which may lead to loss of native phenotype and function (Beltrami et al. 2012; Campisi and di Fagagna 2007).

In order to shorten the ex vivo culture time, a biophysical approach termed macromolecular crowding (MMC) was adopted. MMC increases thermodynamic activities and biological processes by several orders of magnitude (C. Chen et al. 2011; Z. Zhou et al. 2009), as a means to create ECM-rich tissue equivalents. The principle of MMC is derived from the notion that in vivo cells reside in a highly crowded/dense extracellular space and therefore the conversion of the de novo synthesised procollagen to collagen I is rapid (Canty and Kadler 2005). The synthesised procollagen is diluted in culture conditions, which naturally limits the conversion rate of procollagen to collagen (Figure 5-1A). Therefore, the additional inert polydispersed macromolecules were supplemented in the culture medium to facilitate the amplified production of ECM-rich living substitutes (Figure 5-1B).

Figure 5-1 MMC increases thermodynamic activities and biological processes by several orders of magnitude, as a means to create ECM-rich tissue equivalents.

In this body of work, Ficoll™70 & Ficoll™400 (FC), 500kDa dextran sulphate (DxS) and carrageenan (CR) were used to accelerate the ECM deposition in cell culture. According to the preliminary results of WS-1 cells, collagen type I and fibronectin
were highly expressed in the presence of MMC. Fibronectin was also expressed in a fibrillar pattern. The deposited matrix appears to be highly ordered. Therefore, we propose to attain living substitutes with tissue-specific protein composition and structure using custom-made thermoresponsive polymer films, which would open up new avenues in engineering cohesive tissue modules \textit{in vitro}.

\section*{5.2 Methodology}

\subsection*{5.2.1 Materials}

Primary human skin fibroblasts (WS-1; American Tissue Culture Collection), human lung fibroblasts (WI-38; American Tissue Culture Collection) and human corneal keratocytes (HCK; American Tissue Culture Collection) were kindly provided by the Network of Excellence for Functional Biomaterials (NFB), NUI Galway. Dulbecco’s modified Eagle medium nutrient mixture F-12 ham, Ficoll\textsuperscript{TM}70, Ficoll\textsuperscript{TM}400, 500kDa dextran sulphate (DxS) and carrageenan (CR) were purchased from Sigma-Aldrich, human serum (HS) was purchased from Lonza, UK.

\subsection*{5.2.2 Macromolecular crowding for ECM-rich cell assembled constructs production}

WS-1 and WI-38 were cultured in DMEM supplemented with 10\% FBS and 1\% penicillin-streptomycin at 37\(^\circ\)C in a humidified atmosphere of 5\% CO\(_2\). Cells were seeded at a density of 25,000 cells/cm\(^2\) on thermoresponsive coated TCP and were allowed to attach for 24 hours. After 24 hours the medium was exchanged for medium containing macromolecular crowders 75\(\mu\)g/ml CR with 0.5\% HS and re-incubated for 48 hours before further experiments.

HCK were cultured in Dulbecco’s modified Eagle medium nutrient mixture F-12 ham, supplemented with 10\% HS, 1\% penicillin-streptomycin and 1ng/ml of fibroblast growth factor 2 (FGF-2) at 37\(^\circ\)C in a humidified atmosphere of 5\% CO\(_2\). Cells were seeded at a density of 25,000 cells/cm\(^2\) in thermoresponsive coated TCP and were
allowed to proliferate until confluent. Subsequently, the medium was replaced by a medium containing macromolecular crowders (100µg/ml Dxs 500kDa; 37.5mg/ml Ficoll™ 70 and 25mg/ml Ficoll™ 400 (FC); and 75µg/ml CR (Sigma, Ireland)) with 0.5% HS for 48 hours prior to further experiments.

5.2.3 Characterisation of ECM-rich cell-assembled constructs

After MMC treatment, the ECM-rich cell assembled constructs were harvested by simply reducing the environmental temperature. Videos were captured during cell sheet detachment. The cell sheets were then fixed with 4% paraformaldehyde at room temperature for 15min. Subsequently, the cell-sheets were washed three times with PBS and serially dehydrated with 30%, 50%, 70%, 90% and 100% ethanol. The dehydrated cell-sheets were placed on adhesive carbon tabs mounted on SEM specimen stubs and then were dried. The specimens were subsequently scanned by AFM or coated with gold using an Emitech K550 coating system prior to SEM analysis. SEM images were obtained using a Hitachi S-4700 field emission microscope operating with a beam voltage of 15KV.
5.3 Results and discussion

5.3.1 Production of ECM-rich WS-1 cell-assembled constructs

Culture of WS-1 fibroblasts with 0.5% FBS and HS with variable dispersity of macromolecules and subsequent analysis validated that maximum ECM deposition can be achieved by the most polydispersed macromolecule, identifying CR as the most suitable molecule for the cells. The abundance of ECM deposition in the presence of 0.5% HS and 75µg/ml CR, WS-1 was seeded both on spin coated 100nm pNIPAm films and on NIPA-co-NtBAm films. pNIPAm films were not suitable for the attachment of an intact WS-1 cell sheets, even after 2 days in culture, the WS-1 morphology was not as healthy as the control and the cells were clumped together. The more hydrophobic NIPAm-co-NtBAm film facilitated attachment and detachment every time either with or without MMC treatment. The detachment of intact ECM-rich WS-1 fibroblast sheets was carried out after 2 days in the presence of 0.5% HS and 75µg/ml CR, the sheet was able to detach simply by lowering down the ambient temperature below 10°C (Figure 5-2).

Time-lapse microscopy was used to calculate the WS-1 sheet detachment rate both in the presence of MMC and absence of MMC. The detachment rate of WS-1 fibroblast sheets under MMC conditions was slower than that of their non-MMC counterparts; this was expected due to the abundance of deposited ECM (Figure 5-3).
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Figure 5-2 WS-1 cell sheet formation on thermoresponsive films. pNIPAm films were not suitable for the attachment of intact ECM-rich WS-1 cell sheets, even after 2 days in culture. NIPAm-co-NtBAm films facilitated attachment and detachment of intact ECM-rich WS-1 fibroblast sheets after 2 days in the presence of 0.5% HS and 75µg/ml CR. (A. Control; B. WS-1 cell sheet detachment from NIPAm-co-NtBAm film without CR; C. WS-1 adhesion on pNIPAm film; D. WS-1 cell sheets on NIPAm-co-NtBAm film with CR; E. ECM-rich WS-1 cell sheet detached from NIPAm-co-NtBAm film. Scale bar: 500µm)

Figure 5-3 100nm thick spin coated NIPAm-co-NtBAm films were determined appropriate for cell attachment and detachment of intact fibroblast sheets. Due to the
high ECM deposition under MMC conditions, a slower detachment rate was observed using time-lapse analysis (a-f) and subsequent plotting of % detachment area against time (g).

5.3.2 Characterization of WS-1 cell-assembled constructs

After 2 days of culture, the detached cell sheets were fixed and dehydrated for SEM and AFM characterization. SEM analysis of WS-1 cell sheets further corroborates the enhanced ECM deposition, the fibrillar pattern and tissue-like organisation of the cell-sheets produced under MMC crowding conditions (0.5% HS and 75µg/ml CR) was observed within 2 days of culture (Figure 5-4). AFM also confirmed the presence of a more fibrous structure in the intercellular regions on the cell surface under MMC conditions compared to the non-MMC counterparts (Figure 5-5).

Figure 5-4 Scanning electron microscopy analysis of WS-1 cell sheets further corroborate the enhanced ECM deposition, more fibrillar pattern organisation of cell-sheets under MMC crowding conditions was produced (0.5% HS and 75µg/ml CR) within 2 days of culture.
5.3.3 Production of ECM-rich WI-38 and HCK cell-assembled constructs

Apart from WS-1 human skin fibroblasts, WI-38 and HCK cells were also used to attain living substitutes with tissue-specific protein composition and structured constructs. WI-38 attached and became confluent on the 100nm spin coated NIPAm-co-NtBAm film initially, but only 50% of adhered cells detached after 60min of 4°C
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treatment. The whole ECM-rich WI-38 cell sheet constructed in the presence of 0.5% HS and 75µg/ml CR was not preserved during detachment. Therefore, WI-38 is not suitable for ECM-rich cell assembled constructs under thermoresponsive film-MMC conditions.

Thermoresponsive films fabricated in this research were also unstable for optimal HCK cells attachment and proliferation as they did not adhere onto either pNIPAm or NIPAm-co-NtBAm films, even after 2 days in culture. Therefore, cell adhesion promoters were used in accordance with a previous study (Moran et al. 2007b). Collagen and poly-l-lysine were used to coat the 1µm solvent cast pNIPAm films before cell seeding. Collagen was used as it is the main structural protein of various connective tissues in animals and it is mostly found in fibrous tissues in corneas; poly-l-lysine was used as it is recommended by American Tissue Culture Collection from where the cell was bought. As can be seen in Figure 5-6, HCK seeded onto the collagen coated pNIPAm films attached and formed a particle, and the cells did not become confluent even after a prolonged culture period. HCK would adhere and form a monolayer on the poly-l-lysine coated pNIPAm film. Three macromolecule crowders, 100µg/ml DxD; 37.5mg/ml Ficoll™ 70 and 25mg/ml Ficoll™ 400; and 75µg/ml CR with 0.5% HS, were used to treat confluent HCK cell sheets separately for 48 hours. There was no difference between the HCK control and MMC treated HCK cell sheets on the poly-l-lysine coated pNIPAm films.
Figure 5-6 HCK cell sheet formation on thermoresponsive films. pNIPAm films alone were not suitable for the attachment of HCK, even after 2 days in culture. Collagen and poly-l-lysine were used to coat pNIPAm films in order to facilitate the adhesion and detachment of intact ECM-rich HCK sheets, produced after 2 days in the presence of 0.5% HS and macromolecules. (A. HCK seeded onto the collagen coated pNIPAm films; B. HCK seeded onto the poly-l-lysine coated pNIPAm films; C. HCK cell sheet on the poly-l-lysine coated pNIPAm films treated with CR; D. HCK cell sheet on the poly-l-lysine coated pNIPAm films treated with DxS; E. HCK cell sheet on the poly-l-lysine coated pNIPAm films treated with FC; F. HCK cell sheet on the poly-l-lysine coated TCP. Scale bar: 500µm)

MMC treated HCK sheet detachment was achieved by reducing the ambient temperature. As a cell adhesion promoter was used to improve HCK adhesion, HBSS washes had to be used to help cell sheet detachment at room temperature. As a result a comparison of the detachment rate of MMC and non-MMC treated HCK sheets is not possible. Intact HCK sheets were successfully detached from all MMC systems. The overall HCK sheet shrank to 1/5 of the original size (Figure 5-7).
Figure 5-7 37.5mg/ml Ficoll™ 70 and 25mg/ml Ficoll™ 400 treated HCK cell sheet detaching from a poly-l-lysine coated pNIPAm film. Intact cell sheets were maintained as the cell sheet lifts off, the overall HCK sheet shrank to 1/5 of the original size. The cell sheet was washed with HBSS to accelerate detachment. This montage follows the progression of cell detachment upon exposure to cold treatment; (A) 5min, (B) 6mins, (C) 7mins, (D) 9mins, (E) 15mins, (F) 40mins. Scale bar: 500 µm.
5.4 Conclusion

Further immunocytochemistry assessment and complementary fluorescence intensity measurements of the detached ECM-rich cell sheets was carried out, courtesy of the NFB. Data obtained confirmed significant upregulation (p<0.0001) of collagenous proteins; enzymes associated with biogenesis of connective tissue proteins and cross-linking of collagen and elastin; basement membrane proteins; glycoproteins; glycosaminoglycans; and proteoglycans (Figure 5-8).

![Immunocytochemistry assessment of MMC influence on cellular components and biological processes for WS-1 cell sheets.](image)

Figure 5-8 Immunocytochemistry assessment of the MMC (0.5% HS and 75µg/ml CR, 2 days in culture) influence on cellular components and biological processes for WS-1 cell sheets (Images were acquired by collaborators from NFB).

It is confirmed that MMC inclusion in ex vivo experiments is a key modulator of ECM deposition of WS-1, WI-38 and HCK cell sheets. The utilization of MMC, by imitating native tissue localised density, can be utilised to modulate *in vitro* microenvironments and ultimately produce ECM-rich cell substitutes, within hours rather than days or
months in culture, without compromising fundamental cellular functions. Therefore, by seeding fibroblasts onto thermoresponsive systems, the cell sheets produced were of dense and cohesive tissue modules with intact cell-cell and cell-ECM junctions; tissue like morphology; and positively upregulated molecular functions, ECM and cellular components and biological processes. Different cells have different native characteristics, so the identification of the most suitable thermoresponsive substrate for each kind of cell is critical for the ECM-rich cell-assembled constructs. WS-1 prefer the more hydrophobic NIPAm-co-NtBAm copolymer films rather than pNIPAm films. WI-38 is able to adhere and proliferate on thermoresponsive systems, but the cell stick to the substrates even after an extended time in cold treatment. Furthermore, poly-l-lysine has to be used to promote HCK adhesion on pNIPAm films.

After successfully obtaining intact ECM-rich WS-1 and HCK cell-assembled constructs, SEM and AFM confirmed the presence of fibrous ECM under MMC crowding conditions. The fibrous ECM was also visible in the controls which had no MMC crowding, but it was not as dense as that of its MMC counterparts. ICC showed that ECM proteins (e.g. collagen type VII, elastin, fibrillin-1); enzymes associated with collagen maturation and cross-linking (e.g. transglutaminase-2); cytoskeletal proteins (e.g. α-smooth muscle actin, epithelial keratin, tubulin); glycosaminoglycans (e.g. chondroitin sulphate, keratin sulphate, heparin sulphate); proteoglycans (e.g. aggrecan, biglycan); and cytokines (e.g. TEM-1/CD248, IL-10) remained unaffected, indicating that MMC does not affect physiological function. Therefore, by using thermoresponsive films, living substitutes with tissue-specific protein composition and structure were attained. This approach opens up new avenues in engineering cohesive tissue modules in vitro.
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Chapter 6. Differentiation of induced pluripotent stem cells on thermoresponsive films
6.1 Introduction

Induced pluripotent stem (iPS) cells are adult cells that have been genetically reprogrammed to an embryonic stem cell like state by being forced to express genes and factors important for maintaining the defining properties of pluripotent stem cells. Mouse iPS cells were first reported in 2006, and human iPS cells were first reported in late 2007 (K. Takahashi and Yamanaka 2006; K. Takahashi et al. 2007; Yu et al. 2007). iPS cells have become useful tools for regenerative therapy, drug development and the modelling of diseases. For example, human stem cells could have a significant impact on drug development and toxicity tests as a replacement and as a tool to refine animal experiments. In addition to allaying public concern over the use of animals in research, the use of iPS cells could address those instances in which animals have not proven to be appropriate and safe models to predict the efficacy and toxicity of drug candidates in humans (Deng 2010). iPS cells have a unique advantage for disease modelling: they can be made by reprogramming a patient’s own cells to make patient-specific cells in the laboratory.

Previous studies have used thermoresponsive films as platforms for cell preservation; but biomaterials are developed not only to serve as cell carriers providing mechanical support but they can also actively influence cellular response including cell proliferation and differentiation. Therefore, understanding the differentiation of iPS cells in the presence of biomaterials is an important and increseasingly topical issue for biotechnological development. Kuo and Chang used hydrogels comprised of alginate and poly(γ-glutamic acid) (γ-PGA) with a surface neuron growth factor to differentiate iPS cells into neurons (Y. C. Kuo and Chang 2013). Franck et al. studied silk-based biomaterials in combination with ECM coatings for cell-seeded bladder tissue engineering approaches with iPS cells (Franck et al. 2013). Both studies used biological promoters to improve iPS cell differentiation. It is still unknown how iPS cells behave in direct contact with the biomaterials. It is shown in chapter 3 that hMSCs were able to
be preserved on bare solvent cast pNIPAm films. It is desirable that similar coatings were able to support iPS cell regeneration, particularly non-differentiated stem cell regeneration. 1µm thick solvent cast NIPAm-co-NtBAm films were selected for this study and the material-driven induction of iPS cells was investigated.

6.2 Materials and methods

6.2.1 Materials

iPS cells were kindly provided by the REMEDI group in NCBES, NUI Galway. The mTeSR®1 Basal Medium and mTeSR®1 5X supplement for iPS cell culture were purchased from Stemcell Technologies. OCT4 rabbit mAb, Nanog rabbit mAb, SOX2 rabbit mAb, AFP rabbit mAb, ASM mouse mAb, Anti-rabbit IgG, HRP-linked Antibody, Anti-mouse IgG, HRP-linked Antibody, Anti-rabbit IgG (H+L), F(ab')2 Fragment, Anti-mouse IgG (H+L), F(ab')2 Fragment were purchased from Cell Signaling Technology®. Anti-NeuN Antibody, Anti-Nestin Antibody were from Merck Millipore. Anti-Doublecortin antibody - Neuronal Marker was purchased from abcam®. 6-well-plate and 24-well-plate were from NUNC®. All other plastics were purchased from Sarstedt.

6.2.2 iPS cell adhesion and preservation

1) iPS cell culture and imaging technique

Induced pluripotent stem (iPS) cells were maintained in mTeSR®1 Basal Medium supplemented with 20% mTeSR®1 5X supplement, no antibiotic was used in the medium. iPS cells were cultured as colonies in 6-well-plate, and Geltrex coating had to be used to support cell adhesion. For the experiment, the confluent colonies were collected as small pieces using a 10µl tip, which were then seeded in triplicate at even number of colonies with 5 % CO₂ at 37 °C for incubation. The medium was changed every day. In all cases the samples were placed on a thermoplate to maintain a working temperature above the polymer’s LCST. Cell proliferation and cell detachment was
microscopically observed every other day using an Olympus BX51 with Image Pro-Plus analysis system phase contrast microscope.

2) Experimental design for iPS cell and thermoresponsive film interaction

1µm thick solvent cast NIPAm-co-NtBAm films were made for assessment. Half the films were coated with Geltrex in order to compare those with the bare film, Geltrex coated 6-well-plate was used as control. iPS cells were co-incubated with each group for 2 weeks for long term cell-surface interaction effects. iPS cells were also repetitively seeded onto each experimental group 3 times once confluency was reached, this was done to see if cell recovered by temperature controlled detachment had any advantage over those recovered via mechanical detachment.

3) Western blot and Immunocytochemistry of detached iPS cells

For Western blot and Immunocytochemistry analysis, cells were detached from the thermoresponsive films as described. Cells were detached from controls via mechanical scratching. A third of the detached cells were seeded into a 24-well-plate for immunocytochemistry, the rest were lysed for protein which was to be used for western blotting. OCT4 rabbit mAb, Nanog rabbit mAb, SOX2 rabbit mAb, AFP rabbit mAb, Anti-NeuN Antibody, Anti-Nestin Antibody and Anti-Doublecortin antibody were used to test the cell-surface interactions both for Western blotting and immunocytochemistry, as per the manufacturer’s instructions. The Western blot was imaged by FluorChem and analyzed with ImageJ software. Immunocytochemistry staining was captured by confocal microscopy in Anatomy department, NUI Galway.
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6.3 Results and discussion

6.3.1 Long term iPS cell and thermoresponsive film interaction

As previously described, iPS cells were seeded onto the Geltrex controls, Geltrex coated NIPAm-co-NtBAm films and bare copolymer films. The seeded cells were incubated at 37 °C with 5 % CO₂ for 2 weeks and the medium was changed every day. Images of the cells were captured every second day post seeding. iPS cells that adhered on the Geltrex coated films maintained their stem cell morphology similar to the Geltrex controls, but differentiated cells were also found with most neuron-like cells in the inter-colony regions. Most iPS cells seeded on the bare copolymer films failed to attach to the substrate, the adhered cells differentiated very quickly into neuron-like cells as can be observed on the 3rd day after seeding. After one week, there were several very big neuron-like cell colonies on the bare copolymer film (Figure 6-1).
Figure 6-1 2 weeks incubation of iPS cells on NIPAm-co-NtBAm thermoresponsive films. The morphology of iPS cells seeded on Geltrex control has not changed; the majority of cells seeded on Geltrex coated film maintained stem cell morphology, but some iPS cell started to differentiate as early as day 5; cells which adhered the bare NIPAm-co-NtBAm film started to differentiate into neuron like cells on the third day after seeding. Scale bar: 200µm.
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6.3.2 Characterization of long term effects for iPS cells

After 2 weeks of incubation, iPS cells seeded on the control, on the Geltrex coated film and on the bare films were collected for Western blotting and immunocytochemistry assessments. Stem cell markers Nanog, OCT4 and SOX2; Neuron cell markers DCX, Nestin and NeuN; embryonic layer markers α-fetoprotein (AFP) and α isoform of smooth muscle actin (ASM) were used. The proteins which are involved with the self-renewal of undifferentiated embryonic stem cells: Nanog was not or infrequently observed in the Western blotting results, while OCT4 was detected, a higher quantity of SOX2 was detected in the Geltrex controls and Geltrex coated films. The expression of DCX (expressed by neuronal precursor cells and immature neurons in embryonic and adult cortical structures) and ASM was increased on bare NIPAm-co-NtBAm film. There were variations in the cells collected from Geltrex coated film as the duplicated Western blotting lanes showed conflicting results for OCT4, Nanog and ASM (Error! Reference source not found.). This also confirmed the two distinct cell behaviours on Geltrex coated films in Figure 6-1. Further immunocytochemistry assessment was carried out to test the influence of NIPAm-co-NtBAm films on iPS cellular components and biological processes. Data obtained confirmed significant upregulation of neuron cell markers (Nestin, NeuN, DCX) and down regulation of stem cell markers (Nanog, OCT4, SOX2) of the cells collected from the bare copolymer films. The cells collected from the Geltrex coated film expressed both stem cell and neuron cell markers indicating that solvent cast NIPAm-co-NtBAm films may trigger iPS cell differentiation, but the additional Geltrex coating on the films enabled maintaining of iPS cells’ stem status (Figure 6-3).
Figure 6-2 Western blotting results of iPS cells co-incubation with NIPAm-co-NtBAm films. The expression of DCX (expressed by neuronal precursor cells and immature neurons in embryonic and adult cortical structures) and ASM (α isoform of smooth muscle actin) was increased on bare NIPAm-co-NtBAm film. There were variations in the cells collected from Geltrex coated film as the duplicated western blotting lanes showed conflicting results for OCT4, Nanog and ASM.
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Figure 6-3 Immunocytochemistry assessment of iPS cells co-incubation with thermoresponsive NIPAm-co-NtBAm films. Data obtained confirmed significant upregulation of neuron cell markers (Nestin, NeuN, DCX) and down regulation of stem cell markers (Nanog, OCT4, SOX2) of the cells collected from the bare copolymer films. The cells collected from the Geltrex coated film expressed both stem cell and neuron cell markers.

6.3.3 Repetitive iPS cell culture on thermoresponsive films

The 2 weeks of co-incubation of iPS cells and Geltrex coated films yielded both positive and negative results, some cells maintained stem cell status and some cells differentiated. Therefore, it is necessary to assess how iPS cells behave after 3 cycles of reseeding and detachment as was studied for the hMSC. iPS cells were seeded on the Geltrex controls, on the Geltrex coated films and on the bare films as previously described. Cells were detached and reseeded once confluent. The iPS cells seeded on both the Geltrex coated films and the bare films were detached by decreasing the environmental temperature and the control cells were detached by mechanical scraping. The repetitive attachment and detachment of iPS cells on both the Geltrex control and the Geltrex coated films did not affect the cellular activities. While the iPS cells were unable to maintain as stem cell colonies after the first seeding time, more and more
neuron-like cells and cell clamps were found on the 2nd and 3rd seeding time (Figure 6-4).

When the cells became confluent after the 3rd seeding, the cells were collected for Western blotting assessment. The overall tendency is very similar to the 2 weeks co-incubation. The bare film adhered cells expressed more neuron cell markers and less stem cell markers. There was also an increase of embryonic markers in the cells which adhered onto the bare film (AFP & ASM). The cells collected from the Geltrex coated films maintained stem cell markers and expressed a similar amount of proteins in duplications. Control cells showed variations in Western blotting pages especially SOX2 and NeuN expressions (Figure 6-5). Future experiments and immunocytochemistry will have to be done in order to confirm the current results.
Figure 6-4 iPS repetitive attachment and detachment from solvent cast NIPAm-co-NtBAm films. The repetitive attachment and detachment of iPS cells on both the Geltrex control and the Geltrex coated films didn’t affect the cellular activities, iPS cell colonies were found in each passage. While the iPS cells were unable to maintain as stem cell colonies after the first seeding time, more and more neuron-like cells and cell clamps were found on the 2\textsuperscript{nd} and 3\textsuperscript{rd} seeding time.
Figure 6-5 Western blotting results for the repetitive seeding of iPS cells onto NIPAm-co-NtBAm films. The bare film adhered cells synthesis more neuron cell markers and lack of stem cell markers. There was also an increase in embryonic markers in the cells which adhered to the bare film (AFP & ASM) compared to Geltrex control and Geltrex coated film. The cells collected from the Geltrex coated films maintained stem cell markers and expressed a similar amount of proteins in duplications.
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6.4 Conclusion

According to the preliminary results, it can be concluded that bare solvent cast films prepared from NIPAm-co-NtBAm do not support iPS cell preservation. The adhered cells on the bare film differentiated to either neuron cells or mesoderm cells. The utilization of thermoresponsive NIPAm-co-NtBAm films may offer a quicker way for iPS cell differentiation to neuron cells. A Geltrex coating on the films may prohibit iPS cell differentiation, and the coated thermoresponsive films were able to offer a platform for iPS cell preservation and expansion. Such coated films can also be used as an alternative to the traditional detachment method of mechanical scratching, which dramatically reduce the total viability of any cell type. However, further work is needed for iPS-thermoresponsive film evaluation as the incubation time weakens the effect of Geltrex. iPS cells showed signs of differentiation on Geltrex coated copolymer films similar to those on the bare films, only in lower amounts.

iPS cells can be detached simply by reducing the environmental temperature on Geltrex coated films. Even after repetitive attachment and detachment from the coated thermoresponsive films, the iPS cells still retain their stem cell morphology and markers. Bare copolymer films favoured iPS cell differentiation; western blotting results suggested that iPS cells were prone to become cells of neuronal or embryonic lineage on the bare films. The western blotting yielded conflicting results such as in the case of SOX2 from cells gathered from the controls, ASM from coated films as well as the NeuN results. Therefore, further experiments are needed to stabilize the preservation of iPS cells on thermoresponsive systems, more assessments are required to check if the differentiated neuron cells functions similar to normal neuron cells, and additional film modifications have to be used for regulating iPS cell differentiation.
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Chapter 7. General conclusion

Investigations into preparing thermoresponsive cell culture delivery systems have been ongoing over the last couple of decades. A lot of different approaches have been developed for thermoresponsive cell carrier systems; each approach has its own relative advantages and disadvantages. For instance, the electron beam polymerization process is one of the most successful approaches, but it is not suitable for large scale production and it is quite an expensive, inaccessible for most laboratories. The major obstacle to the simple development of thermoresponsive cell delivery systems is that cells adhere poorly onto bulk films. Therefore, this research tries to simplify the fabrication of NIPAm based thin and ultra-thin thermoresponsive films for cell preservation and expansion. The main purpose of the developing these films was to offer an alternative to the technologically complex and expensive grafting techniques and to avoid the use of animal based products to encourage cell adhesion which are expensive, time consuming and a source of possible contamination. Investigation into using the operationally facile spin coating and solvent cast techniques for depositing functional cell culture carries proved to be successful, with a variety of cells grown and detached from thermoresponsive films. Optimisation of the thermoresponsive films for cell adhesion, proliferation and differentiation allowed for the refinement of crucial parameters to thermoresponsive modifications.

- Human mesenchymal stem cells

hMSCs were tested on thermoresponsive films as they hold great potential in regenerative medicine and cell therapies. Thermoresponsive films deposited from the pNIPAm homopolymer via solvent casting further simplified the hMSCs immunophenotype preservation, hMSC immunophenotypic essential surface markers analysis indicated that thermoresponsive surfaces do not induce hMSC differentiation.
Successful hMSC detachment was achieved by simply reducing the environmental temperature. The cells were able to maintain their differentiation potential after three cycles of seeding and non-enzymatic detaching on 1µm thick solvent cast pNIPAm films.

Extensive comparative characterisation of the macrophage-like cells RAW264.7 was carried out as macrophage cells act as both inflammatory mediators and wound heal regulators in the foreign body reaction. Results revealed that there were differences in RAW264.7 behaviours on thermoresponsive films with variable compositions and fabrication methods.

- Macrophage-like cells RAW264.7

Flow cytometry and ELISA studies revealed that RAW264.7 adhered and proliferated better on NIPAm-co-NtBAm films than that on the pNIPAm films. There was not much difference in RAW264.7 behaviour on all types of copolymer films.

RAW264.7 cell responses to the films fabricated by pNIPAm and NIPAm-co-NtBAm showed a distinctive pattern, especially the spin coated pNIPAm films, exhibited immunological activation effects.

Thermoresponsive films developed in this research can be used as an alternative for routine laboratory RAW264.7 cultivation. The adhered RAW264.7 on thermoresponsive films could be detached within 30 min by reducing ambient temperature.

Additionally, the thermoresponsive films were used for ECM-rich assembled cell sheets production.

- Cell sheets engineering

Primary human skin fibroblast cells were successfully grown and detached from 100nm spin coated NIPAm-co-NtBAm films.
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Poly-l-lysine had to be used to coat the 1µm solvent cast pNIPAm films for human corneal keratocytes adhesion, proliferation and detachment.

With the help of MMC, the ECM deposition of collagen, fibronectin in both human skin fibroblast and human corneal keratocyte sheets was significantly increased within a very short time-scale on the thermoresponsive films. The intact cell sheets detachment rate under MMC conditions was slower than their non-MMC counterparts (40 min to 25 min for human skin fibroblasts); this is due to the abundance in deposited ECM.

Post-detachment assessment confirmed the successful construction of ECM-rich assembled cell sheets, which may be useful for wound healing tissue engineering purpose.

The utilization of thermoresponsive films may offer an advantage over the traditional scratch cultivation of iPS cells. This non-destructive cell detachment yields cells which could be used for therapeutic applications.

- Induced pluripotent stem cells

There were significant cell-material interactions observed; NIPAm-co-NIPAm was able to initiate iPS cells neuron differentiation. Cell adhesion promoter may prohibit this interaction, but the effects were not stable.

Successful temperature induced iPS cells detachment was achieved with in 30min. The cells were able to maintain their differentiation potential after three cycles of seeding and thermoresponsive detachment.

The preliminary results are conflicting and require further assessment. Future work will involve stabilization of iPS cells on thermoresponsive systems; regulation of iPS cell differentiation on thermoresponsive films as well as the functional definition of differentiated cells.
Finally, it is possible to manipulate thermoresponsive films not altering adhered cell characteristics. The simplified solvent cast thermoresponsive films offer an alternative to technologically complex and expensive grafting techniques and avoid the damage of traditional cell detachment. The thermoresponsive films developed in this body of research not only offers an advantage of intact cell culture, but also acceptable by everyone in the routine laboratory work.

To completely avoid the use of animal based products to encourage cell adhesion which are expensive, time consuming and a source of possible contamination, it would be ideal to merge thermoresponsive techniques and serum-free medium as cell substrates and test the ability of cells to proliferate in these culture conditions use xeno-free culture conditions.

The thermoresponsive films used in the research were well optimised for the hMSC, iPS cells, RAW264.7 and primary human cells. It is still unknown if new cells/cell lines will adhere onto the thermoresponsive films or not. Therefore, further investigations using wider variety of cells/cell lines are required.
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Appendix I

Thermoresponsive Substrates used for the Expansion of Human Mesenchymal Stem Cells and the Preservation of Immunophenotype

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Abstract The facile regeneration of undifferentiated human mesenchymal stem cells (hMSCs) from thermoresponsive matrices facilitates the collection of stem cells avoiding the use of animal derived cell detachment agents commonly used in cell culture. This communication proposes a procedure to fabricate coatings from commercially available poly(N-isopropylacrylamide) which is both affordable and a significant simplification in alternative approaches used elsewhere. Solvent casting was used to produce films in the micrometer range and successful cell adhesion and proliferation was highly dependent on the thickness of the coating produced with 1-μm thick coatings supporting cells to confluence. 3T3 cell sheets and hMSCs were successfully detached from the cast coatings upon temperature reduction. Furthermore, results indicate that the hMSCs remained undifferentiated as the surface receptor profile of hMSCs was not altered when cells were detached in this manner.

Keywords Thermoresponsive polymers • Human mesenchymal stem cells • Cell expansion and regeneration • Flow cytometry • Stem cell differentiation

Introduction

As evidenced by the literature, the generation of cells for the repair of damaged tissues using stem cell therapy holds much promise due to the pluripotent nature of stem cells. The culture and expansion of human mesenchymal stem cells (hMSCs) which can be used for such therapies is clearly an essential step in realising this potential. For clinical and therapeutic purposes it is desirable to eliminate the use of animal derived products such as tissue, which is routinely used in cell culture, and the use of thermoresponsive platforms capable of hosting and releasing stem cells offers an alternative route for stem cell regeneration. This is becoming increasingly desirable as the regulatory biomedical landscape suggests a shift away from the use of non-animal derived products and the evolution of new techniques that avoids their employment.

A number of factors are capable of influencing or inducing stem cell differentiation including: cell-material interactions, material-surface properties as such chemical composition and energy and cell growth factors [1, 2]. While the ability to differentiate MSCs holds the key to the promise of stem cell therapy, it is also important to be able to culture them for large
scale applications is an undifferentiated state in a reproducible and reliable fashion so that they can be differentiated when desired. There has been extensive research on the use of biomaterials with a view to preserving and differentiating not only hMSCs, but also embryonic stem cells and hematopoietic stem cells [3]. The most extensively investigated biomaterials used for this purpose are collagen, fibrinogen, hyaluronic acid, glycosaminoglycans (GAGs), hyaluronan (HA) in terms of natural materials and polyglactin acid (PGA), poly(lactic acid) (PLA), poly(ethylene glycol) (PEG) and the copolymer poly(lactide-co-glycolide) (PLGA) in terms of synthetic materials. These can take the form of 2-D and 3-D scaffolds, microspheres, hydrogels and coatings etc. [3–5].

Modifications to the cell substrate used to guide hMSC differentiation in vitro indicate that –NH₂ and –SH modified surfaces promote osteogenesis and –OH and –COOH modified surfaces promote and maintain chondrogenesis [2]. Thermoresponsive polymers were also used for hMSC proliferation and multi-differentiation potentials, for example pNIPAm-grafted poly(dimethylsiloxane) (PDMS) and pNIPAm-based polyelectrolyte multilayer films, hMSC cells reserved their potential for differentiation and showed better viability on NIPAm based polymer films than that on the plates surface coated with gelatine [6, 7]. When cells are detached from thermoresponsive polymer coatings the excreted extracellular matrix proteins are preserved which has been shown to facilitate the maintenance of important cell membrane proteins around bone marrow MSCs and human adipose tissue MSCs [8]. However, the film fabrication procedures are complicated and how the cells behave post detachment hasn’t been reported.

With this in mind, the aim of the present study was to develop a simple method to produce pNIPAm coatings with a view to cell regeneration, particularly non-differentiated stem cell regeneration. Recently we published on the fabrication of ultra-thin poly-N-isopropylacrylamide (pNIPAm) based films for cell and cell sheet regeneration via the spin coating method [9–11]. In one of these studies commercially sourced polymer was used to produce pNIPAm solutions from which the films were prepared. A number of different cell lines including 3T3 fibroblasts and hMSCs were successfully grown under standard physiological conditions and were subsequently gently detached upon temperature reduction [10].

Studies have shown that cells attach and therefore proliferate poorly on thick or bulk pNIPAm coatings and many studies report a coating thickness limitation above which there is a dramatic reduction in the number of cells attaching to the polymer surface [12–16]. In studies where pNIPAm was covalently grafted for this purpose, this thickness limitation is in the order of tens of nanometers with the exception of coatings formed via plasma polymerization [12, 17–19]. Our previous studies using spin coated commercially sourced polymer found that there was no relationship between the thickness of the film produced and successful cell attachment and proliferation within the thickness-scale employed (>30 nm to<2,000 nm) [10]. The grafting techniques used to produce thermoresponsive coatings are technologically expensive and economically expensive and many studies seek to investigate less complex and cheaper alternatives. Alternatively there are proprietary thermoresponsive plates available on the retail market but these are expensive and therefore impractical for routine cell culture use.

Our previous investigations include using cell adhesion promoting agents as an over layer on top of thick solvent cast pNIPAm films to improve cell adhesion as well as the aforementioned spin coating approach [10, 20]. This study seeks to offer a significant simplification on methods employed elsewhere by investigating the possible thickness limitation of films produced using the simple solvent casting method. Solvent casting is a very simple, routine and inexpensive technique used to produce polymer films mainly in the micrometer range where a solution of the polymer is prepared in a suitable solvent and is spread around the substrate surface evenly, after which it is dried to remove the solvent. Moreover, solvent casting is a common method used in cell culture protocols for example when depositing cell adhesion promoting layers such as fibronectin or poly-L-lysine and therefore it is a method familiar to most cell culture operation making it a widely accessible approach for film production compared to methods used elsewhere [21, 22]. Previous studies have shown that films produced in this manner are poor cell hosts compared to conventional tissue culture plastic (TCP) substrates but this study indicates that the thickness of the solvent cast films is critical to the cell response with cells attaching and proliferating on thin (1 µm) films similar to TCP and the numbers of cells attaching to films decreasing with increasing film thickness [23, 24]. A schematic illustrating the experimental design is given in Fig. 1. To further simplify the method; commercially sourced pNIPAm was used thus avoiding the expense, equipment and expertise needed for polymerization or grafting procedures which makes the method accessible to all laboratories where non-invasive cell or cell sheet detachment is desirable. While using non-grafting techniques to produce films may lack the elegance of the grafting techniques due to polymer dissolution upon temperature reduction, studies show that pNIPAm is not cytotoxic to cells [24, 25].

We believe therefore, that this method provides a viable and affordable alternative for cell and cell sheet regeneration.

The initial model cell line used was 3T3 fibroblasts for preliminary proof of concept, followed by hMSCs which were characterized post detachment according to the guidelines and standards established by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT).
Appendix I

Experimental Section

Materials

pNIPAm (Mn 20,000-25,000), anhydrous Ethanol (EtOH) (200 proof, 99.5 %), Dulbecco's modified Eagles medium (DMEM), Hank's balanced salt solution (HBSS), penicillin, streptomycin, fetal bovine serum (FBS), phosphate buffered saline solution (PBS), Trypan blue stain, trypsin, trypsin EDTA, Fluorescein-activated cell sorting (FACS) buffer, phosphate buffered saline (PBS) with 1 % FCS and 0.01 % sodium azide were purchased from Sigma Aldrich, phycocyanin (PE) labeled CD10, CD34, CD45 from Biosciences and used as received. For stem cell culture non heat inactivated fetal bovine serum from Hyclone was used to supplement the growth media. 3T3 mouse embryo fibroblast-like cells were kindly provided by University College Cork, hMSCs were kindly provided by the Regenerative Medicine Institute (REMEI) group in the National Centre for Biomedical Engineering Science (NCBES), NUI Galway. Quant-IT PicoGreen dsDNA assay from Invitrogen, alamarBlue assay from BioSource, Thermoshake plastic 25 mm dishes from NUNC, all other plastic consumables from Sarstedt. Fused silica glass disk, 20 mm in diameter from UVG optics.

Film Preparation and Characterization

Films were prepared using the solvent casting method from a 4 % (w/v) of pNIPAm in EtOH on 15 mm Petri dishes, after which they were dried slowly overnight in an EtOH soaked atmosphere dessicator before drying completely in a vacuum oven set to 40 °C and 600 mbar overnight to ensure any residual solvent is eliminated. Films were sterilized under mild UV light for 2 h prior to cell culture experiments. For Fourier transform infrared spectroscopy (FTIR) analysis films were deposited onto aluminum stubs. Samples were stored at room temperature and routinely used within a month of preparation.

Film thickness measurements were performed using the simple scratch test method. Dried polymer film prepared as described above and a sharp blade was used to scratch the surface of the polymer from the underlying substrate and atomic force microscopy (AFM) analysis was used to assess to a height difference between the underlayer and the polymer surface. AFM images were obtained in tapping mode in air using a Dimension 3100 AFM (Digital Instruments, Santa Barbara, CA, USA) and Vecco 1-10 0.05-nm phosphorus (ni doped Si tips and a matrix of 512 × 512 data points along the x-y plane were analyzed in a single scan. Four 100 × 100 μm scans were recorded at a scan rate of 1 Hz on each ablated area to ensure statistical accuracy. The thickness of the films can also be predicted via a convenient formula, Eq. 1

\[ H = \frac{C \nu}{S} \]

Where \( H \) is the thickness of the final film; \( C, \nu \) and \( \rho \) are the concentration, volume and density of polymer solution, respectively; \( S \) is the surface area of the substrate upon which the film was coated on. As can be seen in Table 1 the predicted thickness values and the measured thickness values are in good agreement.

AFM was also used to assess the roughness of the deposited pNIPAm coatings using 10 μm × 10 μm scans. The roughness of the films was reported as root-mean-square (RMS) roughness values, where \( \text{rms} \) denotes the standard deviation of the Z-values along the reference line.

FTIR and X-ray photoelectron spectroscopy (XPS) spectra were acquired using the Hitachi FTIR-8,300 in transmission mode and the AXIS 165 X-ray photoelectron spectrometer respectively.

<p>| Table 1 Predicted solvent cast film thickness versus thickness measured by AFM analysis |
|---------------------------------------------|---------------------------------------------|</p>
<table>
<thead>
<tr>
<th>Predicted Film Thickness (μm)</th>
<th>Measured Film Thickness (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.07 ± 0.07</td>
</tr>
<tr>
<td>2</td>
<td>2.04 ± 0.03</td>
</tr>
<tr>
<td>4</td>
<td>4.02 ± 0.04</td>
</tr>
</tbody>
</table>
Appendix I

Advancing contact angle measurements were performed using the advancing drop method on a home-built goniometer as previously described [10].

Cell Culture, Imaging Techniques and Flow Cytometry of detached hMSCs

3T3 cells were maintained in DMEM, supplemented with 10 % FBS and 1 % penicillin streptomycin antibiotics. The human hMSCs used in these studies were isolated from human bone marrow. For experimentation, the hMSCs used were passaged no more than 5 times. When cells reached 80-90 % confluence, cells were harvested and used for seeding or for experimentation where appropriate.

For experimentation, cells were seeded in triplicate at a density of 40,000 and 20,000 cells/cm² for 2 and 5 days on the pNIPAm films and on TCP controls. In the cases of 3T3 and hMSC cells respectively, incubation conditions were a humidified atmosphere of 95 % air and 5 % CO₂ at 37 °C. In all cases the samples were placed on a thermostatic plate set to 37 °C to maintain a working temperature above the polymer lower critical solution temperature (LCST). The metabolic activity of the cells grown on the prepared samples and the controls were assessed using the alamarBlue assay. Total dsDNA content was also quantified using the Quant-iT PicoGreen dsDNA assay kit.

Cell proliferation and cell detachment was microscopically observed using an Olympus BX51 with Image Pro-Plus analysis system phase contrast microscope. To initiate cell detachment, the warm cell media was removed after which cold HBSS was added and the samples were then placed on a digitally controlled thermal/cooling plate set to 4 °C. Micrographs of the plates were captured frequently on the phase contrast microscope to monitor cell detachment.

For flow cytometry analysis, cells were detached from the thermoresponsive films as described followed by gentle and repetitive pipetting to yield single cells. Cells were detached from TCP controls via conventional trypsinization. The cells were stained with CD90 (also known as Thy-1), CD73 (known as ecto 5'-nucleotidase), CD105 (known as endoglin), CD19 (a marker of B cells that may also adhere to hMSCs in culture and remain vital through stromal interactions), CD34 (a primitive hematopoietic progenitor marker) and CD45 (a pan-leukocyte marker) antibodies, all PE-labeled, as per the manufacturer's instructions and were analyzed by flow cytometry using BD FACS Canto from Biorad and the results were analyzed by Flowjo software [26]. Additionally, half of the cells that were detached from the thermoresponsive surfaces were seeded on TCP and incubated for a further 3 days after which the same marker expressions were checked to define the post-detachment affects. All FAC experiments were performed in triplicate and five positive controls hMSC cells were mixed with 10 % CD45-positive hematopoietic cells (HC) before seeding on TCP to induce MSC differentiation and were incubated for 5 days.

Results and Discussion

The relative roughness of the films was assessed via AFM analysis; the results of which can be seen in Table 2 and all of the films were assessed to be relatively smooth.

The relative hydrophilicity/hydrophobicity of the polymer films was measured by advancing contact angle above the polymer's transition temperature. Distinct stick/slip behavior was clearly evident as the water droplet interacts with the film surface. This type of behavior is described in detail by Gilbreath et al. The stick angle is generally reported as close to the real contact angle of the films in cases where stick/slip behavior is observed, which in this case was an average of 68 ± 2° for 1 μm films, 73 ± 1° for 2 μm and 77 ± 1° for 4 μm films from 500 s to 1,800 s (allowing for the initial calibration of the advancing drop) [27]. While the stick angles of the 1 μm and 2 μm are relatively close in value the 4 μm films are significantly more hydrophobic.

FTIR and XPS analysis confirmed film deposition. FTIR spectra displayed peaks at 1,500 cm⁻¹, 3,300 cm⁻¹, 3,060 cm⁻¹, 1,450 cm⁻¹, 1,500 cm⁻¹, 1,730 cm⁻¹ corresponding to a N-H bending peak, a N-H stretching vibration, a C-H stretching vibration, a C-H asymmetric stretching vibration, a C-H symmetric stretching peak and a C = O stretching peak respectively which correspond to the fingerprint peaks of pNIPAm. The stoichiometry of the IPAM monomer is 75.0 % C, 12.5 % N and 12.5 % O and the elemental composition of the solvent cast PIPAm coatings measured by XPS was 77.4 %, 11.8 % and 10.7 % for C, N and O respectively which is quite close to the monomer stoichiometry thus confirming pNIPAm film deposition.

3T3 cells and hMSCs were seeded on the cast PIPAm films and observed microscopically for cell proliferation after 48 and 120 h incubation respectively. 3T3 cells proliferated and grew on 1 μm films similar to conventional TCP controls but the numbers of cells adhering and proliferating reduced as the thickness of the cast films increased with a modest reduction in cell numbers observed on 2 μm films and a marked reduction in cell numbers on thicker 4 μm films. This was confirmed quantitatively via the PicoGreen

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Solvent cast film RMS roughness as measured by AFM analysis. The roughness of the films was reported as root-mean-square (RMS) roughness values, where RMS denotes the standard deviation of the Z-values along the reference line</th>
</tr>
</thead>
<tbody>
<tr>
<td>Film Thickness (μm)</td>
<td>RMS Roughness (nm)</td>
</tr>
<tr>
<td>1</td>
<td>16.01 ± 0.60</td>
</tr>
<tr>
<td>2</td>
<td>26.01 ± 0.68</td>
</tr>
<tr>
<td>4</td>
<td>28.83 ± 1.35</td>
</tr>
</tbody>
</table>
Appendix I

Fig. 2  a PicoGreen total dsDNA assay results comparing 3T3 dsDNA quantity on TCP controls and solvent cast films of different thickness. b AlamarBlue assay test results comparing 3T3 cell metabolic activity on TCP controls and solvent cast films of different thicknesses. c PicoGreen total dsDNA assay results comparing hMSC dsDNA quantities on TCP controls and solvent cast films of different thicknesses. d AlamarBlue assay test results comparing hMSC metabolic activity on TCP controls and solvent cast films of different thicknesses. 

A dsDNA quantification assay with only 25% of dsDNA compared to TCP controls, Fig 2(a). The metabolic activity AlamarBlue assay results displayed a similar trend with the activity of cells on the 4 μm films only 15% compared to controls, Fig 2(b), which suggests that not only did fewer cells adhere on the thicker substrates, but additionally their metabolic activity was reduced in comparison. hMSC cells were similarly cultured and cell growth was assessed quantitatively and a similar trend was observed for the thickest 4 μm films, but there was a more significant reduction in cell numbers on 2 μm thick films (in comparison to the 1 μm thick films) than in the case of 3T3 fibroblasts, Fig 3 (c). The AlamarBlue assay results reflected this tendency also which suggests that the ideal thickness for cell growth may be cell line dependent, Fig 2(d). Additionally these results indicate that while 1 μm thick films were capable of hosting hMSCs to confluence the proliferation is slower than on the TCP controls i.e. 75% dsDNA compared with TCP controls. The reason why the films become increasingly cell repulsive with increasing film thickness is unclear though it is probably associated with the increased rigidity of the polymer chains with increasing film thickness leading to increased hydration which is non-conducive to protein adsorption which mediates cell attachment, this increased hydrophilicity for the thicker 4 μm thick films was analytically reflected in the advancing contact angle measurements.

3T3 cells were detached under cold treatment and cell to cell contact was maintained and full cell sheets were recovered within 15 min, Figs. 3 and 4. hMSCs were similarly detached from the cast pNIPAm surfaces in less than 30 min, Figs. 5 and 6.
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Fig. 3 3T3 cell sheet detaching from a 1 μm cast pNIPA film. Cell to cell junctions are maintained as the cell sheet lifts off. Cells begin detaching after 1 min with complete detachment achieved after 15 min. This montage follows the progression of cell detachment upon exposure to cold treatment; (a) 0 min; (b) 2 min; (c) 4 min; (d) 5 min; (e) 6 min; (f) 8 min; (g) 10 min; (h) 12 min. Scan bar size 500 μm

As defined by the ISCT, MSC cells must be plastic-adherent in standard cell culture conditions using tissue culture flasks. Secondly, ≥ 95% of the MSC cell surface must express CD105, CD73 and CD90, as measured by flow cytometry. Additionally, these cells must lack the expression (≤ 2% positive) of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA class II. Third, the cells must be able to differentiate into osteoblasts, adipocytes and chondroblasts under standard in vitro differentiating conditions [26]. With this in mind, we tested the cells for 'positive markers' CD73, CD90 and CD105 and for 'negative markers' we tested for CD19, CD34, and CD45 using FACS, as the presence or lack of expression respectively would indicate that the polymer films did not induce hMSC differentiation. Furthermore, half of the detached cells were reseeded and incubated on TCP for 3 days after which the marker expression was checked again to define the post-detachment affects. Prior to testing for the membrane markers, cells were detached from controls via conventional trypsinization, by temperature control from thermoresponsive films and again by trypsinization after reseeding and incubation for 3 days. In terms of the 'positive markers' the expression of all 3 was ≥ 95% which indicates that no

Fig. 4 Complete 3T3 cell sheet detached from a 1 μm thick cast pNIPA film. Scan bar size 200 μm

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hMSC differentiation had occurred in the cases of cells grown and detached from pNIPAm or the controls and moreover no post detachment affects occurred after reseeding and incubation, Table 3. Furthermore, the results indicated that in all cases the expression of all 3 'negative' cell membrane surface markers was ≤2% positive, Table 3. As these markers are rarely expressed on MSC cells these results indicate that no hMSC differentiation had occurred in the cases of cells grown and detached from pNIPAm or the controls, furthermore no post detachment affects occurred after reseeding and incubation.

To assess if this method of expansion and propagation is suitable for maintaining hMSCs in an undifferentiated state over serial passages, cells were seeded and detached as previously described from 1 μm thermoresponsive surfaces and TCP controls, but this time the cells were reseeded and detached through 3 passages. After the final passage the validates pluripotency markers, as described by the ISSCT,
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were assessed by FACS as previously described. It was observed that cells grown and detached on and from 1 μm thick solvent cast films were able to successfully attach and proliferate on subsequent cycles again using 1 μm thick films. The multi-fold expansion of hMSCs on the 1 μm thick films yielded cells which maintained all the 'positive markers' and lacked the so-called 'negative markers' tested for, Table 4, and therefore the cells maintained their multipotentiality in accordance with the prescribed ISCT guidelines.

As in the case of the disassociation enzyme trypsin, there are similar regulatory concerns surrounding the use of serum which is ubiquitously used to supplement cell culture media. Fetal bovine serum (FBS) is the most commonly used cell medium supplement which, as the name implies, is isolated from cow fetal blood and as such it is highly variable consisting of ill-defined bio-components leading to batch-to-batch inconsistencies. Such variability introduces contamination risks such as the inadvertent exposure to adventitious pathogens, which from a clinical application viewpoint is a risk that should be minimized or eradicated.

Taking this into consideration and in light of the results attained using this system to this point, it was decided to seed and incubate hMSCs on 1 μm pNIPAm films as before, but on this occasion in the absence of supplementary serum. Cells attached to the 1 μm surfaces successfully but the proliferation was severely impeded in the absence compared to in the presence of serum, the cells that did attach were easily detached upon temperature reduction in a matter of a few minutes. Future work will expand on using this type of experimental system for multiple passage expansion in reproducible cell culture conditions with a view to establishing a method which would eliminate the use of any animal derived products (serum, trypsin). This will require the use of chemically defined media suitable for the successful support of the hMSCs used. Such a protocol would satisfy regulatory safety requirements consistent with clinical compliance which could be a significant step in the translation of this type of protocol toward practical clinical/therapeutic applications.

It is important to also highlight that the immobilized polymer dissolves upon temperature reduction and is
Appendix I

The toxicity of pNIPAm has been investigated by Malvasi et al. in mice and their studies suggest no detectable toxicity after 28 days using the relatively high concentration of 2,000 mg/kg and studies by Takekawa et al. suggests that there are no cytotoxicity issues under cell culture conditions. Further studies underline that pNIPAm is non-cytotoxic in cell culture conditions at working concentrations higher than the concentrations used in this work [28, 29] While in this study the dissolved polymer did not seem to have any detrimental effect on the cells collected, it is best to remove as much as possible via media exchange or centrifugation before reseeding or further experimentation.

Conclusions

Solvent cast films prepared from commercially sourced pNIPAm offer a convenient, simple and cheap alternative to produce thermoresponsive surfaces with a view to cell and cell sheet regeneration to methods developed elsewhere, with an optimal film thickness of 1 μm. As the thickness of the cast films increased the surfaces became less bioadhesive and the numbers of cells which attached and consequently proliferated declined. This trend was observed for both 3T3 fibroblast cells and the hMSCs. Cell sheet detachment of 3T3 and hMSC cells was achieved through simple temperature control. Most importantly, hMSC immunophenotypic surface profile FACS analysis indicated that 1 and 2 μm thick solvent cast thermoresponsive surfaces do not induce hMSC differentiation i.e. the stem cells maintain their inherent multipotentiality and therefore this protocol offers a gentle and non-destructive approach for cell detachment therapies where the collection of undifferentiated MSCs is desirable. When the method was used for the multifold passaging of hMSCs it was found that cell proliferation through the passaging was similar in all cases and the pluripotent nature of the hMSCs was preserved. Future work will expand on using this type of experimental system for multiple passage expansion in reproducible cell culture conditions with a view to establishing a method which would eliminate the use of any animal derived products (serum, trypsin). Such a protocol would satisfy regulatory safety requirements consistent with clinical compliance which could be a significant step in the translation of this type of protocol toward practical clinical/therapeutic applications. Finally, this simple method of surface preparation allows for the preparation of substrates of differing geometries and sizes which allows for greater flexibility in experimental design, for example, should significant MSC expansion

Acknowledgments We gratefully acknowledge the financial support received from the Irish Research Council under the Embark scheme (Grant No: RS/2010/207). sincere thanks to Dr Sergei Belousov, Materials and Surface Science Institute, University of Limerick, for the XPS analysis.

Disclosures The authors indicate no potential conflicts of interest.

References

Appendix I


Appendix II

Macrophages behavior on different NIPAm-based thermoresponsive substrates

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Received 10 June 2013; revised 8 August 2013; accepted 5 September 2013
Published online 00 Month 2013 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jbm.a.34940

Abstract: Thermoresponsive materials and surfaces are widely used in cell culture applications. There is a lot of research work employing thermoresponsive materials with various structure and compositions. However, little is known about the immunological response to the thermoresponsive materials. Macrophage-like transformed murine cell line RAW264.7 was selected as it is a widely used standard model for immune activation analysis. This study proposes to compare the effects of thermoresponsive films with various compositions on macrophage cells. Thermoresponsive materials are a useful tool to use non-enzymatic harvesting systems for tissue culture. As RAW264.7 cells are difficult to remove from the substrate by enzymatic methods we also explored the possibility to use thermoresponsive materials for the macrophage cultivation. Spin coating and solvent casting was used to produce films of N-isopropylacrylamide-based polymers from the nanometer to micrometer range. Successful cell adhesion and proliferation was highly dependent on the thickness and composition of the coating. RAW264.7 cells were successfully detached from the coatings upon temperature reduction. Furthermore, results indicate that the RAW264.7 cells remained activated as cell secreted cytokine remained at a low level and the surface receptor profile of RAW264.7 was not altered when cells were detached in this manner. © 2013 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 00A000-000, 2013.

Key Words: thermoresponsive polymers, RAW264.7, N-isopropylacrylamide, cell-surface interaction, macrophage activation.


INTRODUCTION

The development of thermoresponsive materials for cell harvesting is currently an increasingly popular research area. Thermoresponsive materials and surfaces are powerful tools for creating tissue-like constructs that imitate native tissue geometry and mimic its spatial cellular organization. A lot of attention is focused on the rational design of biomaterials for cell culture and for further utilization in tissue reconstructions. The interest in thermoresponsive polymers has steadily grown over decades, and a great deal of work has been dedicated to developing temperature-sensitive macromolecules that can be crafted into new smart materials. A recently emerging area is the development of thermoresponsive polymers to cultivate and harvest cells which are able to prevent any animal interference. For instance, we published an article showing that adherent cell films prepared from commercially sourced Poly(N-isopropylacrylamide) (pNIPAm) with an optimal film thickness of 1 μm offer a convenient, simple and cheap alternative for producing thermoresponsive surfaces with a view to cell and cell sheet regeneration. The film was used for the multidimensional passaging of hMSCs. It was found that cell proliferation through the passaging was similar in all cases and the plating expertise of the hMSCs was preserved. Another example is the use of thermoresponsive nanocomposite gels to proliferate and harvest human mesenchymal stem cells.

Additional Supporting Information may be found in the online version of this article.

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Appendix II

(9MECs), which should be effectively cultured in vitro for tissue engineering and regenerative medicine. There are some thermoresponsive products for cell culture available on the commercial market. An example is Nunc UpCell Surface, which supports non-enzymatic harvesting of adherent cells for preservation of cell viability and surface proteins; however, the optimal thermoresponsive coating is still under development.

Some research has been conducted on cell-thermoresponsive material interactions, where cells exhibited different adhesion and proliferation behavior depending on the kind of thermoresponsive polymers due to their individual cell adhesion behavior. An important question to be answered can thermoresponsive materials change the adhered cell’s phenotype? All parameters related to thermoresponsive films should undergo comparative analysis. It is not only chemical composition which can affect the cell behavior but also film thickness. The complete deciphering of the cell-material communication code is still far away.

The present study is based on two popular polymer film deposition methods (spin coating and solvent casting) using pNIPAm and the more hydrophobic material N-isopropylacrylamide (NIPAm). N-isopropylacrylamide-co-N-isopropylacrylamide (NIPAm-co-NIPAm) copolymer was selected because it showed better cell compatibility and had an lower critical solution temperature (LCST) lower than room temperature, which makes the biomaterial much easier for routine lab usage.

Macrophage-like transformed murine cell line RAW264.7 was selected as it is the widely used standard model for modern immune activation macrophages orchestrate the immune response to an implanted biomaterial and play a key role in determining the biocompatible outcomes. Macrophages play a pivotal role in initiating, maintaining, and resolving host inflammatory responses by killing viruses, bacteria, and parasites and acting as scavenger cells. On the other hand, macrophages also exert deleterious effects on the host by inducing pro-inflammatory cytokines. It is also well known that RAW264.7 cells are difficult to remove from certain growth substrates, so thermoresponsive materials are a useful utility. Macrophage adhesion to plasma processed surfaces is distinct from that exhibited by fibroblasts as they tend to establish stronger cell-surface contacts. Incubating cells with too high a tripsy concentration for too long a time period will damage cell membranes and kill the cells. Alternatives have been utilized such as scraping. However, scraping can dramatically reduce the total viability to any cell type and it is not certain if the dead cells will also release intracellular contents that might affect the viable cells.

Based on these considerations, the main goals for this study are: (1) to define the macrophage cell activation level and receptor profile based on different compositions and thicknesses of polymer films, (2) to setup a practical acceptable and simple thermoresponsive substrate for routine lab work. Two thermoresponsive polymers were used for this study: pNIPAm and NIPAm-co-NIPAm (65%/35% molar ratio) copolymer which is more hydrophobic than that of pure poly-NIPAm. RAW264.7 cells were seeded onto both pNIPAm and NIPAm-co-NIPAm thin films ranging from 100 to 4 μm in thickness, and which were fabricated by spin coating and solvent casting. The in vitro response of RAW264.7 cells to these ultrathin and thin films with regards to adhesion, morphology changes, cytokine secretion and cell surface molecule expression were studied.

**Materials and Methods**

Materials

pNIPAm was purchased from Sigma-Aldrich with the LCST at 32°C as stated by supplier. NIPAm-co-NIPAm was synthesised as previously described. The LCST of NIPAm-co-
NIPAm was measured using microcalorimetric method at 16°C in previous studies. The copolymer (65% NIPAm and 35% NIPAm) was synthesised by free radical polymerization using 2,2'-azoisobutylnitrile (0.5 mol%) as an initiator in benzene (10% w/v) under argon. After polymerization at 60°C for 24 h, the mixture was precipitated from acetone to become three times, the product was dried at room temperature in vacuum. Anhydrous ethanol (200 proof, 99.5%), Dulbecco’s modified Eagles medium (MEM), fetal bovine serum (FBS), penicillin-streptomycin, Hank’s balanced salt solution (HBSS), trypsin-EDTA, Lipopolysaccharides (LPS) were purchased from Sigma-Aldrich and used as received. The macrophage-like transformed murine cell line RAW264.7 was kindly provided by the Regenerative Medicine Institute (REMEDi) in NUI Galway. CellTrace CFSE Cell Proliferation Kit was purchased from invitrogen”, Mouse IL-1β, ELISA Ready-SET-Go assay was purchased from e Bioscience. Anti-mouse CD80, Anti-mouse MHCII, Armenian Hamster IgG isotype Ctrl, κ Isotype Ctrl were from Biolegend. All other plastic consumables were from Sartorius.

**Film fabrication and film characterization**

Following films were fabricated for this study: solvent cast pNIPAm (1 μm, 2 μm, 4 μm); spin coated pNIPAm (100 nm, 1 μm); solvent cast NIPAm-co-NIPAm (1 μm, 2 μm, 4 μm); spin coated NIPAm-co-NIPAm (100 nm, 1 μm). The films were prepared on 35 mm Petri dishes. A vacuum oven, which was set to 40°C and 600 mbar was then used to remove the solvent. Films were sterilized by mild UV light for 2 h prior to any cell culture experiment. All samples were freshly made before each experiment. The purpose of this work is to compare the cell behavior on spin coated and solvent cast films. The thickness of the polymer films can be controlled by the application of precise amount of polymer over defined area. The thickness of spin coated film can be controlled by the variation of solution concentration with other parameters fixed. The concentration of polymer solution to obtain a desired thickness was estimated based on our previous work and is reported as expected thickness in Table 1. The spin coated films were fabricated by initially depositing 150 μL aliquot polymer solution onto the Tissue Culture Plastic (TCP) at 150 rpm following final speed 6000 rpm for 30 s, concentration 2% w/v and 10% w/v polymer solutions were used for 100 nm
### Appendix II

#### TABLE I. Theoretically Predicted Film Thickness Versus Thickness Measured by AFM Analysis

<table>
<thead>
<tr>
<th>Film type</th>
<th>Expected thickness</th>
<th>Measured film thickness</th>
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<tbody>
<tr>
<td>pNIPAm</td>
<td>Spin coating</td>
<td>100 nm</td>
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<tr>
<td></td>
<td></td>
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<tr>
<td></td>
<td>1 µm</td>
<td>1.05 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>2 µm</td>
<td>1.06 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>4 µm</td>
<td>2.04 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>4 µm</td>
<td>4.02 ± 0.04</td>
</tr>
<tr>
<td>NIPAm-co-</td>
<td>Spin coating</td>
<td>100 nm</td>
</tr>
<tr>
<td>NIPAm-co-</td>
<td></td>
<td>106.1 ± 0.9</td>
</tr>
<tr>
<td>NIPAm-co-</td>
<td>1 µm</td>
<td>1.16 ± 0.10</td>
</tr>
<tr>
<td>NIPAm-co-</td>
<td>2 µm</td>
<td>2.03 ± 0.08</td>
</tr>
<tr>
<td>NIPAm-co-</td>
<td>4 µm</td>
<td>4.03 ± 0.03</td>
</tr>
<tr>
<td>NIPAm-co-</td>
<td>Solvent casting</td>
<td>1 µm</td>
</tr>
<tr>
<td>NIPAm-co-</td>
<td></td>
<td>1.10 ± 0.10</td>
</tr>
<tr>
<td>NIPAm-co-</td>
<td>2 µm</td>
<td>2.05 ± 0.08</td>
</tr>
<tr>
<td>NIPAm-co-</td>
<td>4 µm</td>
<td>4.03 ± 0.03</td>
</tr>
</tbody>
</table>

#### TABLE III. Advancing Contact Angle Results Obtained by Advancing Drop Method on a Home-built Goniometer

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Stick angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>64.7 ± 0.5</td>
</tr>
<tr>
<td>pNIPAm</td>
<td>51.2 ± 1.0</td>
</tr>
<tr>
<td>pNIPAm</td>
<td>55.3 ± 1.6</td>
</tr>
<tr>
<td>NIPAm-co-</td>
<td>67.6 ± 2.0</td>
</tr>
<tr>
<td>NIPAm-co-</td>
<td>71.8 ± 1.1</td>
</tr>
<tr>
<td>NIPAm-co-</td>
<td>57.3 ± 1.4</td>
</tr>
<tr>
<td>NIPAm-co-</td>
<td>80.2 ± 0.9</td>
</tr>
<tr>
<td>NIPAm-co-</td>
<td>80.4 ± 0.5</td>
</tr>
<tr>
<td>NIPAm-co-</td>
<td>81.3 ± 0.6</td>
</tr>
<tr>
<td>NIPAm-co-</td>
<td>82.4 ± 1.8</td>
</tr>
<tr>
<td>NIPAm-co-</td>
<td>84.6 ± 1.2</td>
</tr>
</tbody>
</table>

and 1 µm, respectively. For solvent cast films, 2% w/v polymer solution in EtOH was spread on 35 mm TCP. The thickness of the solvent cast film can be estimated from total mass of polymer spread over defined area of Petri dish assuming dry polymer density 1 g cm⁻³.²⁹

Fourier transform infrared spectroscopy (FTIR) was used to obtain the films chemical composition. FTIR spectra were acquired using a Shimadzu FTIR-8300 spectrometer in transmission mode. Atomic force microscopy (AFM) was used to measure the film roughness and thickness. AFM images were obtained in tapping mode in air using a Dimension 3100 AFM (Digital Instruments, Santa Barbara, CA) and Veeco 100 nm/cm phosphorus (n) doped Si tips. A matrix of 512 × 512 data points along the x-y plane were analyzed in a single scan. The roughness assessment of the deposited films was taken by 10 × 10 µm scanning. The roughness of the films was reported as root-mean-square (rms) roughness values, where rms denotes the standard deviation of the Z-values along the reference line. For thickness measurement, a sharp blade was used to scratch the surface of the fully dried polymer film, fabricated as described above on the underlying substrate. 100 × 100 µm scanning images were recorded at a scan rate of 1 Hz in the area around the scratch. Contact angle measurements were made in order to access the hydrophobicity of the films. Advancing contact angle measurements were taken on a home-built goniometer as previously described.²⁹

#### TABLE II. Film RMS Roughness as Measured by AFM Analysis, Where the RMS Refers to the Standard Deviation of the Z-values Along the Reference Line

<table>
<thead>
<tr>
<th>Film type</th>
<th>Film thickness</th>
<th>RMS roughness (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pNIPAm</td>
<td>Spin coating</td>
<td>100 nm</td>
</tr>
<tr>
<td>pNIPAm</td>
<td>100 nm</td>
<td>13.5 ± 0.4</td>
</tr>
<tr>
<td>pNIPAm</td>
<td>1 µm</td>
<td>12.8 ± 0.7</td>
</tr>
<tr>
<td>pNIPAm</td>
<td>2 µm</td>
<td>16.0 ± 0.9</td>
</tr>
<tr>
<td>pNIPAm</td>
<td>4 µm</td>
<td>24.0 ± 4.9</td>
</tr>
<tr>
<td>NIPAm-co-</td>
<td>Spin coating</td>
<td>100 nm</td>
</tr>
<tr>
<td>NIPAm-co-</td>
<td>100 nm</td>
<td>12.1 ± 0.8</td>
</tr>
<tr>
<td>NIPAm-co-</td>
<td>1 µm</td>
<td>16.4 ± 1.1</td>
</tr>
<tr>
<td>NIPAm-co-</td>
<td>2 µm</td>
<td>13.4 ± 2.5</td>
</tr>
<tr>
<td>NIPAm-co-</td>
<td>4 µm</td>
<td>15.0 ± 1.7</td>
</tr>
<tr>
<td>NIPAm-co-</td>
<td>Solvent coating</td>
<td>100 nm</td>
</tr>
<tr>
<td>NIPAm-co-</td>
<td>100 nm</td>
<td>18.2 ± 1.4</td>
</tr>
</tbody>
</table>

Cell culture

RAW264.7 cells were maintained in DMEM, supplemented with 10% FBS and 1% penicillin-streptomycin antibiotics. Cells from passage 10 to passage 25 were used for experiments and assays where appropriate.

Briefly, RAW264.7 cells were seeded in triplicate at a density of 40,000 cell/cm² on the pNIPAm and NIPAm-co-NeBAm films. Rock TCP was used as negative control, while LPS (200 ng/mL) was used as positive control. Cells were incubated for 48 h respectively and the incubation conditions were a humidified atmosphere of 95% air and 5% CO₂ at 37°C to maintain a working temperature above the polymer LCST. Care was taken to make sure the sample temperature was maintained above the LCST during any operation to prevent premature cell detachment.

Cell attachment and detachment

Cell adhesion was determined microscopically observed using an Olympus BX51 phase contrast microscope with Image Pro-Plus software. Total DNA content of the cells attached on each kind of film and controls was quantified using the Quanti-IT PicoGreen dsDNA assay kit. Warm HESS was used to wash away the non-adhered cells while the PicoGreen assay was performed after three times of 5°C freeze thaw. Cell detachment was observed using Time Lapse Olympus IX81 motorised inverted microscope with temperature/CO₂ humidified incubation chamber for live cell experiments (Anatomy Department in NU Galway). For initial cell detachment, the original warm medium was removed followed by the addition of cold medium (20°C for pNIPAm films, 4°C for NIPAm-co-NeBAm films) and kept at low temperature for the duration of observation. The samples were then placed under the time lapse microscope immediately for imaging with 20 s per frame.

Cell proliferation and flow cytometry analysis

For cell proliferation assessment, Carbomylation was performed after cell seeding. This ensures that the cells on all substrates had enough time to adhere and proliferate. The seeding density for CFSE staining was reduced to 20,000 cell/cm² in order to prevent cell overgrowth. The cells were incubated for a further 48 h after staining. Flow cytometry was performed...
using BD FACSCanto to test the cell proliferation. For flow cytometry analysis, cells were detached from the thermoresponsive films as described, followed by gentle and repetitive pipetting to yield singular cells. Cells were detached from TCP controls via conventional trypsinization and scraping. The geometric mean values of FITC signal strength summation was then calculated for each kind of sample.

Interleukin 1β assessment

Interleukin 1β (IL-1β) release was assessed by ELISA assay. The cell culture medium was harvested for testing after 48 h of incubation and the standard IL-1β was diluted two fold with the starting point of 1000 pg/ml. The experimental procedure was followed by the standard protocol provided by eBioscience®.

Immunofluorescence staining

Cell surface proteins were also stained and examined by flow cytometry. The cells were stained with CD80 (APC labeled) and MHC II (FITC labeled) antibodies from Biorad, as per the manufacturer’s instructions and were analyzed by the flow cytometry using BD FACSCanto. The

![Image](image_url)

**FIGURE 1.** RAW264.7 cell adhesion on different types of thermoresponsive films. Cells attached successfully on solvent coated pNIPAm films as well as on both spin coated and solvent cast NIPAm-co-NIPAm films; where the cell morphology is the same as the positive control. Cells seeded without serum showed signs of apoptosis, the majority of cells seeded on spin coated pNIPAm film were still floating in the medium with few adhered. (A) RAW264.7 in condition of full medium on TCP; (B) RAW264.7 seeded on 1 µm thick spin coated pNIPAm film; (D) RAW264.7 adhesion on 1 µm thick solvent cast pNIPAm film; (E) RAW264.7 proliferation on 1 µm thick spin coated NIPAm-co-NIPAm film; (F) RAW264.7 adhesion on 1 µm thick solvent cast NIPAm-co-NIPAm film. Scale bar: 200 µm.

**FIGURE 2.** Total DNA content of RAW264.7 attached on each kind of film and control was quantified using the Quan-iT PicoGreen dsDNA assay after 48 h incubation. (A) PicoGreen results confirmed the microscopy image with only 20% DNA contents left on spin coated pNIPAm films. The majority of RAW264.7 cells seeded on spin coated pNIPAm films failed to attach, with only 20% DNA detected. Cell attachment on solvent cast pNIPAm films was decreased when the film thickness increased with the deduction rate around 10%. (B) DNA content on both spin coated and solvent cast NIPAm-co-NIPAm copolymer was similar to that on the TCP control. *: significant low DNA content. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

4 PAN ET AL. MACROPHAGES REACTION ON DIFFERENT NIPAM
Appendix II

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Average signal strength</th>
<th>G_r</th>
<th>Doubling time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>45771 ± 113</td>
<td>0</td>
<td>16.9 ± 0.3</td>
</tr>
<tr>
<td>pNIPAm</td>
<td>6386 ± 143</td>
<td>2.84 ± 0.04</td>
<td>15.3 ± 0.4</td>
</tr>
<tr>
<td>pNIPAm</td>
<td>12674 ± 845</td>
<td>1.38 ± 0.45</td>
<td>25.3 ± 0.4</td>
</tr>
<tr>
<td>1 µm (48 h)</td>
<td>10950 ± 210</td>
<td>2.17 ± 0.03</td>
<td>22.1 ± 0.3</td>
</tr>
<tr>
<td>Spinning</td>
<td>5329 ± 169</td>
<td>3.12 ± 0.06</td>
<td>15.4 ± 0.2</td>
</tr>
<tr>
<td>NIPAm-co-NBAm</td>
<td>6944 ± 124</td>
<td>2.72 ± 0.03</td>
<td>17.8 ± 0.2</td>
</tr>
<tr>
<td>Spinning</td>
<td>4274 ± 248</td>
<td>2.97 ± 0.06</td>
<td>16.8 ± 0.4</td>
</tr>
<tr>
<td>1 µm (48 h)</td>
<td>5221 ± 173</td>
<td>3.13 ± 0.05</td>
<td>15.3 ± 0.3</td>
</tr>
</tbody>
</table>

The signal strength was determined as $\sum_{n}^{N} F(t)$ C(1-n). The values are means.

Results were analyzed by FlowJo software. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

Physical characterization

FTIR confirmed the deposition of the thermoresponsive films. From the FTIR spectra, a C=O bending peak can be observed at around 1150 cm$^{-1}$, a C-H symmetric stretching peak at around 2930 cm$^{-1}$, and a N-H bending peak can be observed at approximately 1500 cm$^{-1}$. A C=O stretching peak is observed at approximately 1730 cm$^{-1}$. The characteristic C-H stretching vibrates at around 2930 cm$^{-1}$. An N-H stretching vibrates can be observed at approximately 3000 cm$^{-1}$. The FTIR spectra of NIPAm film is the same as that of NIPAm-co-NBAm film, as there is only one sub group difference between the NIPAm monomer and NBAm monomer.

The thicknesses of all fabricated films were obtained using AFM by measuring the 2 height distance between the TCP substrate which was exposed by a surgical blade and the polymer layer which was still left on the substrates. The results showed good agreement with the expected theoretical thickness values (Table I). The film roughness was also obtained using the AFM, and the results are shown in Table II.

The relative hydrophilicity/hydrophobicity of the polymer films was measured by advancing contact angle above the thermoresponsive polymer's LST. For PIPAm films we could only compare solvent cast and spin coated films for 1 µm film. Contact angle results indicated that 1 µm solvent cast PIPAm films were slightly more hydrophilic than their spin coated analogous (60° vs 58°, respectively). NIPAm-co-NBAm films were significantly more hydrophobic than PIPAm films (~80°) (Table II). In previous studies of cell adhesion and proliferation we found that copolymerization of NIPAm with more hydrophobic monomer NBAm produces the polymer which are able to support cell adhesion and proliferation better than NIPAm homopolymer.12,24 Same tendency is observed in the present work as it will be shown later.

Cell adhesion on thermoresponsive films

RAW264.7 cells were seeded on the prepared pNIPAm and NIPAm-co-NBAm films and observed microscopically for

![Figure 3](image-url)

FIGURE 3. Proliferation rate of RAW264.7 cells on different substrate films as measured by CFSE staining. Cells adhered to spin coated pNIPAm had higher fluorescence value, which means pNIPAm film fabricated by spin coating prohibited the RAW264.7 cell proliferation. However, cell proliferation on both solvent cast pNIPAm and NIPAm-co-NBAm films was similar or better than that of the control. (A) RAW264.7 CFSE staining measured on pNIPAm films; (B) RAW264.7 CFSE staining measured on NIPAm-co-NBAm films.
Appendix II

RAW264.7 detachment by temperature control
RAW264.7 cells were seeded on the prepared pNIPAm and NIPAm-co-NbRAm films and they were observed microscopically for cell detachment by simply reducing the temperature below the polymers' LCST. RAW264.7 cells started detaching immediately after the temperature dropped below the LCST. The cell morphology of the adhered cells changed dramatically within 5 min of the environmental temperature being dropped. The average attached cell length shrank from over 80 μm to less than 30 μm in 10 min after the temperature dropped, but the rounded up cells would keep sticking and then migrated to reattach under suitable condition.

Cell proliferation on thermoresponsive films
CFSE stain enters the cytoplasm of cells and is converted to the fluorescent ester by removal of its acetate groups by intracellular esterases. CFSE is then retained within cells and covalently coupies via its succinimidyl group to intracellular molecules. The covalently coupled fluorescent CFSE will stay inside cells for extremely long periods and cannot be transferred to adjacent cells. With the proliferation of individual cells, the fluorescent molecules will be divided evenly to each daughter generation, and the signal will become weaker and weaker as the cells passage down. Cells were treated with CFSE 24 h after cell seeding and incubated for a further 40 h before flow cytometry assessment. Statistics in Table IV shows that cells on spin coated pNIPAm proliferate less than control cells. Cells on solvent cast polymers proliferate similar to the control in both cases, but the proliferation on spin coated pNIPAm dramatically reduced compared to spin coated NIPAm-co-NbRAm and control. The cell proliferation on spin coated NIPAm-co-NbRAm is comparable to the control. Cells on the NIPAm-co-NbRAm films and solvent cast pNIPAm films have similar and similar peak readings as the control, while cells seeded on the spin coated pNIPAm film have higher values showing that cell proliferation was prohibited by the under layer substrate (Fig 3).

Cellular immunological response to thermoresponsive films
Interleukin-1 beta, produced by activated macrophages as a proinflammatory cytokine, plays an important role in the body's inflammatory response, while it is also involved in cell proliferation, differentiation and apoptosis. RAW264.7 cells were seeded and kept for 48 h. IL-1β ELISA Ready-SET-Go assay was used to assess the cytokine release. LPS (200 ng/mL) was used to treat RAW264.7 as positive control. Figure 4 shows the cytokine produced by RAW264.7 cells on each kind of sample. The standard deviation of RAW264.7 cells IL-1β release on spin coated pNIPAm films was not correlated to the normalized mean value, therefore no spin coated pNIPAm films were shown in the figure. Cells on NIPAm-co-NbRAm films and solvent cast pNIPAm films showed a low level IL-1β, but the overall cytokine release on pNIPAm type of films was higher than that on the copolymer films. There
Appendix II

![Graphs showing cell surface protein expression](image)

**FIGURE 5.** Surface expression of CD80 on RAW264.7 cells. CD80 surface levels were assessed by direct immunofluorescence and FACS analysis right after the cells detached by trypsin, scraper, and thermoresponsive films. Lower level of CD80 was found on the cell surface which detached by copolymer films, expression was not detectable on the surface which detached by trypsin.

is no significant difference between spin coated and solvent cast NIPAm-co-NBAm films.

By examining the cell surface molecules CD80 and MHC class II, we can know how the cells incubated in the presence of thermoresponsive films trypsinized or scraped can affect the macrophage surface molecules. In general cell culture experiments, RAW cells are always detached with the help of a scraper due to the failure of detachment using trypsin-EDTA. CD80 is a protein found on activated monocytes that provides a co-stimulatory signal, which is necessary for T cell activation and survival. The MHC II molecule is found on antigen-presenting cells and is also required for T cell activation.

It is necessary to analyse RAW264.7 cell surface protein expression under the same conditions for the different modes of detachment and the different polymer surfaces. We assessed CD80 and MHC II expression of cells detached from thermoresponsive films, cells detached by trypsin and scraper using immunofluorescence and flow cytometry. To this end, APC-conjugated CD80 and FITC-conjugated MHC II monoclonal antibody were used to stain the cells. CD80 expressions on cells detached by scraping have wide, multiple peaks, which indicate that this type of detachment could damage the cells. Cells detached from NIPAm-co-NBAm film expressed lower levels of CD80 compared to those detached from pNIPAm films. Spin coated films expressed more CD80 molecules than solvent cast films. Only 60% of attached cells were removed by trypsin after a 24 min treatment, and the expression of CD80 on trypsinised cells were not detectable (Fig. 5). The level of MHC II expression stays low in the presence of all types of polymers compared to control LPS stimulation. The co-incubation of RAW264.7 cells with...
all polymer films does not affect MHC expression (Fig. 6). Overall, the activation of attached RAW264.7 cells on pNIPAm films was a bit higher than that of the TCP control. The activation of cell surface MHC II molecule expression and IL-1β release on NIPAm-co-NBAm films was relatively low. From the results, it was found that IL-1β release was negatively correlated to RAW264.7 cell proliferation. A decrease in proliferation and a higher level of IL-1 serve as the evidence of final differentiation of RAW to macrophage, which means pNIPAm leads to RAW264.7 cell differentiation.

Spin coating and solvent casting film fabrication techniques allowed the preparation of smooth, thin and ultrathin thermoresponsive film coatings on to TCP substrates. Immunological responses to films were then conducted on the RAW264.7 cells which were seeded on the pNIPAm and NIPAm-co-NBAm films. This study also initiates the development of non-invasive monocyte/macrophage cell detachment techniques and could have further tissue engineering applications. It also improved the current strategies to limit macrophage adhesion, fusion and fibrous capsule formation in the foreign body response which have focused on modulating material surface properties.  

RAW264.7 cell adhesion and elongation was clearly found on solvent cast pNIPAm (1, 2, 4 μm) and NIPAm-co-NBAm films (spin coating 100 nm, 1 μm; solvent cast 1, 2,
Appendix II

4 μm), independent of the film hydrophobicity differences. However, the spin coated pNiPAm films (100 nm, 1 μm) failed to support RAW264.7 cell adhesion which indicates that the fabrication method would affect the macrophage adhesion approach. Our previous study has shown that other cell lines could attach on the spin coated films of pNiPAm confirming that attachment is cell line dependent.8 Solvent cast films were able to support RAW264.7 cell adhesion and proliferation better than spin coated films, especially on thinner pNiPAm films. The cell proliferation curve measured by flow cytometry; found a positive correlation for cell adhesion and proliferation on each kind of thermoresponsive film. RAW264.7 cells exhibited not only better cell adhesion, but also better cell proliferation and less IL-1β production on pNiPAm-co-NiBAm copolymer films. This showed that the chemically composition of the films can impact on monocyte/macrophase activation behavior.

The results from the functional response assays of RAW264.7 cells in terms of its IL-18 secretion and cell surface molecule expression showed that RAW264.7 cell do respond to thermoresponsive substrates. IL-1β is a sensitive indicator of immune stimulation that can help monitor the levels of cellular activation induced by different biomaterials.30 CD68 and MHC class II can be used to monitor the progress of an inflammatory response to an implanted material.31 Higher IL-1β release was found on pNiPAm films compared to the planar control and other type of samples. Cellular surface protein expressions can be modified by changing thermoresponsive film composition and fabrication method, which will make sure there is no further multiple step activation to the thermoresponsive substrate. The detachment from thermoresponsive polymer films also preserved the cellular surface molecules comparable to trypsin treatment. This gives the thermoresponsive polymer, especially NiPAm-co-NiBAm, a most promising advantage for macrophage culture and implant coating.

CONCLUSION

In this study the consistent evaluation of polymer films with variable compositions and fabrication methods confirmed that NiPAm-based polymers and copolymers provide a promising non-affecting alternative for RAW264.7 cell detachment. Film induced monocyte/macrophase activation can be controlled by modifying the fabrication method or polymer composition. It is widely known that cell functions such as adhesion, strength, spreading, intracellular signaling, and differentiation potential are affected by the cellular response to the substrate.32-34 Improving the understanding of macrophage cell response to the thermoresponsive films will provide the approaches for tissue cultivation as well as for biocompatible coatings.

The chemical composition, fabrication method, and thickness of thermoresponsive films have a major impact on monocyte/macrophase activation behavior. The cell response to the films fabricated by spin coating and solvent casting showed a distinctive pattern while the cell adhesion to pNiPAm, especially the spin coated one, exhibited negative effects. Small variations in preparation method and chemical composition of thermoresponsive films can significantly affect cell behavior on thermoresponsive substrates. The NiPAm-based copolymer offer a gentle alternative for cell detachment. Successful cell attachment and detachment was achieved by simply controlling the environmental temperature, offering an alternative to trypsin/verse not to be available. This study demonstrates that by modifying biomaterial thermoresponsive films, an easily produced thin film can be used for daily non-invasive macrophage culture as well as further thermoresponsive biomaterial applications.

ACKNOWLEDGMENTS

We gratefully acknowledge the financial support received from The Irish Research Council under the Embark scheme (Grant No.: 14/ST/2010/2807). Sincere thanks to David Connoly, Centre for Microscopy and Imaging at NUI, Galway (CM1) for the image acquisition and analysis. Many thanks to Dr. Kerry Thompson and Deirdre Healy for their kind help with time lapse microscopy. Thanks for the PRT115 funded Flow Cytometry facility in NGBES for the flow cytometry analysis. Thank andrew Kemple for his critical reading and helpful discussion. Finally we would like to dedicate this work to the memory of Dr. William Carroll, may he rest in peace.

REFERENCES


JOURNAL OF BIOMEDICAL MATERIALS RESEARCH A | MONTH 2013 VOL. 105, ISSUE 09
Appendix II


