Controlled particle production by membrane emulsification for mammalian cell culture and release

This item was submitted to Loughborough University's Institutional Repository by the/an author.

Additional Information:

- A Doctoral Thesis. Submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy of Loughborough University.

Metadata Record: [https://dspace.lboro.ac.uk/2134/14922](https://dspace.lboro.ac.uk/2134/14922)

Version: Not specified

Publisher: © Mariana Petronela Hanga

Please cite the published version.
This item was submitted to Loughborough University as a PhD thesis by the author and is made available in the Institutional Repository (https://dspace.lboro.ac.uk/) under the following Creative Commons Licence conditions.

For the full text of this licence, please go to:
http://creativecommons.org/licenses/by-nc-nd/2.5/
Controlled Particle Production by Membrane Emulsification for Mammalian Cell Culture and Release

by

Mariana Petronela Hanga

A thesis submitted to
Loughborough University
for the degree of

DOCTOR OF PHILOSOPHY

Centre for Biological Engineering
Chemical Engineering Department
Loughborough University
March 2014

© by Mariana Petronela Hanga (2014)
Existing commercially available microcarriers are very efficient at encouraging cell attachment and proliferation. However, recovery of the cells is problematic as it requires the use of proteolytic enzymes which are damaging to critical cell adhesion proteins. From this perspective, temperature responsive polymers appear to be a valid option. The current innovative study is to produce and engineer microcarriers in terms of particle size, surface coating and properties, as well as thermo-responsiveness for cell release. All these benefits are based on particle production by membrane emulsification to provide a highly controlled particle size. The polymer of choice is poly N-isopropylacrylamide (pNIPAM) because of the sharpness of its phase transition, biocompatibility and transition temperature close to the physiological value. These characteristics make pNIPAM a very attractive material for Tissue Engineering applications. Cells are cultured on the hydrophobic surface at 37°C and can be readily detached without using proteolytic enzymes from the surface by lowering the temperature to room temperature.

The Dispersion Cell (MicroPore Technologies Ltd, UK) was successfully employed for the production of W/O emulsions. The generated monomer droplets were additionally solidified by applying a free radical polymerisation to manufacture solid pNIPAM microspheres. Additionally, calcium alginate particles were also generated and further functionalised with amine terminated pNIPAM to form temperature responsive core-shell particles by simply taking advantage of the electrostatic interactions between the carboxyl groups of the alginate and amino groups of the modified pNIPAM. Controlled particle production was achieved by varying process parameters and changing the recipe formulation (e.g. monomer concentration, surfactant concentration, pore size and inter-pore spacing, injection rate, shear stress applied at the membrane’s surface). The manufactured particles were then analysed in terms of particle size and size distribution, chemical composition, surface
analysis, shrinkage ratio and thermo-responsiveness and further sterilised and used for cell culture and release experiments.

Swiss Albino 3T3 fibroblastic cells (ATCC, USA) were utilised to show proof-of-concept for this technology. Cell attachment and proliferation were assessed and successfully demonstrated qualitatively and quantitatively. pNIPAM solid particles, uncoated and with different protein coatings were shown to allow a limited degree of cell attachment and proliferation compared to a commercially available microcarrier. On a different approach, uncoated core-shell structures demonstrated improved capabilities for cell attachment and proliferation, similar to commercially available microcarriers.

Having in mind the potential of temperature responsive polymers and the aim of this innovative study, cell detachment from the generated microcarriers was evaluated and compared to a commercially available temperature responsive surface. Necessary time for detachment was recorded and detached cells were recovered and reseeded onto tissue culture plastic surfaces in order to evaluate the replating and reattachment capabilities of the recovered cells. Successful cell detachment was achieved when using the core-shell structures as cell microcarriers, but the necessary time of detachment was of an order higher than that for the commercial temperature responsive surface.

*Keywords: membrane emulsification; pNIPAM; Alginate; monodisperse; microcarriers; non-enzymatic cell harvesting; core-shell particles*
This thesis would feel incomplete without the acknowledgements section.

When I first joined the DTC program in October 2009, I had to move to UK from Romania and start everything from zero. I still remember how scared and alone it felt at the beginning, but as time passed I have learnt to appreciate more and more the opportunity that I was given, to do a PhD in the UK and especially in the growing and very exciting field of Regenerative Medicine.

Moving to a new country with a foreign language and a new life style, as well as with no friends, it was very hard and I am grateful to my family and my friends from Romania who were very supportive and encouraged me to move forward. I thank my dear mom for being there for me whenever I needed, giving me advice and showing me compassion and understanding.

My most gratitude and words can’t express it enough...goes to my boyfriend, recently become my husband, Tudor who was there for me from the beginning even if he was so far away and who always reminded me why all the sacrifices that I have made were worthwhile and what I was here to achieve. I thank you my love for all your support, patience, love, care, advice, for making me laugh when I was sad and frustrated and for being my rock to lean on when I needed the most.

Even if the start was difficult, along the way I have met some extraordinary people that now are my friends Karina, Chris, Mazy, Dave, Mae, Pete, Andy and my ‘UK family’, Andreea and Chris and recently their new addition to the family, baby Olivia. Also, I want to acknowledge our ‘DTC mother’, Liz, who is an amazing person and she was always there to give me advice and listen to me.
My supervisor, Professor Richard Holdich deserves a special thanks and a whole paragraph. I first met him at the interview for the DTC PhD project selections at the end of 2010. Since then, I had a huge amount of respect towards him and that not only because of the vast knowledge that he showed, but also because he is an extraordinary person, very understanding, but demanding at the same time. This is why I became even more ambitious and wanted to prove to Prof. Holdich that I am willing to learn as much as possible during this PhD. And indeed, during these 3 years, I have learnt more from Prof. Holdich than from any other teacher that I had. I want to thank Prof. Holdich for his understanding, especially towards to end of this PhD when it was a very difficult and stressful period for me because of the pressure of the PhD, but also because of some family problems that occurred unexpectedly. Prof. Holdich was not only my supervisor during this period of three years, but also my mentor. I will always be grateful to him!
PUBLISHED WORK

Peer reviewed papers

Hanga, M.P., Holdich, R.G., Membrane emulsification for the production of uniform poly-

Conference papers

Pluripotent stem cell recovery from polymer microcarriers produced by Membrane Emulsification. XX Bioencapsulation newsletter, December 2012

Oral presentations

- 21st – 24th September 2012, XX International Conference on Bioencapsulation, Orillia, Ontario, Canada: In-house controlled particle production by Membrane Emulsification for cell culture and release
- 18th April 2013, DTC Cross Cadre Conference, Loughborough, UK: Microcarrier Engineering: Controlled particles production by Membrane Emulsification for cell culture and release
- 13th May 2013, Health and Life Sciences Research Student Conference III, Loughborough, UK: Controlled temperature responsive particles production by Membrane Emulsification for cell culture and release without the use of enzymes
# TABLE OF CONTENTS

Abstract .........................................................................................................................ii

Acknowledgements ........................................................................................................iv

Published work ..............................................................................................................vi

Table of contents ..........................................................................................................vii

List of figures ................................................................................................................xvii

List of tables ................................................................................................................xxix

List of abbreviations ....................................................................................................xxxiii

1. GENERAL INTRODUCTION ......................................................................................1

1.1. Aims and motivation ...............................................................................................2

1.2. Project methodology .............................................................................................4

1.3. Research questions ...............................................................................................5

2. LITERATURE REVIEW AND BACKGROUND THEORY .........................................6

2.1. Regenerative medicine overview ..........................................................................6

2.2. Biomaterials and their applications in regenerative medicine ................................8

2.2.1. Importance of biomaterials in regenerative medicine ......................................8

2.2.2. Natural Polymers ...............................................................................................9

2.2.2.1. Alginate ........................................................................................................10
# TABLE OF CONTENTS

2.2.2.1. Alginate chemical structure ............................................................. 11

2.2.2.1.2. Production of alginate hydrogels.................................................. 12

2.2.2.1.3. Suitability of alginate hydrogels for regenerative medicine applications .......................................................................................................................... 14

2.2.3. Synthetic polymers .............................................................................. 16

2.2.3.1. Stimuli-responsive polymers............................................................. 17

2.2.3.1.1. poly (N-isopropyl-acrylamide) (pNIPAM) ................................... 21

2.2.3.1.1.1. Chemical structure................................................................. 22

2.2.3.1.2. Phase transition temperature....................................................... 24

2.2.3.1.3. pNIPAM polymerisation methods.............................................. 26

2.2.3.1.4. Crosslinker selection................................................................. 27

2.2.3.1.5. Applied chemistry of choice ...................................................... 28

2.3. Microsphere-based strategies applied to regenerative medicine ........... 33

2.3.1. Advantages over 2D strategies............................................................ 35

2.3.2. Types of microcarriers........................................................................ 37

2.4. Controlled fabrication of microcarriers.................................................. 39

2.4.1. Emulsions............................................................................................ 39

2.4.2. Emulsification methods....................................................................... 42

2.4.3. Membrane emulsification.................................................................... 43
2.4.3.1. Membrane emulsification-based devices .................................................. 44

2.4.3.2. Membranes ................................................................................................. 49

2.4.3.3. Droplet detachment modelling ................................................................. 52

3. GENERAL MATERIALS AND METHODS .............................................................. 56

3.1. Chemicals, reagents, consumables and equipment ........................................ 56

3.2. Dispersion Cell ................................................................................................. 56

  3.2.1. General procedure ...................................................................................... 56

  3.2.2. Metallic membranes .................................................................................. 57

3.3. Particle production .......................................................................................... 58

  3.3.1. Solid pNIPAM particle production .......................................................... 59

  3.3.2. Core-shell pNIPAM-coated Alginate particle production ......................... 60

    3.3.2.1. Materials .............................................................................................. 60

    3.3.2.2. Production of core Calcium Alginate particles ..................................... 62

    3.3.2.3. pNIPAM coating of the core particles .................................................. 63

3.4. Particle characterisation techniques .............................................................. 64

  3.4.1. Interfacial tension measurements ............................................................ 64

  3.4.2. Refractive index measurements .............................................................. 66

  3.4.3. pNIPAM phase transition measurements ................................................ 66
3.4.4. Particle size and size distribution analysis ......................................................... 67

3.4.5. Particles shrinkage ratio ...................................................................................... 68

3.4.6. Fourier Transform Near InfraRed (FT-NIR) analysis ........................................ 68

3.4.7. Scanning Electron Microscopy coupled with Energy-dispersive X-ray spectroscopy (EDX) ........................................................................................................... 69

3.4.8. Water Contact Angle (WCA) measurements ...................................................... 69

3.5. Mammalian cell culture .......................................................................................... 70

3.5.1. Cell line .............................................................................................................. 70

3.5.2. Cell culture procedure ........................................................................................ 72

3.5.2.1. Cell resuscitation ........................................................................................... 72

3.5.2.2. General cell culture procedure ..................................................................... 73

3.5.2.3. Cryopreservation protocol ............................................................................ 74

3.6. Microcarrier cell culture ......................................................................................... 75

3.6.1. Microcarrier preparation ..................................................................................... 75

3.6.1.1. pNIPAM particles preparation ...................................................................... 75

3.6.1.2. pNIPAM-coated Alginate particles preparation ............................................ 77

3.6.1.3. Cytodex-1 (GE Healthcare, USA) particles preparation ............................... 78

3.6.2. Microcarrier general cell culture procedure ....................................................... 79

3.7. Analytic techniques .............................................................................................. 80
TABLE OF CONTENTS

3.7.1. Cell counting and visualisation ...............................................................80

3.7.2. Cell viability/cytotoxicity assessment .................................................80

3.7.3. Cell proliferation assessment ...............................................................81

3.7.4. Determination of metabolite concentration ......................................84

3.8. Statistical analysis ..................................................................................84

4. CONTROLLED pNIPAM PARTICLE PRODUCTION BY MEMBRANE
EMULSIFICATION .........................................................................................85

4.1. Introduction ............................................................................................85

4.2. Aims and objectives ..............................................................................86

4.3. Controlled production of pNIPAM solid particles ...............................87

4.3.1. Effect of crosslinker concentration on particle size .........................87

4.3.2. Effect of surfactant concentration on particle size ............................91

4.3.3. Influence of process parameters .......................................................94

4.3.3.1. Influence of injection flux on particle size ..................................95

4.3.3.2. Influence of shear stress applied at the membrane’s surface .........100

4.3.3.3. Reproducibility of the results .....................................................103

4.4. Solid particle characterisation ...............................................................105

4.4.1. Particle preparation for analysis and further use ............................105
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.4.1.1. Particle drying methods</td>
<td>105</td>
</tr>
<tr>
<td>4.4.1.2. Rehydration of the dried temperature responsive particles</td>
<td>110</td>
</tr>
<tr>
<td>4.4.2. FT-NIR spectrums collection</td>
<td>111</td>
</tr>
<tr>
<td>4.4.3. SEM coupled with EDX analysis</td>
<td>113</td>
</tr>
<tr>
<td>4.4.4. Evaluation of the temperature responsiveness of the generated particles</td>
<td>114</td>
</tr>
<tr>
<td>4.4.4.1. Cloud point measurements by absorbance variation with temperature</td>
<td>115</td>
</tr>
<tr>
<td>4.4.4.1.1. Cloud point measurements of pNIPAM in solution</td>
<td>115</td>
</tr>
<tr>
<td>4.4.4.1.2. Cloud point measurements of pNIPAM crosslinked hydrogels</td>
<td>118</td>
</tr>
<tr>
<td>4.4.4.2. Refractive index measurements for determination of LCST</td>
<td>121</td>
</tr>
<tr>
<td>4.4.4.2.1. Effect of polymer crosslinking degree on the LCST measurements</td>
<td>122</td>
</tr>
<tr>
<td>4.4.5. Changes recorded in response to temperature variation</td>
<td>123</td>
</tr>
<tr>
<td>4.4.5.1. Swelling ratio of gels in different media</td>
<td>123</td>
</tr>
<tr>
<td>4.4.5.2. Shrinkage ratio as a response to temperature changes</td>
<td>125</td>
</tr>
<tr>
<td>4.4.6. Wettability of pNIPAM solid particles in comparison with commercially available temperature responsive surfaces</td>
<td>127</td>
</tr>
<tr>
<td>4.5. Conclusions</td>
<td>130</td>
</tr>
</tbody>
</table>
5. CONTROLLED CORE-SHELL PARTICLE PRODUCTION BY MEMBRANE EMULSIFICATION

5.1. Introduction

5.2. Aims and objectives

5.3. Formulation

5.4. Controlled production of calcium alginate particles

5.4.1. Effect of CaCO₃ concentration

5.4.2. Effect of surfactant concentration

5.4.3. Influence of process parameters

5.4.3.1. Influence of the shear stress and dispersion flux

5.4.3.2. Influence of inter-pore spacing

5.4.3.3. Comparison between disc and annular membrane

5.4.3.4. Reproducibility of the results

5.5. Generation of core-shell temperature responsive particles

5.5.1. Temperature responsive polymer characterisation

5.5.2. Langmuir sorption isotherm

5.5.2.1. Sorption isotherm theory

5.5.2.2. Construction of the Langmuir sorption isotherm of pNIPAM chemisorbed on the calcium alginate particles
TABLE OF CONTENTS

5.5.2.2.1. UV/VIS measurements method ............................................. 160

5.5.2.2.2. Turbidimetry measurements method .................................... 163

5.6. Core-shell particle characterisation .............................................. 166

5.6.1. Visual confirmation of a successful coating ............................ 166

5.6.2. SEM coupled with EDX analysis .............................................. 168

5.6.3. FT-NIR spectrums collection .................................................. 169

5.6.4. Shrinkage ratio as a response to temperature variation .......... 170

5.6.5. Wettability of pNIPAM-coated Alginate .................................. 173

5.7. Conclusions ................................................................................ 175

6. EVALUATION OF GENERATED PARTICLES FOR CELL ATTACHMENT
   AND PROLIFERATION CAPABILITIES ............................................ 177

6.1. Introduction .............................................................................. 177

6.2. Aims and objectives ............................................................... 178

6.3. Experimental procedure ........................................................ 179

6.4. Evaluation of cell attachment and proliferation on pNIPAM solid micro-
carriers ..................................................................................... 180

6.4.1. Comparison of different chemical composition particles performance ........................................ 181

6.4.2. Improvements in cell adhesion and growth ............................ 189
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5. Evaluation of cell attachment and proliferation on core-shell temperature responsive microcarriers</td>
<td>195</td>
</tr>
<tr>
<td>6.5.1. Qualitative and quantitative evaluation of cell growth</td>
<td></td>
</tr>
<tr>
<td>6.5.2. Cell adhesion and growth enhancement</td>
<td>201</td>
</tr>
<tr>
<td>6.6. Conclusions</td>
<td>204</td>
</tr>
<tr>
<td>7. NON-INVASIVE THERMALLY-INDUCED CELL DETACHMENT</td>
<td>207</td>
</tr>
<tr>
<td>7.1. Introduction</td>
<td></td>
</tr>
<tr>
<td>7.2. Aims and objectives</td>
<td></td>
</tr>
<tr>
<td>7.3. Evaluating the commercially available 2D surface for cell detachment capabilities without the use of proteolytic enzymes</td>
<td>209</td>
</tr>
<tr>
<td>7.3.1. Experimental procedure</td>
<td></td>
</tr>
<tr>
<td>7.3.2. Study of cell seeding density effect on cell detachment</td>
<td>211</td>
</tr>
<tr>
<td>7.3.3. Cell detachment recording for different conditions</td>
<td>217</td>
</tr>
<tr>
<td>7.3.4. Cell recovery after external stimuli-induced detachment</td>
<td>219</td>
</tr>
<tr>
<td>7.4. Stimuli-based cell harvesting capabilities of produced temperature responsive particles used as cell microcarriers</td>
<td>221</td>
</tr>
<tr>
<td>7.4.1. Experimental procedure</td>
<td></td>
</tr>
<tr>
<td>7.4.2. Cell harvesting from pNIPAM solid particles</td>
<td>223</td>
</tr>
<tr>
<td>7.4.3. Cell harvesting from core-shell thermo-responsive particles</td>
<td>224</td>
</tr>
</tbody>
</table>
7.5. Discussion and conclusions.................................................................230

8. SUMMARY, CONCLUSIONS AND FUTURE WORK.................................233
  8.1. Summary..............................................................................................233
  8.2. Concluding remarks............................................................................238
  8.3. Future work.........................................................................................241

9. REFERENCES ..........................................................................................245

10. APPENDICES .........................................................................................279
  A. APPENDIX 1.........................................................................................279
    A1. List of equipment and consumable utilised ........................................279
    A2. Motor and pump calibrations............................................................280
    A3. Presto Blue assay calibration curves................................................283
  B. APPENDIX 2.........................................................................................284
    B1. Image J analysis..................................................................................284
    B2. Modelling calculations.................................................................286
LIST OF FIGURES

1. INTRODUCTION

Figure 1-1. Project methodology representation

2. LITERATURE REVIEW AND BACKGROUND THEORY

Figure 2-1. Chemical structures of the two units of alginate (A) and cross-linked alginate by stacking of G-blocks to form an egg-box structure (B)

Figure 2-2. Conformational changes of pNIPAM upon temperature modification (adapted after Chaterji S. et al. (2007))

Figure 2-3. Chemical structure of pNIPAM

Figure 2-4. Cell adhesion to thermo-responsive culture surfaces at 37°C and detachment by lowering the temperature (adapted after Zhang R. (2008))

Figure 2-5. The chemical structure of the compounds participants to the reaction

Figure 2-6. APS has the ability to exist as free radicals when dissolved in water

Figure 2-7. Reaction mechanism between APS and TEMED resulting in the generation of free radicals; TEMED acts as an additional catalyst to the reaction through its ability to exist as a free radical

Figure 2-8. pNIPAM cross-linking reaction mechanism using MBA as cross-linker. The free radicals that initiate the crosslinking reaction are provided by the reaction between APS and TEMED

Figure 2-9. Chemical structure of sorbitan-based surfactants
LIST OF FIGURES

Figure 2-10. Schematic of a typical small scale membrane emulsification apparatus (Nakashima T. et al., 1992)

Figure 2-11. Schematic diagram of membrane emulsification stirred system employing a tube membrane (1) Nitrogen gas tank; (2) Pressure gauge; (3) Dispersion phase storage module; (4) Porous glass membrane; (5) Continuous phase; (6) Magnetic stirrer (You J.O. et al., 2001).

Figure 2-12. Schematic representation of the Dispersion Cell device used for the production of droplets

Figure 2-13. Schematic representation of the array of pores on the metallic membranes employed (Dragosavac M.M. et al., 2008). The scale bar on the image represents 100 µm

Figure 2-14. Representation of forces acting during droplet detachment from the pore of a membrane (adapted from Charcosset C. et al., 2004).

3. GENERAL MATERIALS AND METHODS

Figure 3-1. Reduced and Inherent viscosity plot against polymer concentration. Determination of the intrinsic viscosity of the polymer further used for determination of the polymer’s molecular weight; Values are expressed as means of triplicates.

Figure 3-2. Image of the FT-NIR Antaris II analyser with a reflectance sampling module

Figure 3-3. 3T3 cell morphology when cultured on TCPS at different time points. Scale bars represent 500 µm.
LIST OF FIGURES

Figure 3-4. Presto Blue assay principle: The weakly fluorescent resazurin is reduced when entering the viable cell environment to resorufin, a strongly fluorescent component.

4. CONTROLLED pNIPAM PARTICLE PRODUCTION BY MEMBRANE EMULSIFICATION

Figure 4-1. The effect of crosslinker concentration on 5% wt and 10% wt pNIPAM particles A) size and B) size distribution expressed as span values, when the dispersed phase was injected through an array of 20 µm pores and 80 µm pore spacing at a rate of 1.1 mL/min and a shear stress of 8.5 Pa.

Figure 4-2. Micrographs of temperature responsive particles containing a) 5% wt monomer and b) 10% wt monomer and different concentrations of crosslinker, generated at an injection rate of 1.1 mL/min and a shear stress of 8.5 Pa when employing an array of 20 µm pores and 80 µm pore spacing. Scale bars correspond to 100 µm.

Figure 4-3. The effect of surfactant concentration on 5% wt and 10% wt pNIPAM particle A) size and B) size distribution expressed as span values, when the dispersed phase was injected through an array of 20 µm pores and 80 µm pore spacing at a rate of 1.1 mL/min and a shear stress of 8.5 Pa.

Figure 4-4. a) Variation of particle volume median diameter with dispersed phase injection flux at three different rotational speeds applied. b) Variation of span values with dispersed phase injection rate at three different rotational speeds applied. The membrane employed for this experiment had 20 µm pores and 80 µm pore spacing.

Figure 4-5. Variation of particle volume median diameter (a) and span values (b) with dispersed phase injection flux at three different rotational speeds applied when employing a membrane with 20 µm pores and 200 µm pore spacing.
Figure 4-6. Median particle size dependence on the shear stress applied at the membrane surface when using a 20 μm pore membrane with a) 80 μm and b) 200 μm pore spacing.

Figure 4-7. Median particle diameter obtained from three different sets of experiments when employing a 20 μm pore membrane with an 80 μm inter-pore spacing at different shear stress values and an injection rate of 1.1 mL/min.

Figure 4-8. Median particle diameter obtained from three different sets of experiments when employing a 20 μm pore membrane with a 200 μm inter-pore spacing at different shear stress values and an injection flux of 3.03 mL/min.

Figure 4-9. Photomicrographs of 5% wt low crosslinked thermo-responsive particles before and after freeze drying; the scale bars in the images represent 100 μm. The arrows point out the elongated tail-shaped dried pNIPAM particles.

Figure 4-10. High resolution SEM images of pNIPAM 5% wt low crosslinked freeze dried particles. Scale bars represent: a) 1 mm; b) 100 micrometers; c) 60 micrometers.

Figure 4-11. Photomicrographs of temperature responsive particles air dried from ethanol before and after drying. Scale bars represent 100 μm.

Figure 4-12. High resolution SEM images of the air dried temperature responsive 5% wt low crosslinked particles.

Figure 4-13. Images of re-hydrated freeze dried temperature responsive particles after 24 hours of hydration. Scale bars represent 100 μm. The arrows point to particles that have irreversibly collapsed, thus rendering their re-swelling capabilities.

Figure 4-14. Microscope images of re-hydrated air dried pNIPAM particles. Scale bars represent 100 μm.
LIST OF FIGURES

Figure 4-15. FT-NIR spectrums of generated pNIPAM particles compared to commercially available pNIPAM (Sigma Aldrich, UK) and the NIPAM monomer (Sigma Aldrich, UK).

Figure 4-16. Elemental spectrum of the generated pNIPAM particles obtained by EDX coupled with SEM

Figure 4-17. Absorbance variation with temperature for pNIPAM (Sigma Aldrich, UK) solutions of low concentrations varying from 0.05 mg/mL to 10 mg/mL

Figure 4-18. Absorbance variation with temperature increase for pNIPAM (Sigma Aldrich, UK) solutions of high concentrations varying from 30 mg/mL to 100 mg/mL

Figure 4-19. Absorbance variation with temperature increase from 27°C (below Cloud Point) to 42°C (above Cloud Point), recorded at 560 nm for pNIPAM hydrogels containing 10% wt monomer and with different crosslinking degrees given by the crosslinker concentration used for the preparation of the hydrogels

Figure 4-20. a) Schematic diagram of pNIPAM films distribution in the 12-well plate. b) Photographs of 5% wt and 10% wt pNIPAM hydrogels with different crosslinking degrees above and below LCST showing volume changes in response to temperature increase

Figure 4-21. Refractive index variation with temperature increase for low crosslinked pNIPAM 5% wt hydrogels

Figure 4-22. Phase transition temperatures measurement of pNIPAM 5% wt hydrogels with different crosslinking degrees

Figure 4-23. Swelling ratio variation of 10% wt pNIPAM hydrogels with crosslinker concentration in RO water, below and above LCST; Experimental data plotted
as mean values of three replicates; Error bars are given by standard deviation; 
n=3.

Figure 4-24. Water Contact Angle images and measurements recorded for pNIPAM 10% wt 
hydrogel films compared to TCPS and commercially available two dimensional 
temperature responsive surfaces UpCell (NUNC)

5. CONTROLLED CORE-SHELL PARTICLE PRODUCTION BY MEMBRANE 
EMULSIFICATION

Figure 5-1. Optical photomicrographs of produced calcium alginate particles when 
employing different formulations (F₁ to F₉) (refer to Table 5-1). Scale bars 
represent 100 µm.

Figure 5-2. (a) Optical micrographs of monosized spherical calcium alginate particles 
generated at an injection rate of 0.58 mL/min and a shear stress of 27 Pa (b) SEM 
photographs of dried calcium alginate particles. Scale bars represent: a) 100 µm; b) 100 µm 
(left) and 90 µm (right).

Figure 5-3. Scanning Electron microscopy (SEM) image of calcium carbonate particles used 
to provide internal gelation of alginate

Figure 5-4. The effect of calcium carbonate concentration on median calcium alginate 
particle A) size and B) size distribution expressed as span values

Figure 5-5. Influence of surfactant concentration on particle A) size and B) size distribution 
expressed as span values

Figure 5-6. Effect of peak shear stress at the membrane surface on (a) size and (b) size 
distribution expressed as span values of the generated alginate particles, as a
measure of different flow rates expressed as volume of dispersed phase injected through the membrane’s pores per minute (mL/min); the membrane used in this study had an array of 20 µm pores and 80 µm spacing between pores

Figure 5-7. Comparison of alginate particles size and size distribution expressed as span values generated at 0.58 mL/min with 20 µm pore size with different pore spacing

Figure 5-8. Controlled alginate particle production when employing an annular ring membrane; the effect of process parameters on median particle size

Figure 5-9. The effect of process parameters on particle size distribution expressed as span values

Figure 5-10. Comparative study of the shear stress effect on particle size when employed two different types of membranes: disc and annular

Figure 5-11. Comparative study of the shear stress effect on size distribution when employed two different types of membranes: disc and annular

Figure 5-12. Reproducibility of the results as demonstrated by three sets of experiments performed at different peak shear stress values and an injection rate of 0.58 mL/min when the dispersed phase was passed through the 20 µm pores of a metallic membrane with 80 µm pore spacing

Figure 5-13. Phase transition of amine-terminated pNIPAM (20 ppm) below and above LCST.

Figure 5-14. LCST measurements for amine-terminated pNIPAM at different concentrations; values are expressed as means; the error bars represent the standard deviation obtained from four separate measurements.

Figure 5-15. Effect of pH on the temperature-induced transition of amine-terminated pNIPAM, in a 5 mg/mL aqueous solution; values are expressed as mean; the
error bars represent the standard deviation obtained from four separate measurements

Figure 5-16. Spectrum scans of a 10 mg/mL pNIPAM solution (Sigma Aldrich, UK).

Figure 5-17. Wavelengths scan of pNIPAM aqueous solution before and after contact with calcium alginate particles

Figure 5-18. SEM coupled with EDX analysis of uncoated calcium alginate particles

Figure 5-19. SEM coupled with EDX analysis of pNIPAM-coated calcium alginate particles

Figure 5-20. Calibration curve based on turbidity measurements of amine terminated pNIPAM solutions of different concentrations

Figure 5-21. Mass balance Langmuir type adsorption isotherm of amine terminated pNIPAM onto calcium alginate particles

Figure 5-22. Photographs of pNIPAM-coated alginate A) membranes; B) particles, below and above the phase transition temperature

Figure 5-23. FT-NIR spectrums of pNIPAM-coated Alginate particles compared to alginate and pNIPAM particles

Figure 5-24. Water Contact Angle images recorded for calcium alginate films compared to Tissue Culture Treated Plastic and commercially available temperature responsive surface UpCell (NUNC). Data expressed as mean ± SD.
6. EVALUATION OF PRODUCED PARTICLES FOR CELL ATTACHMENT AND PROLIFERATION CAPABILITIES

Figure 6-1. Viable cell number of 3T3 fibroblasts at passage 18 seeded on pNIPAM solid particles with different chemical compositions and kept in static culture up to 216 hours (equivalent of 9 days) (Mean value ± SD; n=3). Statistical analysis performed by mixed-ANOVA (Within groups, p=0.002<0.05; Between groups, p=0.0005<0.05).

Figure 6-2. Phase contrast images of 3T3 cells on in-house produced pNIPAM solid particles of different chemical compositions after 96 hours in culture: A) 5-0.2; B) 5-0.4; C) 5-0.8; D) 5-1; E) 10-1; F) Cytodex-1; G) Negative control. Scale bars represent 100 µm (G -500 µm)

Figure 6-3. Live/Dead staining of 3T3 fibroblasts after 216 hours of culture on: A) 5-0.8; B) 5-1; C) 10-1; D) Cytodex-1 microcarriers; Scale bars represent 100 µm.

Figure 6-4. Viable cell number of 3T3 fibroblasts at passage 18 seeded onto pNIPAM solid particles coated with different cell adhesion promoters and kept in static culture for: A) 96 hours; B) 216 hours (Mean value ± SD; n=3). Statistical significance was assessed by mixed-ANOVA.

Figure 6-5. Live/Dead staining of 3T3 fibroblasts after 216 hours of culture on: A) 5-0.1; B) 10-1; C) Cytodex-1 microcarriers coated with different cell adhesion promoters. Only merged images of the two fluorescence channels are depicted, where live and healthy cells expressed green fluorescence, while dead or damaged cells expressed red fluorescence. Scale bars represent 100 µm.

Figure 6-6. Glucose (A) and Lactate (B) concentrations in consumed growth medium when 3T3 fibroblasts were kept in static culture for up to 168 hours. Data are the mean metabolite concentration ± standard deviation (n=3). Statistical significance was assessed by mixed-ANOVA method.
7. NON-INVASIVE THERMALLY INDUCED CELL DETACHMENT

Figure 7-1. Phase contrast imaging of cell harvesting from normal TCPS and UpCell surfaces at two different cell seeding densities, Low and High. Scale bars represent 500 µm.

Figure 7-2. 3T3 cell sheet thermal-induced detachment starting from the periphery of the dish towards the centre; Scale bar 100 µm.
LIST OF FIGURES

Figure 7-3. A close-up representation of cell morphology changes experienced during temperature-induced cell harvesting on the UpCell surface. Scale bars represent 100 µm.

Figure 7-4. Live/Dead staining of 3T3 fibroblastic cells during thermal-induced detachment at two different initial cell seeding densities; live and healthy cells are stained in green, while dead or damaged cells in red. Scale bars represent 100 µm.

Figure 7-5. Phase contrast images acquired during 3T3 fibroblasts cell detachment in growth medium at 4ºC when using two different initial cell seeding densities. Scale bars represent 500 µm.

Figure 7-6. Six days in culture monitoring of cell adhesion and proliferation abilities of 3T3 harvested cells in different conditions based on a temperature induced method. Scale bars represent 500 µm.

Figure 7-7. Phase contrast images acquired at 0 min and 240 min after temperature was lowered to 23ºC in order to initialise pNIPAM phase transition and force attached cells to lift off the microcarrier surface. Scale bars represent 100 µm.

Figure 7-8. 3T3 cell morphology assessment when cultured on: A) TCPS; B) and D) commercially available Cytodex-1 microcarriers (GE Healthcare); C) and E) core-shell microcarriers. Phase contrast images are shown in A), B) and C); while Live/Dead staining images are shown in D) and E). Live and healthy cells emitted green fluorescence, while dead or damaged cells emitted red fluorescence. Scale bars represent 100 µm.

Figure 7-9. Thermal-induced cell harvesting evaluation at 23ºC temperature treatment of A) pNIPAM-coated Alginate particles; B) commercially available Cytodex-1 microcarriers (GE Healthcare); Scale bars represent 100 µm.

Figure 7-10. Image acquired after 160 minutes of incubation at 23ºC depicting a 70% confluent microcarrier that barely initiated cell detachment process; Arrows point out the cell detachment pattern starting from the margins of the cell blanket. Scale bar is 100 µm.
Figure 7-11. Cell detachment as cell sheets from core-shell microcarriers started at the periphery of the cell sheet where cells exhibited a rounded morphology indicating detachment from the surface. Arrows point out the cell detachment pattern starting from the margins of the cell blanket. Scale bar represents 100 µm.

Figure 7-12. Thermally-induced cell harvesting when culture on A) pNIPAM-coated Alginate microcarriers; B) Cytodex-1, after incubation at 4°C; Scale bars represent 100 µm

10. APPENDICES

Figure A1. Calibration curve for the motor used for the particle production experiments (refer to Chapters 4 and 5). Data points represent means of triplicate measurements. Error bars represent standard deviation (n=3).

Figure A2. Calibration curve for the peristaltic pump used for all particle production experiments. Data points represent means of triplicate measurements. Error bars represent standard deviation (n=3).

Figure A3. Presto Blue calibration curves for 3T3 fibroblastic cells at passage 30 at two different incubation times. Fluorescent intensity values are plotted as mean values of three separate samples. Error bars are given by standard deviation (n=3).

Figure B1. Image J analysis A) Original phase contrast picture taken with a Nikon Eclipse inverted microscope. Scale bar represents 100 µm. B) 8-bit processed image with particles coloured in black and separated by watershed function. C) Analysed image representing the final outlines of the particles to be counted and measured. The particles touching the edges of the image were excluded from the analysis. D) Feret’s diameter distribution.
LIST OF TABLES

2. LITERATURE REVIEW AND BACKGROUND THEORY

Table 2-1. Characteristics of the considered crosslinking compounds

Table 2-2. Characteristics of commercially available microcarriers

3. GENERAL MATERIALS AND METHODS

Table 3-1. Characteristics of the membranes utilized in this work

Table 3-2. Characteristics of the continuous and dispersed phases used for the production of pNIPAM particles. O – kerosene (without surfactant); O₁ – 2% wt Span80 in kerosene; W₁ – 5% wt NIPAM + 0.2% wt MBA (without APS). Data is expressed as mean ± SD (n=3).

Table 3-3. Characteristics of the continuous and dispersed phases used for the production of core calcium alginate particles. O* – Miglyol 840 (without surfactant); O₁* – 1% wt Span80 in Miglyol 840; W₁* – 1.5% wt sodium alginate with 0.5% wt CaCO₃.

Table 3-4. Volumes of utilised reagents in general cell culture protocol

Table 3-5. Characteristics of Cytodex-1 (GE Healthcare) microcarriers

4. CONTROLLED pNIPAM PARTICLE PRODUCTION BY MEMBRANE EMULSIFICATION

Table 4-1. Elemental compositions of the pNIPAM particles detected by EDX coupled with SEM
Table 4-2. Shrinkage ratio of pNIPAM solid particles calculated using equation [4-3] based on Feret’s diameter values obtained from Image J analysis at a temperature below (23ºC) and above phase transition temperature (38ºC)

5. CONTROLLED CORE-SHELL PARTICLE PRODUCTION BY MEMBRANE EMULSIFICATION

Table 5-1. Different formulations (F₁ to F₉) for calcium alginate particle production

Table 5-2. Full size distribution of calcium carbonate employed for the internal gelation of alginate particles

Table 5-3. Efficiency of Langmuir isotherm

Table 5-4. Efficiency of the Langmuir isotherm for the given absorbate concentrations

Table 5-5. Alginate particles and pNIPAM-coated particles analysed by SEM coupled with EDX spectra elemental analysis demonstrating a nitrogen peak present only in the latter material

Table 5-6. Shrinkage ratio of pNIPAM-coated alginate particles calculated based on Feret’s diameter obtained from Image J analysis of 60 particles at a temperature below (23ºC) and above LCST (38ºC)

Table 5-7. Particle size comparison between laser diffraction method and Image J picture analysis. The measurements for both methods were performed at room temperature (23ºC) below polymer’s phase transition temperature
6. EVALUATION OF PRODUCED PARTICLES FOR CELL ATTACHMENT AND PROLIFERATION CAPABILITIES

Table 6-1. Detailed characteristics of the utilised pNIPAM solid particles

Table 6-2. Expansion data expressed as fold increase at 96 hours and 216 hours in culture for in-house produced pNIPAM particles of different chemical compositions compared to commercially available Cytodex-1

Table 6-3. Expansion data expressed as fold increase at 96 hours and 216 hours in culture for the prior selected in-house produced pNIPAM particles compared to commercially available Cytodex-1 microcarriers when employing different cell adhesion promoters (e.g. Matrigel, gelatine)

Table 6-4. Expansion data expressed as fold increase at 72 hours and 120 hours in culture for the core-shell microcarriers uncoated and coated with gelatine 0.1% wt compared to commercially available Cytodex-1 microcarriers

7. NON-INVASIVE THERMALLY INDUCED CELL DETACHMENT

Table 7-1. Cell detachment time comparison for different sets of conditions

10. APPENDICES

Table B1. General process parameters

Table B2. Formulation specific parameters; F₁ represents the formulation employed for solid pNIPAM particle production, while F₂ is the formulation used for the production of core-shell pNIPAM-coated Alginate particles.
Table B3. Example of droplet size prediction calculations for the formulation $F_1$ employed for the production of solid pNIPAM particles

Table B4. Example of droplet size prediction calculations for the formulation $F_2$ employed for the production of core-shell pNIPAM-coated alginate particles
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2D</td>
<td>Two dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three dimensional</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>ATRP</td>
<td>Atom transfer radical polymerisation</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>CaCO$_3$</td>
<td>Calcium carbonate</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
</tr>
<tr>
<td>DHEA</td>
<td>N,N’-(1,2-dihydroxyethylene) bisacrylamide</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EDX</td>
<td>Energy-dispersive X-ray</td>
</tr>
<tr>
<td>EGDMA</td>
<td>Ethylene glycol dimethacrylate</td>
</tr>
<tr>
<td>EthD-1</td>
<td>Ethidium homodimer-1</td>
</tr>
<tr>
<td>ESR</td>
<td>Electron spin resonance</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FTNIR</td>
<td>Fourier Transform Near Infrared</td>
</tr>
<tr>
<td>G (block)</td>
<td>α-L-guluronic acid</td>
</tr>
<tr>
<td>GDL</td>
<td>D-glucono-δ-lactone</td>
</tr>
</tbody>
</table>
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLP</td>
<td>Good laboratory practice</td>
</tr>
<tr>
<td>GRAS</td>
<td>Generally recognized as safe</td>
</tr>
<tr>
<td>HLB</td>
<td>Hydrophile-Lipophile-Balance</td>
</tr>
<tr>
<td>LCST</td>
<td>Lower critical solution temperature</td>
</tr>
<tr>
<td>M (block) β-D-mannuronic acid</td>
<td></td>
</tr>
<tr>
<td>MBA</td>
<td>N,N’-Methylene-bis-acrylamide</td>
</tr>
<tr>
<td>Mₐ</td>
<td>Average number molecular weight</td>
</tr>
<tr>
<td>Mᵥ</td>
<td>Viscosity-average molecular weight</td>
</tr>
<tr>
<td>NIPAM</td>
<td>N-isopropylacrylamide</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>O/W</td>
<td>Oil-in-Water</td>
</tr>
<tr>
<td>p/Alg</td>
<td>pNIPAM-coated Alginate particles</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PDEAM</td>
<td>poly (N,N-diethylacrylamide)</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PGA</td>
<td>poly glycolic acid</td>
</tr>
<tr>
<td>PLA</td>
<td>poly lactic acid</td>
</tr>
<tr>
<td>PLGA</td>
<td>poly (lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PLL</td>
<td>poly-L-lysine</td>
</tr>
<tr>
<td>PLLA</td>
<td>poly (L-lactic acid)</td>
</tr>
<tr>
<td>PMMA</td>
<td>poly (methyl-methacrylate)</td>
</tr>
<tr>
<td>PNCPAM</td>
<td>poly (N-cyclopropylacrylamide)</td>
</tr>
<tr>
<td>pNIPAM</td>
<td>poly(N-isopropylacrylamide)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PnnPAM</td>
<td>poly (N-n-propylacrylamide)</td>
</tr>
<tr>
<td>PTFE</td>
<td>poly (tetrafluoroethylene)</td>
</tr>
<tr>
<td>PVA</td>
<td>polyvinyl alcohol</td>
</tr>
<tr>
<td>PVP</td>
<td>polyvinyl pyrolidone</td>
</tr>
<tr>
<td>RAFT</td>
<td>Reversible addition-fragmentation chain transfer</td>
</tr>
<tr>
<td>REDOX</td>
<td>Reduction-oxidation</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-Glycine-Aspartic acid</td>
</tr>
<tr>
<td>RO</td>
<td>Reverse Osmosis</td>
</tr>
<tr>
<td>Rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SPG</td>
<td>Shirasu Porous Glass</td>
</tr>
<tr>
<td>TCPS</td>
<td>Tissue culture plastic surface</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N’,N’-Tetramethylethylenediamine</td>
</tr>
<tr>
<td>UCST</td>
<td>Upper critical solution temperature</td>
</tr>
<tr>
<td>ULA</td>
<td>Ultra low cell attachment</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VPTT</td>
<td>Volume phase transition temperature</td>
</tr>
<tr>
<td>W/O</td>
<td>Water-in-Oil</td>
</tr>
<tr>
<td>WCA</td>
<td>Water contact angle</td>
</tr>
<tr>
<td>YIGSR</td>
<td>Tyrosine-Isoleucine-Glycine-Serine-Arginine</td>
</tr>
</tbody>
</table>
1. INTRODUCTION

Therapeutic applications require a high cell number per patient treatment depending on the cell type required and condition to be treated. The number of cells required can vary from $10^4$ up to $10^8$ cells. For example, age-related macular degeneration requires $8-16 \times 10^4$ retinal pigment epithelial cells per patient treatment, while insulin-dependent diabetes can require up to $10^9$ islet cells (Mason C. and Dunnil P., 2009). One of the major current issues is the lack of a large scale system for stem cell manufacturing, capable of culturing stem cells while still preserving their properties. Ideally, this would be a system in which cells are cultured in an *in vivo* mimicking environment.

Current culturing methods generally employ two-dimensional surfaces such as treated polystyrene or treated glass which are successful at achieving cell attachment and proliferation, but are labour intensive and inefficient in achieving high numbers of cells due to surface limitations. Also, 2D surfaces are lacking in mimicking the *in vivo* environment, resulting in significantly different complex biological responses such as receptor expression, transcriptional expression, cellular migration and even apoptosis. It is well known that cells cultured using a three-dimensional culture method mimic more closely the biochemical and physiological cues present in natural tissues or organs than cells grown on 2D surfaces (Haycock J.W., 2011).

Three-dimensional culture models can be grouped into the study of whole animals and organotypic explant cultures (including embryos), cell spheroids, microcarrier cultures and tissue-engineered models. Not all 3D culture models require the use of scaffolds, but these structures have become more and more popular in the last decade (Pampaloni F. *et al.*, 2007). From the perspective of the work carried out in this thesis, the interest is focused on suspension culture using microcarriers as 3D models.
CHAPTER 1. INTRODUCTION

Existing commercially available microcarriers (such as Cytodex 1 – GE Healthcare; Hillex II – SoloHill) are very efficient at encouraging cell attachment and proliferation. However, recovery of the cells is problematic as it requires the use of proteolytic enzymes which are damaging to critical cell adhesion proteins (Baumann H. et al, 1979). From this perspective, there is a growing need for the development of a 3D construct (e.g. microcarrier) that not only can be used for cell manufacturing, but also has the advantage of a non-damaging and non-enzymatic cell harvesting potential.

1.1. Aims and motivation

Successful cell therapies require the ability to utilise large numbers of well-defined viable cells with intact properties. As such, one of the weakest points of current technologies is the lack of a well-defined up-scaling system for cell manufacturing. 3D structures such as microcarriers in conjunction with bioreactors have been previously employed successfully for the production of large numbers of cells, but the major disadvantage arose in the final step of cell harvesting performed by either employing damaging proteolytic enzymes or by destroying the microcarrier support leaving an impure product. This work seeks to explore techniques that will help to address this issue by designing a new type of microcarrier capable of allowing cell attachment and proliferation, as well as cell harvesting with intact cell-cell junctions by employing an external stimuli-responsive material.

Accordingly, the aim of this doctoral thesis was to produce temperature responsive particles in a controlled manner and explore their applications and suitability for 3D cell harvesting without the use of generally employed proteolytic enzymes. The existing 2D temperature responsive surfaces (e.g. NUNC UpCell) have been successfully used for cell harvesting,
but as with any 2D surface they require labour intensive protocols and have major limitations in terms of the cell number that can be achieved due to the limited surface area. As a different approach to overcome these limitations, 3D surfaces under the form of microcarriers show great potential. The aim of this work was realised in three parts, as described in detail in Figure 1-1 and section 1.2.

Figure 1-1. Project methodology representation.
1.2. Project methodology

The initial step in microcarrier preparation consisted of selecting the appropriate material (e.g. polymer) based on the final application. Referring to the aims and objectives of this thesis (section 1.1), the polymer of choice was the temperature responsive synthetic polymer, poly (N-isopropylacrylamide) (pNIPAM). Once the material has been selected, membrane emulsification was applied on a device with a very simple design and two different chemistries have been tested until an optimal recipe was found. Once the optimal formulation was established, polymer particles were produced in a controlled manner by varying process parameters. The generated polymer particles were then characterised in terms of particle size, size distribution, temperature responsiveness and potential for promoting cell adhesion and proliferation (refer to Chapter 4 and Chapter 5).

Once all the desired properties were achieved, the produced temperature responsive microcarriers were further used for 3D cell culture in static conditions only (refer to Chapter 6). In parallel, mammalian cells (Swiss Albino 3T3 mouse fibroblasts, ATCC, USA) were cultured in a 2D system following protocols described in detail in section 3.5 until a required number of cells was achieved, followed by cell harvesting by using proteolytic enzymes and cell seeding onto temperature responsive microcarriers. 3T3 mouse fibroblasts cell line was selected as a model cell line for demonstrating proof-of-concept for the novel type microcarrier. During the 3D microcarrier cell culture (carried out by following the protocols described in detail in section 3.6), cell attachment capabilities were assessed together with cell viability and cell proliferation on the selected chemistries.

As the final part of this thesis, the suitability of the selected polymer and selected chemistry was demonstrated by performing cell release experiments from the 3D surfaces provided (refer to Chapter 7). In theory, cell harvesting should be possible without employing
proteolytic enzymes by simply lowering the temperature below a certain value when using temperature responsive polymers. The cells harvested employing this method were then evaluated in terms of survival and replating capacities. The cell release tests from temperature responsive microcarriers were compared to commercially available 2D temperature responsive surfaces.

1.3. Research questions

This doctoral thesis seeks to address several research questions with a great impact on current research issues in the Regenerative Medicine and Tissue Engineering fields.

• Can temperature responsive microcarriers be produced in a controlled manner?
• Can alginate particles of appropriate sizes be generated for use as microcarrier cores?
• Are the developed temperature responsive particles capable of allowing cell attachment and growth?
• Can thermally induced cell harvesting from 3D surfaces (e.g. microcarriers) be achieved?

These research questions will be addressed and answered as well as possible in the following chapters.
2. LITERATURE REVIEW AND BACKGROUND THEORY

2.1. Regenerative medicine overview

Regenerative medicine, as viewed today, is an emerging interdisciplinary area that applies the principles of engineering and life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ (Langer R. & Vacanti J.P., 1993; Sipe J.D., 2002). This shows great promise for the treatment of a large number of conditions from birth defects (Saxena A.K., 2010) to diabetes (Montanya E., 2004), heart disease (Nugent H.M. and Edelman E.R., 2003) or even spinal cord injuries (Nomura H. et al, 2006; Yucel D. et al, 2013), thus having the ability to improve the quality of life for many.

Regenerative medicine aims to repair or replace lost or damaged cells. This could be achieved by the stimulation of the natural regenerative potential of cells or by cell therapy and tissue engineering of several cell types, including stem cells (Mason C. and Dunnil P., 2008). Typical tissue engineering employed strategies require the combination of a scaffold, adequate cells and bioactive molecules for the production of hybrid constructs that can be implanted into patients to induce tissue regeneration or replace failing or malfunctioning organs (Mano J.F. et al, 2007). Typically, the scaffold is required to serve as a temporary template for the formation of extracellular matrix (ECM) by accommodating and guiding the growth and proliferation of cells in a three dimensional environment (Mota C. et al, 2012). A successful scaffold should meet some basic requirements, generally involving non-toxicity, biocompatibility, biodegradability, promoting favourable cell interactions while still maintaining mechanical and physical properties (Sipe J.D., 2002; Mano J.F. et al, 2007; Mota C. et al, 2012). Cell-scaffold constructs have been successfully employed in engineering \textit{in vitro} thin avascularised tissues such as skin (Groebler F. et al, 2011),
CHAPTER 2. LITERATURE REVIEW AND BACKGROUND

cartilage (Wimpenny I. et al, 2012) or even bladder (Atala A. et al, 2006; Atala A., 2011). However, their use in fabrication of more complex tissues (e.g. liver, kidney) still faces numerous challenges. Some human cell therapies are readily available and have been clinically successful. For example, highly-complex cell-based liver-assist devices have been explored for outside the body use to aid liver failure patients (Chamuleau R.A.F.M., 2009; Sussman N.L. et al, 2009). Similarly, renal-assist devices have been developed to assist patients with acute renal injuries (Humes H.D. et al, 2013; Issa N. et al, 2007).

Although some commercially available tissue-engineered products have proven to be clinically successful, their production generated high prices resulting in disappointing volumes of sales. Therefore, prices must be reduced to ensure a larger market penetration and consequent patient benefit which can only be achieved by developing an effective and reproducible manufacturing bioprocess, capable of increased production volumes (Williams D.J. and Sebastine I.M., 2005). From a manufacturing point of view, cost-effective production could be enabled by employing bioreactor systems that provide a precisely controlled environment for stem cell expansion. In addition to creating a suitable physiological environment, bioreactors allow for maintenance of sterility, reduction in labour and the elimination of the need for sterile repackaging resulting in lower maintenance costs (Naughton G.K., 2002).

A big challenge is choosing the polymer with properties that closely match the characteristics required for a specific application. For applications such as cell culture, the polymer is required to be biocompatible in order to avoid an immune response and it is preferred to be biodegradable as its removal will not be needed. Because polymers are complex long chain molecules, their properties are also complex and in order to select the appropriate polymer, its properties and chemistry must be understood.
CHAPTER 2. LITERATURE REVIEW AND BACKGROUND

2.2. Biomaterials and their applications in regenerative medicine

2.2.1. Importance of biomaterials in regenerative medicine

Biomaterials are synthetic materials with applications in replacing parts of living systems or which function in an intimate contact with the living tissue. Although biomaterials have primarily medical applications, they have been employed for growing cells (Ratner B.D. et al, 1996; Mieszawska A.J. and Kaplan D.L., 2010; Ramalingam M. and Tiwari A, 2010) or as delivery systems of drugs, proteins or other important substances (Buckles R.G., 1983; Goldberg M. et al, 2007; Chen R.R. et al, 2003; Gombotz W.R. et al, 1995). In medical applications, biomaterials are rarely used on their own as simple materials, but more commonly are integrated in devices such as substitute heart valves (Filova E. et al, 2009), dental implants (Muddugangadhar B.C. et al, 2011; Bayne S.C., 2005) or artificial hip joints (Navarro M. et al, 2008). Typically, biomaterials are classified into four groups: metals, ceramics, polymers and composites (Ratner B.D. et al, 1996A; Saha N. et al, 2011).

The work described in this thesis only focused on polymeric biomaterials. Polymers can be polysaccharides (e.g. cellulose, chitin, alginate, hyaluronate), proteins (e.g. collagen, gelatine, casein, albumin) and /or synthetic and natural polymers (e.g. polyvinyl alcohol (PVA), polyvinyl pyrrolidone (PVP), polyethyleneglycol (PEG), poly-L-lactic acid (PLLA)) (Saha N. et al, 2011). It is generally agreed that a biomaterial to be used in tissue engineering needs to possess certain material characteristics such as biocompatibility, suitable surface chemistry, interconnected porosity, desired mechanical properties and biodegradability. Ideally, the polymeric biomaterial employed should create not just a physical, but also a chemically favourable environment by promoting protein absorption, thus ensuring the presence of ligands (functional groups) that specifically bind to cell receptors, promoting a desirable interaction between cells and surface. A lot of progress was
done in producing polymers containing specific hydrophilic or hydrophobic components, biodegradable repeating units, engrafted peptides or even multifunctional structures that can be used as points of generating three dimensional networks (Peppas N.A. and Langer R., 1994). Biomaterials can be decorated with different functional groups (e.g. amine, carboxyl and hydroxyl) for promoting cell adhesion and for directing cells functionality.

2.2.2. Natural polymers

Even though naturally derived polymers are characterised by poor mechanical properties and batch-to-batch variability (Yang S. et al. 2001), they have gained a widespread attention owing to their favourable attributes, such as biocompatibility, availability and low cost (Ko H-F. et al, 2010). These characteristics and the ability to be fabricated into hydrogels into an easy manner, makes natural polymers appealing choices for production of scaffolds. Natural polymers offer the advantage of containing macromolecules recognizable by the biological environment. Extracellular matrix (ECM) is the material that surrounds cells in tissue and provides mechanical support, controls cell functionality and guides the complex multicellular processes of tissue formation and regeneration (Davies J.A., 2001). Owing to their similarity with the ECM, polymeric biomaterials derived from natural sources (e.g. purified protein components from animal tissue) have several advantages over synthetic biomaterials because of their inherent properties of biological recognition due to the receptor-binding ligands that they contain (Naderi H. et al, 2011; Mano J.F. et al, 2007).

Naturally derived polymers have the ability to form gels that typically have high water content within the network. As a result, they have been referred to as ‘hydrogels’ (Ko H-F. et al, 2010; Ratner B.D. and Bryant S.J., 2004) and were found to have widespread
applications in biomedical engineering due to their highly hydrated state similar to natural
tissues and tunable mechanical, chemical and biocompatibility properties similar to the
native ECM (Geckil H. et al, 2010; Ratner B.D. and Bryant S.J., 2004). Various gelation
mechanisms have been employed depending on the selected polymer, such as ionic
crosslinking, thermal or pH-induced gelation, crystallization or solvent exchange or by
simply modifying the polymer viscosity (Gutowska A. et al. 2001). Among all the naturally
derived polymers, polysaccharides composed of sugar-ring building blocks have been of
increased interest for various tissue engineering applications. Commonly employed
polysaccharides include cellulose, chitosan, alginate and agarose (Ko H-F. et al., 2010). In
regards to the work presented in thesis, the interest was focused on alginate employed for
production of core-shell microcarriers (see details in Chapter 5).

2.2.2.1. Alginate

Alginate is a collective term for a family of naturally occurring, anionic and hydrophilic
linear polysaccharide, primarily derived from brown seaweed and bacteria (Melvik J.E. et
al., 2004; Fang Y.P. et al., 2007) being one of the most abundant biosynthesised materials.
Alginate has been used extensively for encapsulation and transplantation of cells (Hunt N.C.
et al., 2009; Hunt N.C. and Grover L.M., 2010; Bhat A. et al, 2013) due to its
biocompatibility, non-toxicity to cells, low price and simplicity in fabrication of cell
constructs. Its biocompatibility is related to its purity (Orive G. et al., 2002). Alginate has
been approved by the Food and Drug Administration (FDA) for human use as food additive
and wound dressing material (Cho S.H. et al, 2005; Cho S.H. et al, 2009), thus alginate is a
suitable biomaterial for diverse regenerative medicine applications.
2.2.2.1.1. Alginate chemical structure

The monovalent salt of alginic acid is a linear co-polymer composed of 1,4-linked α-L-guluronic (G) and β-D-mannuronic (M) acid residues (Figure 2-1A) (Bucke C., 1987; Cho S.H. et al., 2009). The residues are arranged in a block-wise pattern with G-blocks and M-blocks interspersed with MG alternating blocks (Pasut E. et al., 2008). The overall composition of M and G residues and their distribution patterns within the alginate vary with the species of seaweed (Fang Y.P. et al., 2007).

![Chemical structures of the two units of alginate (A) and cross-linked alginate by stacking of G-blocks to form an egg-box structure (B).](image)

Figure 2-1. Chemical structures of the two units of alginate (A) and cross-linked alginate by stacking of G-blocks to form an egg-box structure (B).

The G-residue exhibits a high affinity for divalent ions (e.g. calcium, strontium, barium etc.) at room temperature (Cho S.H. et al., 2005). The affinity for divalent ions increases in the
CHAPTER 2. LITERATURE REVIEW AND BACKGROUND

order: $\text{Mg}^{2+} < \text{Ca}^{2+} < \text{Sr}^{2+} < \text{Ba}^{2+}$ (Fang Y.P. et al., 2007). Literature generally reports that gelation with $\text{Mg}^{2+}$ does not occur due to the lack of strong polymer-ion interactions (Donati I. et al., 2006; Donati I. et al., 2009). However, magnesium alginate gels have been successfully prepared and it was strongly dependent on the alginate chemical composition (Topuz F. et al., 2012).

2.2.2.1.2. Production of alginate hydrogels

Gelation of alginates occurs when the carboxyl groups present in its structure are crosslinked with cations and/or polyelectrolytes. Thus, in an aqueous solution of cations, alginate crosslinks by forming a three-dimensional gel network via the stacking of the G-residues to form an “egg-box” structure (Figure 2-1B) (Cho S.H. et al, 2005; Cho S.H. et al, 2009; Bucke C., 1987; Dhoot N.O. et al, 2004). In the alginate molecule only the GG-blocks and the GM-blocks can bind $\text{Ca}^{2+}$ in the form of “egg-box”, while MM-blocks act as the solvating moiety (Fang Y.P. et al., 2007). Hydrogels of varying mechanical strength, elasticity and swelling characteristics can be produced and are dependent on the nature of the crosslinking cation, the length of the polymer chains, the M-to-G ratio and percentage of the block structures (Zimmermann H. et al., 2005).

Calcium is the most frequently used gelling cation. Practically, the preparation of alginate hydrogels simply involves dripping sodium or potassium alginate solution into an aqueous solution of the crosslinking calcium cations typically made from calcium chloride ($\text{CaCl}_2$). This approach is advantageous as it is performed under mild conditions (e.g. aqueous solution, room temperature, pH ~ 7.2 - 7.4) while avoiding the use of toxic reagents (Dhoot N.O. et al, 2004). However, a major disadvantage of using $\text{CaCl}_2$ as a gelling agent consists
in a rapid and difficult to control gelation rate resulting in non-uniform structures and polymer concentration gradients (Bienaime C. et al., 2003; Brun-Graeppi A.S. et al, 2011). Therefore, the alginate gels prepared by employing CaCl₂ as a source of divalent ions have limited use due to the lack of gel stability and potential regions of gel failure as a result of the concentration gradients formed.

Alternatively, calcium carbonate (CaCO₃) could be employed as a crosslinking agent. Unlike CaCl₂ which relies on diffusion to crosslink alginate, CaCO₃ determines internal gelation through the release of Ca²⁺ in situ. Poorly soluble CaCO₃ is homogenously mixed with sodium or potassium alginate and the Ca²⁺ are then solubilized by addition of a catalyst such as glacial acetic acid (Liu X.D. et al., 2003) or D-glucono-δ-lactone (GDL) (Kuo C.K. et al., 2007; Lu L. et al., 2010; Nunamaker E.A. et al., 2011) that lowers the pH thus releasing the divalent ions to determine alginate gelling. CaCO₃ determines a slower gelation rate compared to CaCl₂ used on regular basis, due to its low solubility in water (Cho S.H. et al, 2009; Brun-Graeppi A.S. et al, 2011). Calcium ions sourced from CaCO₃ bind to the nearest available carboxylic groups, thus crosslinking the alginate in a spatially uniform manner (Nunamaker E.A. et al., 2011).

Another divalent cation that could be employed is Barium (Ba²⁺) (Grohn P. et al, 1997) which has a higher affinity to alginate crosslinking binding sites compared to commonly used divalent ions (e.g. Ca²⁺). Thus, barium ions form stronger gels with alginate compared to calcium, but the affinity is highly dependent on the alginate composition. As for example, it was found that high G-alginates form stronger and more stable gels with barium ions compared to calcium ions (Brun-Graeppi A.S. et al, 2011; Morch Y.A. et al., 2012; Morch Y.A. et al., 2006). However, barium- alginate gels exhibit a lower permeability when
compared to calcium-alginate gels and their use *in vitro* is limited due to barium leakage over time reaching possibly toxic levels (Morch Y.A. *et al.*, 2012; Morch Y.A. *et al.*, 2006).

Ionically crosslinked alginites tend to lose their mechanical properties over time *in vitro*, which can be attributed to an outward flux of crosslinking ions into the surrounding medium (Rowley J.A. *et al.*, 1999; Hunt N.C. *et al.*, 2010). Calcium- and barium-alginate gels were found to be sensitive towards chelating agents (*e.g.*, phosphate, citrate, EDTA (Grohn P. *et al.*, 1997) and non-gelling agents (*e.g.*, Na\(^+\) and Mg\(^{2+}\)) (Brun-Graeppi A.D. *et al.*, 2011). To address this issue, stable covalent crosslinked units could be introduced into the gels, such as peptides (Dhoot N.O. *et al.*, 2004), allowing a greater control over the mechanical and swelling properties of the gels (Rowley J.A. *et al.*, 1999). Alginate capsules have been stabilized with a polycation such as poly-L-lysine (PLL) which during the gelation, binds to mixed sequences of G and M. The generated complexes did not dissolve in the presence of chelators or non-gelling cations. Since charged PLL is known to be immunogenic, the PLL-alginate capsules had to undergo a final incubation step in alginate to form alginate-PLL-alginate structures (Brun-Graeppi A.S. *et al.*, 2011).

2.2.2.1.3. Suitability of alginate hydrogels for regenerative medicine applications

Because of its properties, natural and modified alginate hydrogels are a very attractive choice as a matrix for encapsulation of drugs (Hua S. *et al.*, 2010) or cells (Cho S.H. *et al.*, 2009) for therapeutic applications, as scaffolds for tissue engineering (Grohn P. *et al.*, 1997; Kwon Y.J. *et al.*, 2002; Cho S.H. *et al.*, 2005), wound healing materials (Augst A.D. *et al.*, 2006; Pereira R. *et al.*, 2013; Pereira R. *et al.*, 2013A), bridges for resected peripheral nerves (Dhoot N.O. *et al.*, 2004; Frampton J.P. *et al.*, 2011) or even as synthetic extracellular...
material (Rowley J.A. et al, 1999). Despite its extensive use, alginate lacks appropriate adhesion sites to facilitate cell attachment and it cannot be broken down enzymatically in mammals (Augst A.D. et al, 2006; Kwon Y.J. et al, 2002; Baldwin A.D. et al, 2010). The cell adhesion resistance of alginate gels could be attributed to the increased hydrophilicity, thus lacking the ability to adsorb proteins generally required for cell adhesion. In this regard, one approach to ensure cell adhesion is to couple cell adhesion proteins, such as gelatine (Kwon Y.J. et al, 2002), collagen (Grohn P. et al, 1997) or laminin (Dhoot N.O. et al, 2004; Frampton J.P. et al, 2011). However, this approach can lead to non-specific binding and is limited by the long-term instability of proteins (Dhoot N.A. et al., 2004; Bhat A. et al., 2013).

As a different approach, covalently modified alginates have been shown to allow cell attachment (Baldwin A.D. et al, 2010; Rowley J.A. et al., 1999; Rowley J.A. et al., 2002). For example, Rowley J.A. et al (1999 and 2002) had modified calcium-crosslinked alginate gels by using aqueous carbodiimide chemistry to covalently graft cell adhesion peptides containing an RGD (Arginine-Glycine-Aspartic acid) sequence for promoting cell attachment and spreading. Rowley J.A. et al (1999) demonstrated that myoblasts adhere, proliferate and fuse when cultured on RGD-modified alginate gels. By using the same aqueous carbodiimide chemistry, Dhoot N.O. et al (2004) modified the alginate gel by covalent attachment of YIGSR (Tyrosine-Isoleucine-Glycine-Serine-Arginine) peptide to the carboxylic groups of alginate. The same group showed in their study that cell adhesion is a function of peptide density on the alginate gel surface. The RGD sequence is present within several major ECM constituents that provide integrin-binding sites for cell adhesion (Bhat A. et al., 2013). Unlike large proteins that are subject to degradation and denaturation, peptide sequences have the advantage of being more stable (Hern D.L. et al., 1998). Despite the fact that it is well known that alginate gels do not sustain cell attachment and
proliferation, modified alginate gels might be valid alternatives for cell culture substrates because of their important properties (e.g. biocompatibility, permeability, easy dissociation of cation crosslinked gels and release of cells without the use of proteolytic enzymes).

2.2.3. Synthetic polymers

Despite their advantages, natural biomaterials are difficult to purify, the variation between batches is high and the risk of pathogen contamination could be an issue (Lutolf M.P. et al., 2005; Peppas N.A. and Langer R., 1994; Cho S.H. et al., 2005). These limitations lead to a greater attention given to synthetic materials where a greater control over material properties and tissue responses could be achieved (Lutolf M.P. et al., 2005; Peppas N.A. and Langer R., 1994).

Many biomaterials used for clinical applications were not originally designed as such, but were off-the-shelf materials. For example, materials used for artificial hearts were originally based on commercial-grade polyurethanes. Despite allowing the easy fix of serious medical problems, these off-the-shelf materials also introduced serious complications (Peppas N.A. and Langer R., 1994) resulting in an urgent need for developing and synthesizing new biocompatible synthetic materials with tunable properties. The first generation of synthetic biomaterials was specifically designed to maintain long-term integrity of bulk properties as well as preventing toxic side effects, being intended for use as prostheses or medical implant devices. These materials were thus designed to be biologically inert, that is, to perform their function without aiding the natural healing process (Vladkova T.G., 2010; Chan A. et al., 2013). The requirement for biomaterials to promote healing shifted towards the design of bioactive materials that integrate with biological molecules or cells to aid tissue regeneration.
CHAPTER 2. LITERATURE REVIEW AND BACKGROUND

(Chan A. et al., 2013; Vladkova T.G., 2010; Hench L.L., 2006; Hench L.L. et al., 2010). For example, a bioactive material known as BioGlass was developed for bone tissue engineering and it stimulated cellular response by leaching an inorganic phase as a result of a controlled chemical reaction (Hench L.L., 2006; Hench L.L. et al., 2010; Gerhardt L-C. et al., 2010).

For the work presented in this thesis, the interest was focused on synthetic polymers that have been used in conjunction with cells for different applications in the biomedical field. Synthetic polymers could be highly useful since their properties such as porosity, degradation rate and mechanical characteristics could be tailored for specific applications. Moreover, synthetic polymers are often cheaper than natural ones as they can be produced in large quantities, are readily available and generally have a longer shelf life (Dhandayuthapani B. et al., 2011). Amongst the most commonly used synthetic polymers, polyesters such as poly lactic acid (PLA), poly glycolic acid (PGA) and poly (lactic-co-glycolic acid) (PLGA) copolymers have been extensively used for tissue engineering applications. As a result of their biocompatibility and biodegradability, the above mentioned polymers have also been approved by FDA for use as degradable sutures (Kukarni R.K. et al., 1971; Agrawal C.M. et al., 1995; Athanasiou K.A. et al., 1996; Gunatillake P.A. et al., 2003) or for implantation in humans (Croll T.I. et al., 2004; Houchin M.L. et al., 2008).

2.2.3.1. Stimuli- responsive polymers

A special class of synthetic polymers is represented by stimuli-responsive polymers. In the last couple of decades, stimuli-responsive polymers have exhibited an increased interest due to their fast response to external stimuli making them suitable for applications in different biotechnology related fields, such as drug delivery systems (Bawa P. et al, 2009) or cell
culture platforms (Yang L. et al., 2012). From a biomedical point of view, the ‘smart’ polymeric systems of importance are pH or temperature-sensitive. pH-sensitive polymers are a class of polyelectrolytes that present ionisable weak acidic or basic moieties attached to a hydrophobic backbone. Upon ionization, the polymer coiled chains extend dramatically responding to the electrostatic repulsions of the generated charges (anions or cations), thus reversibly or irreversibly changing polymer solubility or conformation in aqueous solutions (Aquilar M.R. et al., 2007; Dai S. et al., 2008; Huh K.M. et al., 2012). In the case of temperature-sensitive ‘smart’ polymers, their structure contains a fine hydrophobic-hydrophilic balance. Small temperature changes around the critical temperature determine the polymer chains to collapse or to expand as a response to the new adjustments of the hydrophobic and hydrophilic interactions between the polymeric chains and the aqueous media (Bromberg L.E. et al., 1998; Aquilar M.R. et al., 2007).

Both pH-responsive and temperature-responsive polymers have been previously reported for non-enzymatic cell harvest. However, only a reduced number of studies have been performed using pH-responsive polymers for cell-based applications due to the limited pH range (6.8 to 7.4) required for normal cell functions (Patel N.G. and Zhang G, 2013). A recent publication has shown that pH-responsive surfaces formed by alternate layering of cationic and anionic layers had the ability to induce cell sheet detachment by either local or global pH lowering. A pH value in the range of 5 to 7.4 had no effect on cell detachment. However, when cells were exposed to pH 4, complete cell sheet detachment occurred within 2-3 minutes. The group reported that the detached cells retained viability and differential potential (Guillaume-Gentil O. et al., 2011). Despite the optimistic results reported, cell sensitivity to pH changes differs with cell type and it strongly dependent on exposure time. In the light of these aforementioned reasons, pH-responsive surfaces have limited potential to cell-based applications. As a result, the work presented in this doctoral thesis was focused
only on temperature responsive polymers as they offer several advantages (refer to section 2.2.3.1.1) with potential to be applied in cell expansion methods.

There are two main types of temperature responsive polymers, one type that presents a lower critical solution temperature (LCST) and a second type that exhibits an upper critical solution temperature (UCST). In contrast to the large number of reports on LCST polymers, only a limited number of UCST polymers were reported, such as poly (methyl-methacrylate) (PMMA) (Pietsch C. et al., 2010). LCST-exhibiting polymers are water soluble at low temperatures, unlike UCST polymers which exhibit a reverse solubility in water, i.e. being insoluble at low temperatures and dissolving upon heating (Ward M.A. et al., 2011; Pietsch C. et al., 2010). Once the critical solution is reached, individual polymer chains collapse prior to aggregation, increasing scattering of light in the solution resulting in cloudiness, thus leading to LCST also being referred to as ‘cloud point’ (Bromberg L.E. et al., 1998; Ward M.A. et al., 2011). Upon reaching the ‘cloud point’ two different phases are generated; one containing the collapsed polymer that expelled most of the associated water from its structure and the other being the water itself. The polymer collapse at LCST is an entropy-driven process. In contrast to the LCST transition, UCST transition is mostly enthalpy driven causing a broader transition compared to the LCST transition (Bromberg L.E. et al., 1998; Pietsch C. et al., 2010; Ward M.A. et al., 2011). Temperature sensitive polymer behaviours in solution at LCST and UCST critical points are described by the Flory-Huggins theory (Pietsch C. et al., 2010). When polymers are dissolved in water, three types of intermolecular interactions are possible: between polymer molecules, between polymer and water and between water molecules (Li L. et al., 2002). In the case of LCST exhibiting polymers, temperature increase results in a negative free energy defined by the Gibbs equation ($\Delta G = \Delta H - T\Delta S$) which does not favour water-polymer interactions over the two other types of possible interactions. The negative free energy ($\Delta G$) is determined
by the higher entropy term ($\Delta S$) with respect to the enthalpy term ($\Delta H$). At temperatures above LCST, the entropy term dominates the otherwise exothermic enthalpy of the hydrogen bonds formed between the polymer polar groups (e.g. hydroxyl) and water molecules. The entropy increase with temperature increase is determined by the water-water associations generated in the system (Schild H.G. et al., 1992; Bromberg L.E. et al., 1998; Klouda L. et al., 2008). This behavioural phenomenon is generally referred to as the ‘hydrophobic effect’ (Schild H.G. et al., 1992; Southall N.T. et al., 2002).

Poly (N-alkyl (meth)-acrylamide)s are perhaps the most studied class of temperature responsive polymers. Poly (N-isopropylacrylamide) (pNIPAM) has gained significant attention for biomedical applications due to its sharp phase transition at around 32°C with the possibility of easily adjusting this value to near body temperature. However, despite its advantages, pNIPAM also possesses some inherent disadvantages, such as questionable biocompatibility, phase transition hysteresis and a significant end group influence on thermal behaviour (Roy D. et al., 2013). Other thermo-responsive polymers have also been previously investigated, such as poly (N-cyclopropylacrylamide) (PNCPAM) or poly (N-n-propylacrylamide) (PNnPAM). However, these two polymers have significantly different phase transitions with PNCPAM having a transition temperature of 53°C, while PNnPAM having a transition temperature of 10°C (Ito D. and Kubota K., 1999; Roy D. et al., 2013). Temperature-responsive polymers with transition temperatures close to pNIPAM have been previously reported. One such is poly (N, N-diethylacrylamide) (PDEAM) with a reported thermal transition at 33°C. However, this value has been shown to be tacticity-dependent (Kobayashi M. et al., 2000).
2.2.3.1.1. poly (N-isopropyl-acylamide) (pNIPAM)

Amongst the numerous temperature-responsive polymers, poly (N-isopropyl-acrylamide) (pNIPAM) has been widely employed for biomedical applications because of its biocompatibility and sharp phase transition at a value (e.g. 31°C - 32°C) close to the physiological temperature (Heskins M. and Guillet J.E., 1968). Above this temperature referred to as LCST, pNIPAM hydrogels exhibit a hydrophobic surface allowing cells to attach and proliferate. By lowering the temperature below this value, the surface becomes gradually hydrophilic allowing cell detachment without any enzymatic treatment usually required for cell harvesting (Kwon O.H. et al., 2000; Tang Z. et al., 2012; Liu D. et al., 2012). By employing this method, the harvested cell sheet maintains intact cell-to-cell junctions and extracellular matrix underneath the cell sheet (Kushida A. et al., 1999) making pNIPAM surfaces very suitable and attractive materials for the construction of three-dimensional tissue-like structures. Despite the aforementioned favourable properties, pNIPAM hydrogels have two major limitations, i.e. poor mechanical properties and slow response to temperature changes. The slow response to temperature is believed to be caused by the formation of a dense skin layer of polymer as a result of the strong hydrophobic interactions created between the isopropyl groups of pNIPAM. Thus, when above the LCST, the outward diffusion of water molecules is retarded resulting in a slower swelling rate (Zhang X-Z and Zhuo R-X., 2000; Zhang X-Z. et al., 2008).

For the aforementioned reasons, bulk pNIPAM hydrogels may not be suitable for a range of biomedical applications, such as cell culture, drug delivery or on-off switches which require a more robust response (Zhang X-Z and Zhuo R-X., 2000; Zhang X-Z. et al., 2008). However, according to the Tanaka-Fillmore theory that the response time of the hydrogel is proportional to the square of the hydrogel size (Tanaka T. and Fillmore D.J., 1979),
pNIPAM under the form of microgels (e.g. microcarriers) have a very fast response rate to temperature as a result of the small size of around micrometers (Lin S.Y. et al., 1999; Zhang X-Z. et al., 2008). In regards to pNIPAM poor mechanical properties, many attempts for improvement have been done. Some of the strategies included synthesis of a heterogenous structure using mixed solvents (Zhang X-Z. et al., 2008); the use of hydrophilic co-polymers to increase hydrogen bonding and generate a faster response (Schild H.G., 1992; Ebara M. et al., 2001; Zhang J. et al., 2007); crosslinking (Alli A. and Hazer B., 2008) or the use of porogens, such as polyethylene glycol (PEG) (Zhang X-Z and Zhuo R-X., 2000; Zhuo R.X. and Li W., 2003; Zhang X-Z. et al., 2005). In this thesis, the focus was set on the chemical crosslinking which is one of the simplest and most versatile tools for controlling mechanical properties and response rate of hydrogels (Varga I. et al., 2001; Alli A. and Hazer B., 2008).

Taking advantage of these properties of pNIPAM surfaces, pNIPAM-based culture substrates under the form of plane two-dimensional membranes (Wang T. et al., 2011; Liu D. et al., 2012) or even 3D scaffolds (Rayatpisheh S. et al., 2014) have been previously produced. However, to the best of knowledge, no temperature responsive surfaces under the form of microcarriers with applications in non-enzymatic cell harvesting have been previously reported. The advantages of employing culture substrates under the form of microcarriers are presented in detail in section 2.3.

2.2.3.1.1.1. Chemical structure

pNIPAM in water demonstrated remarkable hydration-dehydration changes in response to changes in temperature. Thus, pNIPAM is fully hydrated with an extended chain conformation below 32°C and gets extremely dehydrated and compact above this
temperature (Figure 2-2) (Chaterji S. et al., 2007). These distinctive properties of pNIPAM hydrogels are determined by its chemical structure (Figure 2-3) consisting of repeat units of hydrophilic (–CONH-) and hydrophobic (–CH(CH₃)₂) groups (Schmaljohann D, 2006; Zhang R. et al., 2008). Below the LCST, the hydrophilic moieties (–CONH-) may interact with water molecules through hydrogen bonding leading to water uptake by the hydrogel. However, as the temperature increases above LCST, the hydrogen-bonding interactions are destroyed and the hydrophobic moieties (–CH(CH₃)₂) become dominant, thus determining the release of water molecules entrapped in the polymer network (Zhang X-Z. et al., 2008).

Figure 2-2. Conformational changes of pNIPAM upon temperature modification (adapted after Chaterji S. et al. (2007)).

Figure 2-3. Chemical structure of pNIPAM.
Cells are cultured on the hydrophobic surface at 37°C, above the LCST, and can be readily detached from the surface by lowering the temperature to room temperature. The hydration and expansion of the polymer chains on the surface as a function of the temperature reduction are responsible for the cell detachment without the need of proteolytic enzymes (Figure 2-4) (Canavan H.E. et al., 2005; DaSilva R.M.P. et al., 2007).

Figure 2-4. Cell adhesion to thermo-responsive culture surfaces at 37°C and detachment by lowering the temperature (adapted after Zhang R. (2008)).

2.2.3.1.1.2. pNIPAM phase transition

The LCST is defined as the temperature value below which the components of a mixture are miscible at all compositions (IUPAC definition). Referring to pNIPAM, the first report of such a parameter was published by Heskins M. and Guillet J.E. (1968) that suggested that in aqueous solutions, pNIPAM prepared using free-radical initiators exhibited a LCST at around 31-32°C in aqueous solutions (Heskins M. and Guillet J.E., 1968; Tauer K. et al.,
2009). Aqueous pNIPAM solutions exhibit a reversible temperature-induced phase separation. Below the ‘cloud point’, pNIPAM is soluble in water due to the macromolecule’s ability to form hydrogen bonds with the water molecules via the amide groups. However, above the ‘cloud point’, the polymer becomes insoluble in water and precipitates generating a two-phase system. The polymer’s insolubility in water above the ‘cloud point’ is determined by the hydrogen bonds created between the adjacent groups of the macromolecule (Ottaviani M.F. et al., 2001).

LCST usually refers to aqueous polymer solutions, but in the work presented in this thesis, crosslinked pNIPAM hydrogel particles were produced. The same phase transition is applicable for the crosslinked pNIPAM hydrogels, but the critical temperature of the hydrogel is known as the volume phase transition temperature (VPTT) (Zhu X. et al., 2012). Usually, the concentration of the polymer solution used for the determination of the LCST by calorimetric or absorbance studies is low (0.5 – 1% w/v) (Constantin M. et al., 2011). Generally the value obtained for the LCST determined at low concentration is considered to be the same as the VPTT of the hydrogel. This assumption doesn’t take into consideration the fact that the physical and chemical crosslinked hydrogels with applications in biomedical field are generally prepared from high concentrated polymer solutions, ranging from 5% to 10% w/v (Ramkissoon-Ganorkar C. et al., 1999). Thus, crosslinked hydrogels in the swollen state (below VPTT) could be considered as a concentrated polymer solution related to the amount of water retained by the hydrogel. Moreover, a recent study (Constantin M. et al., 2011) showed that the VPTT of a pNIPAM crosslinked hydrogel agrees with the LCST value obtained for the polymer solution and even more, the authors concluded that the ‘cloud point’ method utilised for the determination of LCST was more reliable than the calorimetric method as it takes into consideration both the dehydration and the hydrophobic interactions of the polymer chains.
2.2.3.1.1.3. pNIPAM hydrogel polymerisation methods

By definition, hydrogels are crosslinked polymer networks with hydrophilic properties, generally prepared from hydrophilic monomers. Hydrogels may be synthesized by either physical or chemical methods. Physically crosslinked hydrogels are spontaneously formed under appropriate conditions without the use of external crosslinking agents. The structure of such hydrogels is held together by the highly organised aggregated chain segments and secondary molecular forces such as hydrogen bonding, ‘Van der Waals’ forces or hydrophobic interactions. On a different approach, chemically crosslinked hydrogel synthesis involves the use of three components: monomer, crosslinker and initiator (Ilic-Stojanovic S. et al., 2011). A number of chemical methods including one-step procedures or multiple-step procedures have been previously reported for synthesis of hydrogels (Ahmed E.M., 2013).

pNIPAM hydrogels were of interest for the work presented in this doctoral thesis. pNIPAM polymer could be produced by radical polymerisation including methods such as atom transfer radical polymerization (ATRP) (Ye J. and Narain R., 2009; Lu X. et al., 2007) and reversible addition–fragmentation chain transfer (RAFT) (You Y-Z. et al., 2007; Isoda K. et al., 2011) controlled polymerisation, free radical polymerisation (Sayil C. and Okay O., 2002; Alli A. et al., 2008; Haraguchi K. et al., 2011; Seddiki N. and Aliouche D., 2013) or even UV photopolymerisation (Kurecic M. et al., 2012). Methods such as ATRP and RAFT polymerization allow synthesis in a controlled manner of polymer chains with different functionalities while maintaining narrow molecular weight distributions (Ye J. and Narain R., 2009; Isoda K. et al., 2011). However, these methods are generally employed to attach pNIPAM hydrogels to solid supports. The most commonly reported method to synthesize pNIPAM hydrogels is free radical polymerisation through the use of a structurally-similar
monomer (e.g. MBA) as a crosslinker (Sayil C. and Okay O., 2002; Seddiki N. and Aliouche D., 2013).

2.2.3.1.4. Crosslinker selection

The crosslinker employed in the polymerisation of pNIPAM particles is methylene-bis acrylamide (MBA) which has a similar structure to the NIPAM monomer. MBA is the most commonly used crosslinker for the production of pNIPAM hydrogels, but other crosslinkers with similar monomer-like structures, have been considered, such as ethylene glycol dimethacrylate (EGDMA) (Obeso-Vera C. et al., 2013) or N,N’-(1,2-Dihydroxyethylene) bisacrylamide (DHEA) (Nayak S. et al, 2005). Their properties are provided below in Table 2-1.

Table 2-1. Characteristics of the considered crosslinking compounds

<table>
<thead>
<tr>
<th>Crosslinker</th>
<th>Molecular formula</th>
<th>Molecular mass (g/moL)</th>
<th>Chemical structure</th>
<th>Water solubility (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBA</td>
<td>C₇H₁₀N₂O₂</td>
<td>154.17</td>
<td><img src="image" alt="Methylenbisacrylamide" /></td>
<td>20*</td>
</tr>
<tr>
<td>EGDMA</td>
<td>C₁₀H₁₄O₄</td>
<td>198.22</td>
<td><img src="image" alt="Ethylene glycol dimethacrylate" /></td>
<td>5*</td>
</tr>
<tr>
<td>DHEA</td>
<td>C₈H₁₂N₂O₄</td>
<td>200.2</td>
<td><img src="image" alt="N,N’-(1,2-Dihydroxyethylene) bisacrylamide" /></td>
<td>50**</td>
</tr>
</tbody>
</table>

* - at 20°C according to the MSDS sheet provided by the manufacturer (Sigma Aldrich)
Based on the properties provided in Table 2-1, the crosslinker with the best water solubility is DHEA, but this compound is a reversible crosslinker that has been proven useful in the production of hollow thermo-responsive microgels (Nayak S. et al., 2005). This property does not make it useful for achieving the objectives of this work. Based on this consideration, the remaining two crosslinkers are MBA and EDGMA, from which the best water solubility is attributed to MBA, making it appropriate to be used as a crosslinker in the formulation employed in this work.

2.2.3.1.1.5. Applied chemistry of choice

The crosslinking reaction of choice for the production of pNIPAM particles was free radical polymerization that produces "linear" strings of monomer units by a chain reaction started by the reduction-oxidation (redox) system consisting of ammonium persulfate (APS) and N,N,N’,N’-tetraethylenediamine (TEMED). The selected crosslinker based on the selection provided in section 2.2.3.1.4 was MBA which is very effective since it contains two unsaturated bonds in its structure, both of which are susceptible to free radical addition. As a result of its favourable chemical structure, MBA displays a higher reactivity than the monomer (NIPAM) which contains only one vinyl group in its structure. The chemical structures of the reaction participants are provided in Figure 2-5. Free radical methods were used since they do not require the use of complex chemicals or metals, employed in controlled polymerisations that might be difficult to eliminate and could potentially present a source of toxicity when used with cells (Chen Z. et al., 2006; Chang C.W. et al., 2009).
The reaction is a vinyl addition polymerization initiated by a free radical-generating system (Chrambach A., 1985). APS has the ability to exist as free radicals when dissolved in water at elevated temperature (e.g. 60°C-70°C) (Hsu S-C. et al., 2002) as shown in Figure 2-6, thus having the ability to polymerise NIPAM. The disadvantage of using only APS as a source of free radicals is that the reaction takes a longer time to occur which increases the probability of free radicals to be consumed by oxygen that enters the polymerisation system, oxygen acting as free radicals scavenger (Chrambach A., 1985). In order to avoid the loss of free radicals and decrease the reaction time, temperature has to be increased to 70°C, but for the system employed here, this approach has no advantages and it is not recommended as the oil phase used for the production of one type of polymer droplets is kerosene with a flash point of around 70°C.
As a different approach, the polymerization could be initiated by a redox system formed by APS and TEMED. The reaction mechanism between APS and TEMED resulting in the generation of free radicals is provided in Figure 2-7. TEMED reacts with APS initially forming a coordinative bond between the two molecules and finally resulting in an unpaired valence electron. TEMED has the ability to exist as a free radical, thus acts as an additional catalyst to the reaction and minimises the time necessary for the generation of the free radicals. The persulfate free radicals convert acrylamide-derived monomers to free radicals which react with inactivated monomers to begin the polymerization chain reaction (Shi Q. and Jackowski G., 1998). It is very important to remove the oxygen from the system as it is a radical scavenger and it interferes with the polymerisation (Chrambach A., 1985).

The produced free radicals combine with NIPAM and further on with MBA, as shown in Figure 2-8. The reaction starts with the additions of the free radicals onto the NIPAM, thus elongating the polymer chain, followed by the addition of the monomer-like structured crosslinker (MBA), thus connecting two linear chains covalently between the nitrogen atoms of the amide groups by a methylene group. The chain propagation continues forming a large polymer network until the free radicals generated are consumed.
Figure 2-7. Reaction mechanism between APS and TEMED resulting in the generation of free radicals; TEMED acts as an additional catalyst to the reaction through its ability to exist as a free radical.
Figure 2-8. pNIPAM cross-linking reaction mechanism using MBA as cross-linker. The free radicals that initiate the crosslinking reaction are provided by the reaction between APS and TEMED.
2.3. Microcarrier-based strategies applied to regenerative medicine

Microcarriers are small particles / beads with diameters in the range of micrometres. Van Wezel A.L. (1967) introduced the term ‘microcarrier’ for the first time in 1967 in his work describing the use of Diethyl-amino-ethyl (DEAE) Sephadex A-50 ion exchange medium as carriers for mammalian cell culture. In microcarrier culture, cells grow as monolayers on the provided surface (Nasser A. and El-Moghaz, 2010) or as multilayers inside the pores of macroporous structures that are usually suspended in culture medium by gentle stirring (Nilsson K. et al., 1986; Rasey J.S. et al., 1996).

Several factors have been identified to be crucial for the successful use of microcarriers in Regenerative Medicine applications. It has been long recognized that cell inoculation is the most critical stage of microcarrier cell culture (Kim B.S et al., 1992). Since the proliferation of anchorage-dependent cells is dependent on cell attachment to suitable culture substrates (Grinnell F., 1980), the microcarrier surfaces have to be specifically designed to promote and enhance cell attachment.

Cell adhesion to surfaces is mediated by protein adsorption onto the provided surfaces. The adsorption of cell adhesion-mediating ECM molecules in appropriate amount, spectrum, spatial conformation, flexibility and accessibility for integrin receptors is strongly influenced by the physical and chemical properties of the material surface. Such properties are wettability, surface charge, surface roughness and topography, mechanical properties (rigidity or elasticity), crystallinity, porosity, solubility, pH or the presence of certain atoms or chemical functional groups, e.g. carbon, amine groups or oxygen groups (Bacakova L. et al., 2004). The optimum protein adsorption and implicit cell adhesion is typically obtained at mild, intermediate values of wettability. For example, Tamada Y. and Ikada Y. (1986) claimed that a polymer surface with a water contact angle of 70º gave the most suitable
surface for cell adhesion. Highly hydrophobic surfaces are often referred to as non-fouling materials as they possess cell anti-adhesive properties, despite their ability to allow protein adsorption even in relatively large amounts (Heitz J. et al., 2003; Bacakova L. et al., 2004; Parizek M. et al., 2009). Cells have the ability to detect very sensitively the mechanical properties of the provided substrate and regulate their adhesion accordingly (Bacakova L. et al., 2004). For example, if the substrate is very rigid and non-deformable, cells are not able to reorganize these molecules in order to access the suitable ligands for the integrin receptors (Groth T. et al., 1999; Bacakova L. et al., 2004). On the other hand, if the substrate is highly flexible, elastic and irreversibly deformable, cells are not able to adhere even though ligands are easily accessible for integrin-binding. As a result of the weak mechanical properties, this type of substrate is not able to withstand the cell tractional forces generated by the cytoskeleton assembling during proliferation (Engler A. et al., 2004; Bacakova L. et al., 2004). Similar to wettability and mechanical properties, other surface properties, such as roughness, surface charge and charge density, have to be in an optimal range in order to promote cell adhesion and proliferation. However, the optimal range is specific to the material type, as well as cell type.

More importantly, after inoculation, a minimum number of cells per microcarrier are required for normal cell growth to occur (Hu W.S. et al., 1985; Nilsson K., 1989; Kim B.S. et al., 1992). Initial cell adhesion to the microcarriers could be improved by manipulating different process parameters. For example, it has been suggested that by using a reduced volume of medium during the first hours of microcarrier cell culture resulted in an increase in the rate and proportion of attached cells. By employing a reduced volume of medium the chance of contact between cells and microcarriers increased together with the conditioning effect of the medium on the microcarrier surface (Bluml G., 2007). In addition, the number of cells that attach to the provided surface is dependent on the microcarrier diameter,
typically in the range of 100 µm to 400 µm. From an engineering and manufacturing point of view, the size distribution of the provided microcarriers should be as narrow as possible in order to ensure a homogeneous culture environment. An uneven size distribution favours cell attachment to the smaller beads as a result of a minimal cell contact with the larger microcarriers that possess a higher sedimentation rate (Nilsson K., 1989; Malda J. and Frondoza C.G., 2006). Moreover, the specific density of the microcarriers should be slightly higher than that of the culture medium (typically in the range of 1.02 to 1.10) in order to achieve suspension culture by gentle agitation (Malda J. and Frondoza C.G., 2006).

The chemical composition of the microcarriers has a major impact on the harvesting of viable cells. Generally, enzymes such as trypsin or collagenase (Heng B.C. et al., 2009; Bajpai R. et al., 2007; Heng B.C. et al., 2007; Hybbinette S. et al., 1999; Batista U. et al, 2010) are required, but cell recovery efficiency could be unsatisfactory and is dependent on the microcarrier chemical composition and degree of porosity (Malda J. and Frondoza C.G., 2006). Moreover, the chemical composition of microcarriers determines biodegradability. This is particularly important for cell-seeded microcarriers intended for direct tissue site delivery which are required to have optimal biodegradation rates to ensure sufficient in vivo longevity (McGlohorn J.B. et al., 2003; Malda J. and Frondoza C.G., 2006; Tan H. et al., 2009).

2.3.1. Advantages over 2D strategies

The use of microcarriers in Regenerative Medicine applications offers numerous advantages over traditional cell culture strategies. Standard cell culture methods are time and labour intensive and cost ineffective. These limitations could be improved by using microcarriers
for cell culture as they provide a large surface area for monolayer cell growth during propagation in a homogenous suspension culture system. Microcarrier cell cultures are characterized by a high surface-to-volume ratio with the possibility to accommodate higher cell densities than those obtained in static cultures. The provided surface area available for cell growth could be easily adjusted by increasing the amount of microcarriers (Nilsson K., 1989; Rodriguez M.E. et al., 2014). From an industrial / manufacturing point of view, microcarrier-based systems possess a tremendous potential as they are space saving and cost effective by minimising the consumption of materials required for cell culture (e.g. medium, additives such as serum) (Levine D.W. et al., 1977; Malda J. and Frondoza C.G., 2006; Martin Y. et al., 2011). Thus, microcarrier cell culture introduced new possibilities and, for the first time, made possible the practical high-yield culture of anchorage-dependent cells.

By employing microcarriers in suspension culture systems, the cell culture procedure could be easily scaled-up (Van Wezel A.L., 1967; Kim B.S. et al., 1992). For example, the suspension culture system comprising of microcarriers used in conjunction with bioreactor systems could be specifically designed to allow a precise control over pH, dissolved oxygen and level of shear applied (Rafiq Q.A. et al., 2013), while facilitating a more efficient gas-liquid oxygen transfer and maintenance of crucial physical, biological and chemical environment conditions. Moreover, when utilising these systems, minimal disruption is required for periodic analysis and small samples of cells are needed without sacrificing the bulk of the cell-seeded microcarriers (Malda J. and Frondoza C.G., 2006). In cell culture, the risk of contamination is related to the number of handling steps (e.g. opening and closing culture vessels) required to produce a given quantity of cells. Microcarrier cell culture reduces the number of handling steps resulting in a much-reduced risk of contamination (Crespi C.L. and Thilly W.G., 1981).
2.3.2. Types of microcarriers

Generally, the term ‘microcarrier’ refers to spherical shaped particles since they are prepared by an emulsion technique. However, non-spherical shaped microcarriers have been reported, such as DEAE-cellulose in the form of elongated cylinders (Reuveny S. et al., 1982; Nilsson K., 1989; Chen A. K-L. et al., 2011). While on beaded-shaped microcarriers, cells grow as monolayers, on elongated cylinder-shaped microcarriers cells grow in aggregates and form cell bridges between the provided microcarriers (Nilsson K., 1989; Chen A. K-L. et al., 2011). Microcarriers are generally classified as solid or porous. Solid microcarriers are characterised by the presence of small pores (micropores) that only allow cells to attach and proliferate on their external surface (Nilsson K., 1986; Rodriguez M.E. et al., 2014). Alternatively, porous microcarriers possess large pores (macroporous) that allow cell colonization of the inner surface, thus mimicking more closely the in vivo microenvironment. Thus porous microcarriers offer an increased surface area available for cell growth resulting in an improved cell density and productivity. Moreover, porous microcarriers create a protected environment for the cells offering protection against shear stress (Nilsson K., 1986; Del Guerra S. et al., 2001; Abdulrhman A.A., Abdurrahim A.E, 2012; Rodriguez M.E. et al., 2014).

The most commonly used microcarriers are composed of a dextran matrix (e.g. Cytodex 1) which facilitates attachment and proliferation of a wide range of cells, such as chondrocytes (Cetinkaya M. et al, 2011), mesenchymal stem cells (Schop D. et al, 2010; Weber C. et al., 2007) or even human embryonic stem cells (Chen A. K-L. et al, 2011). However, a wide variety of materials with good biocompatibility such as cellulose, chitosan, collagen, gelatine, polystyrene etc. have been used for production of microcarriers. Currently, there is a wide range of commercially available microcarriers (Table 2-2).
Table 2-2. Characteristics of commercially available microcarriers

<table>
<thead>
<tr>
<th>Microcarrier</th>
<th>Manufacturer</th>
<th>Core material</th>
<th>Surface coating</th>
<th>Size /µm</th>
<th>Surface charge</th>
<th>Macroporous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytodex 1</td>
<td>GE Healthcare</td>
<td>DEAE-crosslinked dextran</td>
<td>NO</td>
<td>147-248</td>
<td>Positive</td>
<td>NO</td>
</tr>
<tr>
<td>Cytodex 3</td>
<td>GE Healthcare</td>
<td>DEAE-crosslinked dextran</td>
<td>Collagen</td>
<td>141-211</td>
<td>Positive</td>
<td>NO</td>
</tr>
<tr>
<td>Cytopore</td>
<td>GE Healthcare</td>
<td>DEAE-crosslinked cellulose</td>
<td>NO</td>
<td>200-280</td>
<td>Positive</td>
<td>YES</td>
</tr>
<tr>
<td>Cultispher G/S</td>
<td>Percell Biolytica</td>
<td>Highly crosslinked gelatine</td>
<td>None</td>
<td>130-380</td>
<td>NO</td>
<td>YES</td>
</tr>
<tr>
<td>Hillex</td>
<td>Solohill</td>
<td>Modified polystyrene</td>
<td>Cationic trimethyl ammonium</td>
<td>90-212</td>
<td>Positive</td>
<td>NO</td>
</tr>
<tr>
<td>Hillex II</td>
<td>Solohill</td>
<td>Modified polystyrene</td>
<td>Cationic trimethyl ammonium</td>
<td>160-180</td>
<td>Positive</td>
<td>NO</td>
</tr>
<tr>
<td>Glass</td>
<td>Solohill</td>
<td>Crosslinked polystyrene</td>
<td>High silica glass</td>
<td>90-150</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>Plastic</td>
<td>Solohill</td>
<td>Crosslinked polystyrene</td>
<td>None</td>
<td>90-150</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>ProNectin</td>
<td>Solohill</td>
<td>Crosslinked polystyrene</td>
<td>Recombinant fibrinogen</td>
<td>90-150</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>HyQSphere</td>
<td>Thermo Scientific</td>
<td>Modified polystyrene</td>
<td>Cationic triethyl ammonium</td>
<td>160-180</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>DE52 / 53</td>
<td>Whatman</td>
<td>DEAE-cellulose</td>
<td>NO</td>
<td>N/A</td>
<td>Positive</td>
<td>N/A</td>
</tr>
<tr>
<td>2D MicroHex</td>
<td>NUNC</td>
<td>Cell culture grade polystyrene</td>
<td>NU/nclon surface modification</td>
<td>Side-length: 125 µm, Thickness: 25 µm</td>
<td>N/A</td>
<td>NO</td>
</tr>
</tbody>
</table>
2.4. Controlled fabrication of microcarriers

2.4.1. Emulsions

Emulsions have been intensively utilized in different industries such as food, cosmetics and pharmaceutical industries. The size of the droplets contained in emulsions determines some of the most important properties of the emulsion-based products, such as shelf-life, appearance, texture (McClements D.J., 1999). An emulsion is a mixture of two immiscible or poorly miscible liquids where small droplets of one liquid (dispersed phase) are dispersed into the continuous phase represented by the other liquid. The simplest type of emulsion is the monodisperse (single) emulsion which can be very useful for fundamental studies due to its simplicity compared to the polydisperse one. Single emulsions could also be applied to biomaterials of great importance for the production of monosized particles to be used as drug delivery vehicles (Meyer R.F., 2010) or as cell carriers (Hanga M.P. and Holdich R.G., 2014). Depending on the mean droplet diameter, emulsions can be divided into nano- (10–100 nm) (Solans C. et al., 2005), mini- (50–500 nm) (Miller C.M. et al., 1994) and macro-emulsions (greater than 1000 nm) (Sharma M.K. and Shah D.O., 1985). The work reported in this thesis will tackle only macro-emulsions. Common types of emulsions include oil-in-water (O/W) (e.g. milk) and water-in-oil (W/O) (e.g. butter) (Schramm L.L., 2005).

Emulsions are generally thermodynamically unstable and tend to lose their stability due to coalescence which is an irreversible process that leads to the fusion of two or more droplets. The thermodynamic instability of the emulsions is determined by the positive Gibbs free energy (ΔG) of formation accumulated at the interface between the two phases. The interface free energy increases by increasing the interfacial area resulting in the thermodynamic instability of the system (Becher P., 2001; Tadros T.F., 2009). The change in interfacial free energy can be expressed by the equation [2-1]:
\[
\Delta G_{\text{form}} = \gamma \Delta A - T \Delta S
\]  

[2-1]

Where \( \Delta G \) is the formation Gibbs free energy, \( \gamma \) is the interfacial tension; \( \Delta A \) is the created interfacial area; \( \Delta S \) is the entropic term. The dominant term is the surface contribution \( (\gamma \Delta A) \), while the entropic term \( (T \Delta S) \) is insignificant unless the droplet size is small (Tadros T.F., 2009).

High interfacial area in emulsions leads to thermodynamic instability manifesting as various destabilizing mechanisms such as creaming, sedimentation, coalescence or flocculation as a result of the system attempts of lowering the interfacial area (Becher P., 2001; Tadros T.F., 2009). Coalescence refers to the process of thinning and disruption of the liquid film between the droplets resulting in the fusion of two or more droplets to form larger droplets. The driving force for coalescence is the film fluctuations resulting in the close approach of the droplets with their separation being impaired by the strong van der Waals forces (Tadros T.F., 2009). Coalescence could be reduced by adding a suitable emulsifying agent which migrates to the liquid-liquid interface stabilising the emulsion (McClements D.J., 1999; Schramm L.L., 2005). The addition of surfactant to the emulsion results in lowering the interfacial tension between the two phases, thus reducing the Gibbs free energy formed (Schramm L.L., 2005). The reduction of interfacial tension is owing to the distinct structure of surfactants.

A surfactant also known as surface active agent is an amphiphilic molecule comprising of two structurally distinct parts: a hydrophilic head and a hydrophobic tail for water-soluble surfactants or oleophilic head and oleophobic tail for oil-soluble surfactants. In the great majority of surfactants, the hydrophobic part consists of a hydrocarbon chain usually with an average length of 12 to 18 carbon atoms and it may include aromatic rings (Cullum D.C.,
Surfactants could be classified according to the nature of their hydrophilic parts in: anionic (negatively charged), non-ionic (uncharged), cationic (positively charged) or amphoteric (both positively and negatively charged) (Cullum D.C., 1994). Another classification of surfactants was firstly introduced by Griffin W.C. (1949) based on the hydrophilic-lipophilic balance (HLB). The HLB number represents an empirical numerical correlation of the emulsifying and solubilizing properties of different surface active agents (Griffin W.C., 1949; Corin K.C. and O'Connor C.T., 2014). The suitability of a surfactant to be used in the production of a specific emulsion is verified based on the HLB value. Thus, surfactants with HLB number in the range of 3 to 6 are suitable to be used as emulsifiers for W/O emulsions, whereas the surfactant with HLB values in the range of 8 to 18 are suitable for the preparation of O/W emulsions (Becher P., 2001). The surfactant of choice for the work presented in this thesis was a sorbitan-based non-ionic surfactant (e.g. Span 80) with a HLB value of 4.3 which is in the range recommended for W/O emulsifiers (Becher P., 2001; Tadros T.F., 2009). The structure of the sorbitan-based surfactant selected is presented in Figure 2-9.

Figure 2-9. Chemical structure of sorbitan-based surfactants.
2.4.2. Emulsification methods

The production of emulsions requires an external source of energy that usually is represented by the shear stress applied. Conventional methods involve the use of stirring equipment, colloid mills, homogenizers or ultrasonic equipment for generating high shear stresses to deform and disrupt large droplets (Charcosset C. et al., 2004). The biggest disadvantage of the conventional methods is that the shear stress generated is not uniform across the system resulting in emulsions with inconsistent uniformity of the droplets and making impossible direct control over droplet size (Sotoyama K. et al., 1999; Shah R.K. et al., 2008). Due to this disadvantage, new techniques for producing emulsions have been developed and optimized to gain a better control over the droplet size, such as: flow focusing (Utada A.S. et al., 2005), microchannel emulsification (Kawakatsu T. et al., 2001; Neves M.A. et al., 2008), shear rupturing (Mason T.G. et al., 1997) and membrane emulsification (Katoh R. et al., 1996; Sotoyama K. et al., 1999; Dragosavac M.M. et al., 2012; Holdich R.G. et al., 2013). Membrane emulsification has the potential of producing droplets at higher throughputs than can be obtained in other microengineered systems such as microchannel or microfluidic devices, but at the expense of a lower degree of monodispersity (Vladisavljevic G.T. et al., 2012). Thus, according to Vladisavljevic G.T. et al. (2012), membrane emulsification generates droplets with coefficients of variation (CV) in the range of 10-20%, while microchannel devices can generate droplets with a CV less than 5% and microfluidic and flow focusing devices of less than 3%. However, the droplet monodispersity obtained by membrane emulsification is still in the range of interest for the production of microcarriers. For example, commercially available microcarriers have a size coefficient of variation of typically 18%. In the light of the aforementioned reasons, the work presented in this thesis was focused on membrane emulsification (refer to section 2.4.3).
2.4.3. Membrane emulsification

Membrane Emulsification is a relatively novel and highly attractive technique with numerous advantages over conventional emulsification methods or devices, including the possibility of producing very fine emulsions of controlled droplet sizes and with a narrow size distribution, as well as simplicity of design and reduction in the consumption of the surfactant required. By employing membrane emulsification, low shear stress is generated at the membrane surface allowing the use of shear-sensitive compounds, such as proteins. Typically, membrane emulsification involves using a low pressure to force the dispersed phase to permeate through a membrane having uniform pore-size distribution into the continuous phase. Membrane emulsification technique is applicable to both O/W and W/O emulsions (Dickinson E., 1994; Schroder V. et al., 1998; Joscelyne S.M. and Tragardh G., 2000; Vladisavljevic G.T. et al., 2000; Charcosset C. et al., 2004; Egidi E. et al., 2008). The size of the generated droplets is determined by several parameters, such as membrane properties (e.g. pore size, wettability, pore shape, distance between pores) and process parameters (e.g. shear stress, DP injection rate, viscosity of the phases, interfacial tension, surfactant type and concentration) (Stillwell M.T. et al., 2007; Kosvintsev S.R. et al., 2008; Egidi E. et al., 2008). Up-to-date, different types of membranes have been employed for membrane emulsification.

The types of membranes employed and their properties are described in detail in section 2.4.3.2. The role of surfactant in membrane emulsification is to rapidly adsorb to the newly formed oil–water interface to facilitate droplet detachment and stabilise the formed droplets by reducing the interfacial tension between the phases. As a rule, the faster the surfactant molecules adsorb to the newly formed interface, the smaller the droplet size of the resultant emulsion becomes. Surfactant molecules should not adsorb to the membrane surface, since
otherwise the membrane can become fouled by the surfactant molecules and the dispersed phase can spread over the membrane surface (Schroder V. et al., 1998). Droplet detachment from the membrane pores can be achieved by two mechanisms depending on the presence of shear stress at the membrane surface and are described in detail in section 2.4.3.3. In addition to shear stress, the rate at which the dispersed phase is passed through the membrane plays a crucial role. If dispersed phase flux is increased, the droplet volume prior to detachment increases resulting in an increase in droplet size (Peng S.J. and Williams R.A., 1998; Vladisavljevic R.A. and Schubert H., 2003; Stillwell M.T. et al., 2007). Another phenomenon linked to the dispersed phase flux is the change in dynamic interfacial tension which increases due to the creation of fresh interface as the droplet expands, thus lowering the surfactant coverage per unit area (Rayner M. et al., 2005). This is a kinetic process, as the interfacial tension will be lowered by new surfactant adsorbing from the continuous phase to the surface, but the faster the dispersed phase flux the lower will be the overall effective surfactant concentration during drop formation at the interface between the two phases. Lastly, the increase in injection rate may lead to a higher number of active pores, or it may cause a transition from a dripping regime to a continuous outflow regime (Egidi E. et al., 2008).

2.4.3.1. Membrane emulsification-based devices

Membrane emulsification has been first introduced in 1986 by Nakashima and Shimizu for the production of highly-uniform sized kerosene-in-water and water-in-kerosene emulsions by employing a glass membrane referred to as Shirasu Porous Glass (SPG) (Nakashima T. et al., 1992; Vladisavljevic G.T. and Williams R.A., 2005; Egidi E. et al., 2008). A series of membrane emulsification devices have been introduced over the years for the production of...
emulsions. Based on the methods employed for the generation of the shear stress at the membrane surface, membrane emulsification devices could be classified as: cross-flow systems (Nakashima T. et al., 1992; Schroder V. et al., 1998; Williams R.A. et al., 1998; Nakashima T. et al., 2000; Ho T.H. et al., 2013), stirred systems (You J.O. et al., 2001; Kosvintsev S.R. et al., 2005; Dragosavac M.M. et al., 2008) or dynamic systems based on rotating (Vladisavljevic G.T. and Williams R.A., 2006) or vibrating (Holdich R.G. et al., 2010) membrane. Typical cross-flow membrane emulsification systems involve the continuous phase flowing through the tubular membrane parallel to the membrane surface, while the pressurised dispersed phase flows radially across the membrane wall (Schroder V. et al., 1998). Crossflow systems are easily scalable and provide a constant shear stress along the membrane surface (Schroder V. et al., 1998; Vladisavljevic G.T. et al., 2012).

The first membrane emulsification apparatus described by Nakashima T. consisted of a tubular microfiltration membrane, a pump, a feed vessel and a pressurized (N\textsubscript{2}) oil container and is presented in Figure 2-10 (Nakashima T. et al., 1992; Joscelyne S.M. and Tragardh G., 2000; Charcosset C. et al., 2004). The dispersed phase was pumped under nitrogen gas (N\textsubscript{2}) pressure through the pores of the microporous SPG membrane into the continuous phase which was circulated through the middle of the membrane. Droplets grew at the pore outlets until reaching a critical size when droplet detachment occurred as a result of reaching a balance between several forces acting at the membrane’s pore (Schroder V. et al., 1998; Charcosset C. et al., 2004). Droplet detachment mechanism and the forces acting at the membrane pore are described in more detail in section 2.4.3.4.
Figure 2-10. Schematic of a typical small scale membrane emulsification apparatus (Nakashima T. et al., 1992).

In order to ensure a regular droplet detachment from the membrane’s pores, shear stress has to be generated at the membrane – continuous phase interface and this could be achieved by recirculating the continuous phase by using a low shear pump or by applied agitation (Vladisavljevic G.T. and Williams R.A., 2006). The continuous phase recirculating approach is typically used in cross-flow systems. The main disadvantage of this approach consists in additional drop breakage occurring inside the pump and the tubes (Vladisavljevic G.T. and Williams R.A., 2006; Egidi E. et al., 2008). The other alternative is to employ an agitation-induce shear which requires a rate of mixing high enough to provide the required shear value on the membrane surface, but not too excessive in order to prevent further droplet break-up (Vladisavljevic G.T. and Williams R.A., 2005). From this perspective, stirred systems are viable alternatives to crossflow membrane emulsification systems. Stirred membrane emulsification systems are typically simpler in design and easier to operate and employ tubular (You J.O. et al., 2001) or flat membranes (Kosvintsev S.R. et al., 2005; Dragosavac M.M. et al., 2008). An example of a stirred emulsification system with a
tube–shaped porous glass membrane is presented in Figure 2-11. The dispersion phase was pressed through the membrane’s pores under nitrogen gas pressure into the continuous phase. The shear stress at the membrane surface was ensured by a magnetic stirrer placed below the membrane (You J.O. et al., 2001).

![Figure 2-11. Schematic diagram of membrane emulsification stirred system employing a tube membrane](image)

Figure 2-11. Schematic diagram of membrane emulsification stirred system employing a tube membrane (1) Nitrogen gas tank; (2) Pressure gauge; (3) Dispersion phase storage module; (4) Porous glass membrane; (5) Continuous phase; (6) Magnetic stirrer (You J.O. et al., 2001).

An increased interest was given to stirred membrane emulsification systems employing flat membranes (Kosvintsev S.R. et al., 2005; Kosvintsev S.R. et al., 2008; Dragosavac M.M. et al., 2008; Egidi E. et al., 2008; Dragosavac M.M. et al., 2012). An example of such a device is represented by the Dispersion Cell (MicroPore Technologies Ltd., UK). The Dispersion Cell has a simple and user-friendly design as shown in Figure 2-12. The device is made of a poly (tetrafluoroethylene) (PTFE) base that houses an inlet for the dispersed phase and the metallic disc-shaped flat membrane. On top of the membrane, a glass cylinder is screwed
and it contains the continuous phase. The Dispersion Cell uses a 24V DC motor to drive a paddle-blade stirrer that provides the shear stress at the membrane’s surface necessary for drop detachment. The dispersed phase is injected at the base of the device with the help of a peristaltic pump or injection pump through the uniform pores of the disc shaped membrane into the continuous phase thus forming an emulsion.

Figure 2-12. Schematic representation of the Dispersion Cell device used for the production of droplets.

From an industrial point of view, the Dispersion Cell device is not suitable for use due to an incompatible design for up-scaling, but it is excellent for testing formulations, studying the effect of process parameters on particle size and size distribution and obtaining small quantities of material. The knowledge obtained from the Dispersion Cell could be transferred to a novel system based on an oscillating membrane (Holdich R.G. et al, 2010) functioning on the same principle that has been reported by our group for the production of uniform droplets with applications at higher manufacturing scales.
Another approach consists of systems in which the droplet detachment is stimulated by the rotation (Vladisavljevic G.T. and Williams R.A., 2006) or vibration (Holdich R.G. et al., 2010) of the membrane within a stationary CP. The shear stress generated could be controlled by the rotation speed of the membrane (Vladisavljevic G.T. and Williams R.A., 2006) or the frequency and amplitude of the membrane oscillation (Holdich R.G. et al., 2010). These systems possess the major advantage of scalability as they could be easily integrated into systems with large membrane surface area. The work presented in this thesis focused only on the use of the Dispersion Cell for the production of W/O emulsions.

2.4.3.2. Membranes

Up to present time, a broad range of types of membranes have been employed for the production of emulsions, such as SPG (Nakashima T. et al., 1992; Katoh R. et al., 1996; ), ceramic (Schroder V. and Schubert H., 1999; Wu J. et al., 2006), metallic (Kostvintsev S.R. et al., 2005; Stillwell M.T. et al., 2007; Egidi E. et al., 2008; Dragosavac M.M. et al., 2012) or polymeric (Vladisavljevic G.T. et al., 2000) membranes. The membranes typically employed for membrane emulsification can be classified in two main categories: tortuous pore channel membranes (e.g. SPG, α-Al₂O₃, silica etc.) and microengineered sieves (e.g. metallic). The main disadvantage of the tortuous membranes is the low flux (Vladisavljevic G.T. and Schubert H., 2003) as a result of the membrane thickness and a smaller number of active pores. On the opposite, microengineered sieves can achieve high fluxes (Holdich R.G. et al., 2010) as a result of a higher number of active pores than compared to tortuous membranes. Based on these advantages, the work presented in this doctoral thesis involved the use of microengineered metallic disc-shaped membranes fabricated by galvanic deposition of nickel onto a template formed by a photolithographic technique.
Depending on the membrane hydrophilicity or hydrophobicity and the composition of the phases employed, O/W or W/O emulsions could be produced (Vladisavljevic G.T. and Williams R.A., 2005; Seo M. et al., 2007). Regardless of the type of membrane employed, the dispersed phase should not wet the membrane pores. Thus, hydrophilic membranes are suitable for preparing O/W emulsions, while hydrophobic membranes could be employed for W/O emulsions. Nakashima T. (1992) studied the effect of membrane wetting on the monodispersity of the produced emulsions and found that by employing a hydrophobized SPG membrane for preparation of O/W emulsions, a polydispersed emulsion with a larger average droplet size was generated compared to when hydrophilic SPG membranes were employed for the production of the same type of emulsion. Similar results were obtained for the production of W/O emulsions when employing both hydrophilic and hydrophobic membranes (Nakashima T. et al., 1992). Hydrophilic membranes could be made hydrophobic by different chemical surface treatment methods. However, this approach requires the use of coupling agents (e.g. silane) that limits the use of membrane emulsification for the production of products with applications in industries such as food industry. Also, the membrane surface treatment is required to be repeated after each cleaning cycle due to possible damage incurred during the cleaning step (Joscelyne S.M. and Tragardh G., 2000). However, the chemical treatment approach could be avoided by simply pre-soaking the membrane in the continuous phase (Katoh R. et al., 1996).

The porosity of the membrane plays an important role in the droplet size distribution. The porosity determines the distance between adjacent pores. The closer the pores are together, the higher the porosity is and the greater is the likelihood of droplet coalescence at the membrane’s surface before detachment (Joscelyne S.M. and Tragardh G., 2000). The metallic membrane porosity was calculated based on the fact that all the membranes employed have a perfectly ordered hexagonal array of pores with a pore in the centre as
shown in Figure 2-13. Taking this into consideration, the membrane’s porosity could be calculated by using equation [eq. 2-2] (Dragosavac M.M. et al., 2008).

\[ \varepsilon = \frac{3 \times \text{Apore}}{A_{\text{hexagon}}} = \frac{3 \left( \frac{\pi d_p^2}{4} \right)}{\frac{3 \sqrt{3}}{2} L^2} = \frac{\pi}{2 \sqrt{3}} \left( \frac{d_p}{L} \right)^2 \]  

[2-2]

Where \( d_p \) is pore size;

\( L \) is pore spacing.

The number of pores found on the disc membrane can then be calculated by using eq. [2-3]:

\[ n = \frac{4 \varepsilon A_m}{\pi d_p^2} \]  

[2-3]

Where \( n \) is pore number;

\( \varepsilon \) is porosity;

\( A_m \) is membrane area;

\( d_p \) is pore size.
2.4.3.3. Droplet detachment modelling

Two main droplet detachment mechanisms have been previously described in literature: spontaneous transformation-based droplet formation (Sugiura S. et al., 2002; Kosvintsev S.R. et al., 2008) and shear-induced droplet formation (Kosvintsev S.R. et al., 2005; Egidi E. et al., 2008). The first mechanism generally refers to droplet detachment from the membrane surface in the absence of shear when droplets could be generated at low dispersed phase flow rates as a result of the buoyancy force counteracting the capillary force that retains the drop at the membrane surface (Sugiura S. et al., 2002; Kosvintsev S.R. et al., 2008). The droplet at a pore tends to form a spherical shape under the action of interfacial tension. However, some distortion may occur and is dependent on the continuous phase flow rate and the contact angle between the droplet and the membrane surface (Peng S.J. and Williams R.A., 1998). Droplet detachment from the membrane is dependent on several main forces acting at the membrane’s surface. The relative magnitude of these forces changes continuously as the droplet increases in size. These forces could be divided in two main groups: (a) forces holding the droplet and (b) forces detaching the droplet from the pore (Figure 2-14). The theoretical model for predicting the generated droplet size was obtained by calculating the overall forces assuming a rigid and spherical shaped droplet. The capillary force ($F_{ca}$) is the main force holding the droplet on the membrane surface and represents the effect of the dispersed phase adhesion around the edge of the pore opening. Another force occurs during droplet detachment due to the density difference between the dispersed and continuous phases. This force is the buoyancy force ($F_b$) and it can be neglected for droplets smaller than 500 µm (Kosvintsev S.R. et al., 2008). The main detaching force is the viscous drag force ($F_d$) which is created by the continuous phase flowing past the droplet parallel to the membrane surface. The other forces involved in droplet detachment are the static pressure difference force ($F_{neck}$) which is created by the pressure difference between the
dispersed and continuous phases, the dynamic lift force \( (F_L) \) which is caused by the asymmetric velocity profile of the continuous phase and the inertial force \( (F_i) \) induced by the dispersed phase mass flowing out of the pore into the forming droplet (Joscelyne S.M. and Tragardh G., 2000; Charcosset C. et al., 2004).

However, Zhu J. and Barrow D. (2005) suggested that at small interpore distances an additional force is generated and is causing droplet detachment. They have found that the droplet size increases direct proportional to the dispersed phase flow rate up to a maximum and then decreases when the pore distance is at the finer end of the pore spacing range. By video microscopy, the same research group observed that as the droplets grew in size, they interacted with the droplets produced at the adjacent pores resulting in deformation from the preferred spherical shape, followed by detachment under the influence of a ‘push-off’ force (Zhu J. and Barrow D., 2005). When the ‘push-off’ force is present, smaller droplets with greater size distribution are expected to form (Egidi E. et al, 2008).

![Figure 2-14. Representation of forces acting during droplet detachment from the pore of a membrane (adapted from Charcosset C. et al., 2004).](image)

53 | Page
The equations used in this work were introduced in a previous study (Kosvintsev S.R. et al., 2005). Briefly, the model consisted in calculating the droplet size from a balance of the capillary force and the drag force acting on a strongly deformed droplet at a single membrane pore:

\[ x = \frac{\sqrt{18\tau^2 r^2 p^2 + 2\sqrt{81\tau^4 r^4 p^4 + 4\tau^2 r^2 p^2 \gamma^2}}}{3\tau} \]  
[2-4]

Where \( r_p \) is the pore radius, \( \tau \) is the shear stress, \( \gamma \) is the interfacial tension and \( x \) is the drop diameter.

\[ r_{\text{trans}} = \frac{D}{2} 1.23(0.57 + 0.35 \frac{b}{T})^0.036 nb^{0.116} \frac{Re}{1000+1.43Re} \]  
[2-5]

For the Dispersion Cell, equation [2-5] can be used to calculate the location of the transitional radius along the paddle-blade radius. The transitional radius is the point at which the rotation changes from a forced vortex to a free vortex where \( b \) is the blade height, \( T \) is the tank width, \( D \) is the stirrer width, and \( nb \) is the number of blades. The Reynolds Number is defined by \( Re = \frac{\rho \omega D^2}{2\pi \eta} \), where \( \rho \) is the continuous phase density, \( \omega \) is the angular velocity and \( \eta \) is the continuous phase coefficient of dynamic viscosity. For the experimental equipment used in this study, \( H = 16 \text{ cm}, D = 3.1 \text{ cm}, nb = 2, b = 1.1 \text{ cm} \) and \( T = 3.5 \text{ cm} \). The boundary layer thickness, \( \delta \), is defined by the Landau- Lifshitz equation [2-6] (Landau L.D. and Lifshitz E.M., 1959).

\[ \delta = \sqrt{\frac{\eta}{\rho \omega}} \]  
[2-6]

\[ \tau = 0.825\eta \omega r^{\frac{1}{\delta}} \]  \hspace{1cm} \text{for} \ r < r_{\text{trans}} \]  
[2-7]

\[ \tau = 0.825\eta \omega r_{\text{trans}} \left(\frac{r_{\text{trans}}}{r}\right)^{0.6} \frac{1}{\delta} \]  \hspace{1cm} \text{for} \ r > r_{\text{trans}} \]  
[2-8]
CHAPTER 2. LITERATURE REVIEW AND BACKGROUND

The shear stress in the boundary layer above the membrane surface varies according to equations [2-7] and [2-8], for radial positions less than the transitional radius and greater than the transitional radius, respectively. In this work, the maximum shear stress [2-9] is calculated using equations [2-7], or [2-8], with \( r = r_{\text{trans}} \) for a given rotation speed and continuous phase viscosity.

\[
\tau_{\text{max}} = 0.825 \eta \omega r_{\text{trans}} \frac{1}{\delta} \quad \text{for } r = r_{\text{trans}} \quad [2-9]
\]

The maximum shear stress is then used in equation [2-4] to provide the predicted drop size, which is then compared to the experimental values obtained in the presented work where shear stresses and continuous phase viscosity were varied. The approach based on equations [2-4] to [2-9] will be referred to as Model A. An alternative approach specifically designed for the Dispersion Cell where the shear stress profile varies across the whole surface of the membrane (Dragosavac M.M. et al., 2008) is based on using average shear stress values for calculating the predicted drop size [eq. 2-10].

\[
\tau_{\text{av}} = \int_{0}^{r_{\text{trans}}} 0.825 \eta \omega r \frac{1}{\delta} (2\pi r) dr + \int_{r_{\text{trans}}}^{Dm/2} 0.825 \eta \omega r_{\text{trans}} (r_{\text{trans}} \frac{0.6}{\delta} (2\pi r) dr

\]

\[
= \frac{6.6}{Dm^2} \eta \omega \frac{1}{\delta} \left\{ r_{\text{trans}}^3 \frac{3}{3} + r_{\text{trans}}^{1.6} \frac{1.4}{1.4} \left[ \left( \frac{Dm}{2} \right)^{1.4} - r_{\text{trans}}^{1.4} \right] \right\} \quad [2-10]
\]

The model based on average shear stress [eq. 2-10] will be referred to as Model B. Based on the shear profile at the membrane’s surface in the Dispersion Cell, model B is considered to be more accurate as it takes into account the variation of the shear stress at the membrane surface.
3. GENERAL MATERIALS AND METHODS

3.1. Chemical reagents, consumables and equipment

All the chemicals used in this work were acquired from Sigma Aldrich, UK unless otherwise stated. All the water used for preparation of aqueous solutions was obtained from a Reverse Osmosis system (Millipore, UK). A list of equipment utilised for achieving this work is provided in Appendix A1.

3.2. Dispersion Cell

3.2.1. General procedure

Membrane emulsification was employed for the production of Water-in-Oil (W/O) emulsions and applied on the Dispersion Cell device (MicroPore Technologies, UK). The working principle of the Dispersion Cell device is described in detail in section 2.4.3.1. The metallic disc shaped membranes used in this work are described in detail in section 3.2.2. All the calibration curves of the equipment employed in this set-up (mechanical stirrers, peristaltic pump and syringe pump) are provided in Appendix A2. Regardless of the emulsion formulation chosen, the following general procedure can be employed when utilizing the Dispersion Cell presented in Figure 2-12.

Prior to each experiment, the base of the device was filled with a small volume of continuous phase and the air bubbles trapped in the system were removed with the help of a glass syringe. It is important to remove the air trapped in the system as it could interfere with droplet production having an influence on the size distribution of the generated droplets.
and implicit particles. Once all the air bubbles were removed from the system, the metallic membrane was then placed with the shiny side up at the base of the device and a PTFE sealing ring was positioned above the membrane to ensure that no leakage will take place during operation of the device. The PTFE sealing ring can also be used with continuous phases such as kerosene or toluene as it does not swell in contact with these phases. Once the sealing ring was placed, the glass cell was then inserted at the base and the desired volume of continuous phase was added, followed by the positioning of the paddle blade stirrer on top. Once the device was prepared, the dispersed phase was injected at the base of the Dispersion Cell.

Stirrer speed settings ranged from 2 V to 10 V and were expressed as maximum shear stress at the transitional radius (equation [2-9]) (refer to section 2.4.3.3). The dispersed phase was injected through the membrane at a constant controlled rate using either a syringe pump (KDS Scientific model 101) with injection rates ranging between 0.58 mL/min and 3.3 mL/min or a peristaltic pump (Watson Marlow 101U) with injection rates ranging between 1.6 mL/min and 17 mL/min. The continuous phase volume used for all experiments was 100 cm³ and for each experiment 10 cm³ of dispersed phase was injected.

3.2.2. Metallic membranes

The disc shaped metallic membranes used in this work were employed for production of W/O emulsions. In order to be suitable for this type of emulsion, the membranes were kept in the oil phase without any surfactant overnight and at least 30 minutes before each experiment, in order to increase their hydrophobicity. After use, the membranes were soaked in soapy water and placed in a sonication bath for 20 minutes, followed by washing with
Reverse Osmosis (RO) water, air drying and soaking in the oil phase without surfactant for 30 minutes.

The main membranes used in this work were disc shaped metallic arrays of 20 µm pores with 80 µm, and alternatively 200 µm pore spacing with an effective surface area of 8.54 cm². The characteristics of all the membranes used in this work are presented in Table 3-1. The membrane porosity and number of pores were calculated based on the equations provided in section 2.4.3.2.

Table 3-1. Characteristics of the membranes utilized in this work

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Pore size/ µm</th>
<th>Inter-pore Distance / µm</th>
<th>Membrane Area / cm²</th>
<th>Effective diameter/ cm</th>
<th>Porosity / %</th>
<th>Number of pores</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTFE-coated (disc)</td>
<td>20</td>
<td>200</td>
<td>8.54</td>
<td>3.3</td>
<td>0.9</td>
<td>24,477</td>
</tr>
<tr>
<td>Nickel (disc)</td>
<td>20</td>
<td>80</td>
<td>8.54</td>
<td>3.3</td>
<td>5.66</td>
<td>153,937</td>
</tr>
<tr>
<td>Nickel (annular)</td>
<td>20</td>
<td>200</td>
<td>1.79</td>
<td>2.52</td>
<td>0.51</td>
<td>3,264</td>
</tr>
</tbody>
</table>

3.3. Particle production

Regardless of the particle type, there are two steps in particle production: droplet production by membrane emulsification, followed by crosslinking of the monomer droplets generated. Monomer droplets were produced by using the Dispersion Cell described in section 3.2. Once the W/O emulsion containing the monomer droplets is obtained, the emulsion is
transferred to a clean, dry, 250 mL beaker and under stirring the crosslinking step is initiated based on the chemistry applied.

3.3.1. Solid pNIPAM particle production

The monomer (NIPAM), the crosslinker (MBA), the initiator (APS), the accelerator (TEMED), low odour kerosene and sorbitan monooleate (Span 80) were all supplied by Sigma Aldrich, UK.

The dispersed phase was obtained by dissolving the monomer in RO water to achieve either a 5% wt or 10% wt concentration depending on the experiment, followed by the dissolving of the crosslinker to achieve concentrations varying from 0.2% wt to 1% wt. Prior to each experiment, the initiator was added to the dispersed phase in a concentration of 10 mg per mL of dispersed phase. The continuous phase was obtained by dissolving Span 80 into kerosene under vigorous stirring and heating up to 50ºC (below the flash point of kerosene) to achieve a concentration of 2% wt.

Prior to the emulsification step, the continuous phase is stirred for 15 minutes under a Nitrogen (Oxygen free) atmosphere to remove the air trapped in the system that interferes with the free radical polymerisation chemistry described in more detail in section 3.3.1.2. The dispersed phase is then injected with a peristaltic pump or a syringe pump through the uniform pores of the disc shaped metallic membrane into the continuous phase to form a W/O emulsion containing monomer droplets. Once the W/O emulsion was formed, the resultant emulsion was transferred to a clean, dry beaker and under continuous stirring at 300 rpm while nitrogen (oxygen free) was continuously purged and 0.5 mL of accelerator (TEMED) was added to initiate the free radical polymerisation. The reaction was stirred at
room temperature under a nitrogen atmosphere for 40 minutes, after which the stirring was stopped and the emulsion containing the pNIPAM particles was left to settle. After some time, phase separation occurred. Two sharp layers were observed: top layer containing the oil phase and the bottom layer containing the solid pNIPAM particles. The top layer was decanted and the particles were then washed with soapy water, followed by water dialysis for several days in order to remove any unreacted reagents, as well as oil traces. The washed particles were stored in deionized water at room temperature or dried and stored as a powder. Depending on the type of particles, several drying methods were attempted and are described in detail in Chapters 4 and 5.

3.3.2. Core-shell pNIPAM-coated Alginate particle production

3.3.2.1. Materials

Sodium alginate (food grade) was acquired from Kalys Gastronomie (France). Its viscosity average molecular weight, $M_V$, was determined by applying the Mark-Houwink equation [eq. 3-1]:

$$[\eta] = k M^\alpha$$

Where: $[\eta]$ represents the intrinsic viscosity

$M$ is the viscosity-average molecular weight of the polymer

$K$; $\alpha$ are parameters that depend on the particular polymer-solvent system and temperature used.
The viscosity-average molecular weight of the sodium alginate used in this work was determined in 0.1M NaCl at 25°C by using an Ostwald capillary viscometer and applying the equation given by Martinsen A. et al. (1991) [eq. 3-2] for *Laminaria hyperborea* origin sodium alginates.

\[
[\eta] = 6.9 \times 10^{-4} M^{1.13}
\]  

[3-2]

By employing an Ostwald capillary viscometer, the coefficients of viscosity of sodium alginate solutions of different concentrations were determined, followed by calculation of the reduced and inherent viscosities of the polymer solutions. Based on the reduced and inherent viscosities, the intrinsic viscosity of the polymer was determined, as shown in Figure 3-1.

Figure 3-1. Reduced and Inherent viscosity plot against polymer concentration. Determination of the intrinsic viscosity of the polymer further used for determination of the polymer’s molecular weight; Values are expressed as means of triplicates.
Based on the above calculations, the viscosity-average molecular weight of sodium alginate was found to be equal to $1.59 \times 10^5$ g/mol and is comparable to the value of $1.4 \times 10^5$ g/mol found by Cheaburu C.N. et al. (2013). The food-grade alginate employed in this study is not ideal as no information on M-to-G ratio is available and there are limitations in regard to its purity. High purity alginates with well defined characteristics are available from specialised companies. However, these are available at high prices.

Pharmaceutical grade Miglyol 840 was obtained from Sasol, Germany. According to the manufacturer’s description, Miglyol 840 is a propylene glycol diester of saturated plant fatty acids with a viscosity of 10 mPa s. Span 80 was supplied by Sigma Aldrich, UK, glacial acetic acid from Fisher Scientific, UK, and amine-terminated pNIPAM from Sigma Aldrich, UK with an average number molecular weight $M_n$ of 5500 (used as received).

3.3.2.2. Production of core calcium alginate particles

The dispersed phase was prepared by dissolving sodium alginate using magnetic stirring and heating to 60°C in Reverse Osmosis (RO) water to achieve a concentration of 1.5% wt. The solution was then left to cool down to room temperature after which calcium carbonate as a source of calcium ions is added to achieve a final concentration of 0.5% wt. Before use, the dispersed phase was left to deaerate for at least one hour at room temperature in order to remove any air bubbles that might interfere with the droplet formation. The continuous phase consisted of 1% wt Span 80 in Miglyol 840 and was obtained by dissolving Span80 in the oil phase under vigorous stirring and heating at 60°C.
Once the W/O emulsion was formed, the resultant emulsion was transferred to a clean, dry beaker and under continuous stirring at 300 rpm, glacial acetic acid was added to dissolve the calcium carbonate and release Ca\(^{2+}\) to initiate the internal gelation of the alginate droplets, thus forming alginate microgel particles. The gelation was maintained for 30 minutes, after which the core particles were washed successively with acetone and then deionized water to remove any oil traces.

### 3.3.2.3. pNIPAM coating of the core particles

Amine terminated pNIPAM was dissolved in RO water at room temperature to form solutions of different concentrations ranging from 0 ppm to 1000 ppm. The solubility of PNIPAM in cold RO water at room temperature can be attributed to the ability of the macromolecule to form hydrogen bonds with the water molecules via the amide functional groups.

For the coating step, 0.5 g of dried alginate particles were weighed and suspended in 20 mL solutions of different concentrations of amine terminated pNIPAM and then placed on a shaking platform at room temperature for 24 h. It is believed that the amino groups from the amine terminated pNIPAM form polyelectrolyte complexes with carboxylic acid groups in the alginate network. The coated beads were retrieved by filtration and used for further experiments.

In order to quantify and demonstrate successful coating, a Langmuir sorption isotherm was constructed and it is described in detail in section 5.5.2.2. The amounts of polymer adsorbed onto the calcium alginate particles were determined by measuring the residual polymer
concentrations in the solutions after the contact time. This was achieved by retrieving the residual polymer solutions after filtration of the coated beads and further filtered through a 0.2 µm filter (Sartorius) to insure the removal of any coated alginate beads that might have escaped the first filtration and might interfere with the further measurements.

3.4. Particle characterisation techniques

3.4.1. Interfacial tension measurements

The surface tension and interfacial tension measurements of the continuous phase and dispersed phase were performed by using the Du Nouy ring method with an electronic tensiometer (White Electronic Instruments; DB 2KS). The method involves slowly lifting a ring, made of platinum, from the surface of a liquid or interface between two liquids. The force required to raise the ring from the liquid's surface is measured and related to the liquid's surface tension.

The surface tension and interfacial tension measurements of the continuous and dispersed phases employed for the production of pNIPAM solid particles and pNIPAM-coated alginate particles are provided in Tables 3-2 and 3-3. The values shown in both tables are the average measurement values of three different samples.
Table 3-2. Characteristics of the continuous and dispersed phases used for the production of pNIPAM particles. O – kerosene (without surfactant); O₁ – 2% wt Span80 in kerosene; W₁ – 5% wt NIPAM + 0.2% wt MBA (without APS). Data is expressed as mean ± SD (n=3).

<table>
<thead>
<tr>
<th>Aqueous phase</th>
<th>Oil phase</th>
<th>Surface Tension / Interfacial tension / mN/m (Mean ±SD; n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>/</td>
<td>O</td>
<td>27.6 ± 0.1</td>
</tr>
<tr>
<td>/</td>
<td>O₁</td>
<td>27 ± 0.06</td>
</tr>
<tr>
<td>W₁</td>
<td>/</td>
<td>51 ± 0.2</td>
</tr>
<tr>
<td>W₁</td>
<td>O</td>
<td>16.4 ± 0.2</td>
</tr>
<tr>
<td>W₁</td>
<td>O₁</td>
<td>2.5 ± 0.1</td>
</tr>
</tbody>
</table>

Table 3-3. Characteristics of the continuous and dispersed phases used for the production of core calcium alginate particles. O* – Miglyol 840 (without surfactant); O₁* – 1% wt Span80 in Miglyol 840; W₁* – 1.5% wt sodium alginate with 0.5% wt CaCO₃.

<table>
<thead>
<tr>
<th>Aqueous phase</th>
<th>Oil phase</th>
<th>Surface Tension / Interfacial tension / mN/m (Mean ± SD, n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>/</td>
<td>O*</td>
<td>30.4 ± 0.2</td>
</tr>
<tr>
<td>/</td>
<td>O₁*</td>
<td>27.3 ± 0.2</td>
</tr>
<tr>
<td>W₁*</td>
<td>O₁*</td>
<td>18.8 ± 3.8</td>
</tr>
</tbody>
</table>
3.4.2. Refractive index measurements

In order to be able to measure the size of the produced particles by using a particle analyser device that uses laser diffraction, the refractive index is an important parameter of the samples that has to be known. In this work, particles with different formulations have been prepared, so refractive index measurements had to be performed for each formulation. The refractive index was measured for hydrogel membranes prepared by following the same recipe as for the different formulated particles by using an automatic digital refractometer (Rudolph Research J357). Refractive index measurements with temperature increase were performed to monitor absorption in the range of 20ºC to 40ºC representing temperature values above and below the pNIPAM phase transition temperature found to be approximately 32ºC (Heskins M. and Guillet J.E., 1968; Tauer K. et al., 2009).

3.4.3. pNIPAM phase transition measurements

Following the same recipe previously described in section 3.3.1., pNIPAM thin films were produced. The transition temperature of the pNIPAM films with different formulations was determined by either measuring the absorbance at 560 nm with temperature variation between 27ºC and 40ºC on a microplate reader (BMG Labtech Omega) with a waiting time of 10 minutes between measurements to allow the samples to reach temperature equilibrium or either by measuring the refractive index changes in response to temperature increase in the range of 27ºC to 40ºC by employing an automatic digital refractometer (Rudolph Research J357). The temperature induced transition of the amine terminated pNIPAM (Sigma Aldrich, UK) was determined following the same protocol and was measured for 5
mg/mL solutions at different pH values. Triplicate samples were produced and measured for each experiment.

3.4.4. Particle size and size distribution analysis

Particle size and size distribution were measured by employing laser light scattering (Malvern Instruments Mastersizer 2000). The Mastersizer measures the intensity of light scattered as a laser beam passes through a dispersed particulate sample (Mie light scattering). The data collected is then analysed to calculate the size of the particles that created the scattering pattern. In order to be able to perform the size measurements of a specific material, the refractive index value is required. For all the different samples, the refractive index was measured as described previously in section 3.4.1.

The uniformity of the size distribution is expressed as the span which is defined according to equation [3-3].

\[
Span = \frac{D(0.9)-D(0.1)}{D(0.5)}
\]  

[3-3]

Where D(0.9), D(0.5) and D(0.1) are the particle sizes at which 90%, 50% and 10% of the volume distribution lies on the cumulative curve. Span values of less than 1 are usually reported to be monosized distributions (Williams R.A. et al, 1998), but the lower the span the more monosized the distribution is.
3.4.5. **Particles shrinkage ratio**

A Nikon Eclipse Ti inverted microscope was used for visualizing the solid particles. The shrinkage of the thermo-responsive particles was obtained by increasing the temperature from 23°C (room temperature) to 38°C with a five minutes resting period in order to reach the temperature equilibrium. The temperature was increased by using a Heating/Cooling Stage – Linkam DC60 coupled to the Nikon microscope. Pictures of the particles at room temperature and at 38°C were taken with a 10x objective and then analysed by using Image J software. Particle shrinkage was defined as the ratio between the volume of the particles at 23°C and the volume of the same particles at 38°C. Image J is a public domain, Java-based image processing program developed at the National Institutes of Health, USA. The Image J protocol applied for the analysis is described in detail in Appendix B1.

3.4.6. **FT-NIR analysis**

Taking into consideration that in the process of pNIPAM particle production starts from the monomer, Fourier Transform Near InfraRed (FT-NIR) spectroscopy was employed to confirm the presence of the crosslinked polymer in the generated particles. A FT-NIR analyser (Thermo Scientific Antaris II) with reflectance sampling was utilised. The temperature responsive particles were dried prior to analysis. pNIPAM particles were dried by freeze-drying technique, while the core-shell type particles were air dried from acetone. Dried particles were placed in a clear, dry glass vial that was then positioned on the reflectance sampling module as shown in Figure 3-2. The same empty glass vial was used for calibration of the device prior to analysis and background measurement.
3.4.7. Scanning Electron Microscopy (SEM) coupled with Energy-dispersive X-ray spectroscopy (EDX)

The surface of the generated particles was investigated by Scanning Electron Microscopy operated at 10 kV (Cambridge Instruments Stereo Scan 360). Samples were dried from acetone over a period of several days and then Gold /Palladium sputtered before SEM analysis. The elemental analysis and chemical characterisation of the generated particles were achieved by employing SEM coupled with EDX analysis.

3.4.8. Water Contact Angle (WCA) measurements

The wettability of the generated surfaces was measured by employing Water Contact Angle technique achieved by using a Kruss DSA4 sessile drop shape analysis system. Polymer films were prepared using the exact same chemistry and recipe as the one applied for polymer bead production. A drop of ultrapure water was gently placed onto the sample.
surface using a micro-syringe and an image was taken. The software analyses and fits the drop profile using the Young/Laplace equation, followed by using a linear regression to estimate the initial WCA at the point the drop first contacted the surface. Measurements were taken from several different samples.

3.5. Mammalian cell culture

All cell culture procedures were carried out in accordance with Good Laboratory Practice (GLP) regulations in HERA safe class 2 microbiological safety cabinets (Thermo Scientific, UK) utilising aseptic techniques.

3.5.1. Cell line

The cell line employed for this work was the Swiss albino 3T3 cell line (ATCC, USA). The 3T3 cell line was established by Todaro G. and Green H. in 1962 from disaggregated Swiss mouse embryos and has become the standard fibroblast cell line used as "feeder cells" in human embryonic stem cell research (Todaro G. and Green H., 1963). The cells were received as a frozen suspension at a concentration of 2.5x10^6 cells/mL in a cryovial and resuscitated following the protocol described in detail in section 3.5.2.1. Following resuscitation, the cells were seeded on tissue culture plastic surfaces (TCPS) and cultured for several passages, following cell passaging and cryopreservation to form a master cell bank and working cell banks. The Swiss Albino 3T3 cells exhibited typical fibroblast morphology, as shown in Figure 3-3. All images were visualised using a Nikon Ti Eclipse inverted microscope at varying magnifications.
Figure 3-3. 3T3 cell morphology when cultured on TCPS at different time points. Scale bars represent 500 µm.
3.5.2. Cell culture procedure

The Swiss albino 3T3 fibroblasts were cultured using a high glucose Dulbecco’s Modified Eagle’s Medium (DMEM, 4500 mg/L glucose, with L-glutamine and sodium bicarbonate, sterile-filtered, Sigma Aldrich, UK) supplemented with 10% v/v foetal calf serum, heat inactivated (FCS, Sigma Aldrich, UK). This complete growth medium was made into 20 mL and 40 mL aliquots stored at 2 - 8ºC and used within one month of preparation.

3.5.2.1. Cell resuscitation

A cryovial containing the cell suspension in freezing medium (refer to section 3.5.2.3) stored in liquid nitrogen (vapour phase) was safely removed and thawed quickly in a water bath set at 37ºC until a flake of ice was left and then was rapidly transferred to the safety cabinet. 9 mL of complete growth medium was added to a 15 mL centrifuge tube and pre-warmed in the water bath set at 37ºC. 0.5 mL of pre-warmed complete growth medium was added dropwise to the cryovial to dilute the cell suspension in order to avoid osmotic shock and minimize cell death. The diluted cell suspension was then transferred to the 15 mL centrifuge tube containing the remaining pre-warmed complete growth medium (8.5 mL), drop by drop to further avoid osmotic shock. The tube containing the cell suspension was then centrifuged at 250 g for 5 minutes at room temperature (23ºC) using a Sigma 3-16PK bench top centrifuge in order to remove any traces of cryoprotectant that is toxic to the cells. After centrifugation, the supernatant was gently and carefully aspirated and the resulting cell pellet was resuspended in 10 mL of pre-warmed complete medium. The cell suspension was then plated onto pre-labelled TCPS (e.g. T-flasks, multi-well plates etc.) depending on the experiment. In accordance with good laboratory practices, all the TCPS used in this work
were labelled as it follows: cell line, cell passage (usually previous number plus one), seeding density and date.

3.5.2.2. General cell culture procedure

Cells were seeded on TCPS at a seeding density of $5 \times 10^3$ cells/cm$^2$ (following manufacturer instructions) and then placed in a humidified incubator at 37°C and 5% CO$_2$ controlled environment. Depending on the surface area of the TCPS used, the volumes of complete growth medium, DMEM without Phenol Red used for washes and enzyme are different and are provided in Table 3-4. The cells were kept in culture until >80% confluency was observed under an inverted phase contrast microscope, typically at day 5 in culture. Complete medium exchange was performed every 72 hours of culture with fresh pre-warmed medium unless otherwise stated.

Table 3-4. Volumes of utilised reagents in general cell culture protocol

<table>
<thead>
<tr>
<th>TCPS</th>
<th>Provided surface area / cm$^2$</th>
<th>Volume of complete growth medium / mL</th>
<th>Volume of DMEM without Phenol Red (used per one wash) / mL</th>
<th>Volume of TrypLE Select / mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-175 flask</td>
<td>175</td>
<td>50</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>T-75 flask</td>
<td>75</td>
<td>25</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>6-well plate</td>
<td>9.6</td>
<td>3</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td>12-well plate</td>
<td>3.8</td>
<td>2</td>
<td>1</td>
<td>0.25</td>
</tr>
</tbody>
</table>
Upon reaching confluency, cells were passaged using the following protocol. Consumed medium was gently aspirated and cells were washed twice with DMEM without Phenol Red (1 g/L D-glucose, without L-glutamine; Sigma Aldrich, UK) in order to remove any traces of serum that might interfere with the enzymatic activity of the proteolytic enzyme employed for lifting the cells from the surface. After gentle aspiration, a proteolytic enzyme (Gibco TrypLE Select, Invitrogen, UK) was added and cells were incubated for 5 minutes at 37ºC and 5% CO₂. TrypLE Select is a recombinant enzyme derived from microbial fermentation and a non-animal alternative for commonly used porcine Trypsin. Like trypsin, TrypLE Select cleaves peptide bonds on the C-terminal sides of lysine and arginine. However, because of its purity; it exhibits increased specificity due to the action of a single enzyme which reduces the damage caused by cleavage from multiple enzymes present in trypsin. After incubation, the enzyme was inactivated by adding a volume of fresh pre-warmed complete growth medium equivalent of three times the volume of enzyme used. The cell suspension was then centrifuged at 250 g for 5 minutes at room temperature. The supernatant was gently aspirated and the cell pellet was resuspended in 10 mL of fresh complete medium. The cells were then counted as described in detail in section 3.7.1 and cells were then seeded at a seeding density of 5x10³ cells/cm² on TCPS.

3.5.2.3. Cryopreservation protocol

Cells at a density of 2.5 x 10⁶ cells/mL were cryopreserved by using the protocol described below. Following cell passaging described in section 3.5.2.2., the same steps are followed until a cell pellet is obtained after centrifugation. After gentle aspiration of the supernatant, the cell pellet was resuspended in a known volume of complete medium and a cell count was performed. Once the cell number was known, the cell suspension was centrifuged again
at 250 g for 5 minutes and the obtained cell pellet was then resuspended in a known volume of cryopreservation medium comprising of high glucose DMEM supplemented with 10% v/v cryoprotectant (dimethylsulfoxide, DMSO; Sigma Aldrich, UK) and 20% v/v serum. One mL of cell suspension in the cryopreservation medium was added per cryovial. The cryovials were then rapidly transferred to a Mr. Frosty (Nalgene; Sigma Aldrich, UK) and placed in a -80°C freezer for 24 hours, after which the cryovials were transferred and stored in liquid nitrogen in the vapour phase at -180°C.

3.6. Microcarrier cell culture

3.6.1. Microcarrier preparation

All microcarrier cell culture was carried out in static conditions in Corning CoStar Ultra Low cell attachment 6 and 12-well plates, in order to ensure that cell attachment will only occur on the provided microcarrier surfaces. Prior to cell culture, the microcarriers employed were sterilised by using different techniques as described below.

3.6.1.1. pNIPAM particles preparation

Once produced, pNIPAM particles were intensively washed in RO water for several days in order to remove any traces of oil or unreacted reagents. The particles stored in RO water were then sterilised by autoclaving at 121°C for 15 minutes using a Systec VX-95 autoclave (Systec, Germany). After autoclaving, the vessel containing sterile pNIPAM particles was transferred to the safety cabinet and washed several times with Dulbecco’s Ca²⁺ and Mg²⁺
free Phosphate Buffer Saline (D-PBS, Gibco Invitrogen). The sterile particles suspension in D-PBS were left to settle, the PBS was aspirated and pNIPAM particles were resuspended in complete growth medium and incubated at 37°C for at least one hour prior to cell seeding, a step known as conditioning of the microcarriers, allowing the pNIPAM particles to become hydrophobic and to permit proteins to adsorb on their surfaces, thus facilitating and promoting cell adhesion.

For a specific set of experiments, the sterile pNIPAM particles were further coated with either porcine gelatine (Sigma Aldrich, UK) or Matrigel basement membrane matrix (BD BioSciences, UK). Porcine gelatine dry powder was weighed and dissolved in RO water under stirring and heating at 60°C to obtain a solution of 0.1% wt concentration. Once dissolved, the 0.1% wt gelatine solution was sterilised by autoclaving at 121°C for 15 minutes. The previously-sterilised pNIPAM particles suspended in D-PBS were let to settle, the PBS was gently aspirated to minimise loss of particles and the particle pellet was then resuspended in a known volume of 0.1% wt gelatine solution and placed in a shaking incubator (Thermo Scientific MaxQ Mini 4450) set at 37°C and 200 rpm for 12 hours. After 12 hours, the shaking was stopped, the coated particles left to settle, the residual gelatine solution was aspirated and the coated particles re-suspended in complete growth medium and conditioned at 37°C for at least one hour prior to use. Matrigel basement membrane matrix is a solubilized basement membrane matrix extracted from the EHS mouse tumour, rich in basement membrane proteins. Prior to Matrigel preparation, the received product was thawed at 4°C overnight and then partitioned in aliquots of 0.3 mL according to manufacturer’s instructions (batch dependent), taking special measures to avoid gelling that occurs above 4°C. Each 0.3 mL aliquot was then resuspended in 50 mL of cold DMEM. Sterile pNIPAM particles were then resuspended in the cold mixture of DMEM-Matrigel and placed in a shaking incubator set at 37°C and 200 rpm for 12 hours. After 12 hours, the
shaking was stopped, the coated particles left to settle, the residual solution was aspirated and the Matrigel-coated pNIPAM particles re-suspended in complete growth medium and conditioned at 37°C for at least one hour prior to use.

3.6.1.2. pNIPAM-coated Alginate (p/Alg) particles preparation

The produced particles were first dialysed in RO water for several days in order to remove any traces of reagents and oil and then were washed three times with Ca²⁺ and Mg²⁺-free D-PBS. The washed and hydrated particles were then transferred to a 100 mm Petri dish (Corning, USA) and placed at the centre of the safety cabinet where UV sterilisation was applied continuously for three hours. A sterility test was performed before using the particles in cell culture. The test consisted of resuspension of freshly UV-sterilised particles in complete growth medium and incubation at 37°C for at least 24 hours. If contamination is present, then the growth medium exhibits a colour change from red to different shades of orange-yellow depending on the degree of contamination. The medium colour change is given by the Phenol Red present in the DMEM which is a pH indicator exhibiting a gradual colour change from yellow to red over the pH range of 6.8 to 8.2. From the sterility test, it was found that three hours of continuous UV sterilisation was sufficient to ensure sterility of particles. Once particle sterilisation was achieved, re-suspension of particles in complete growth medium was performed, followed by their incubation at 37°C for at least one hour prior to cell seeding in order to condition the particles and facilitate cell adhesion to the given surfaces.

For a series of experiments, cell growth enhancement was studied when employing a cell adhesion promoter such as porcine gelatine (Sigma Aldrich, UK). The sterilised pNIPAM-
coated Alginate (p/Alg) particles suspended in D-PBS were let to settle, the PBS was gently aspirated to minimise loss of particles and the particle pellet was then resuspended in a known volume of 0.1% wt gelatine solution pre-sterilised by autoclaving and placed in a shaking incubator (Thermo Scientific MaxQ Mini 4450) set at 37ºC and 200 rpm for 12 hours. After 12 hours, the shaking was stopped, the gelatine coated particles left to settle, the residual gelatine solution was aspirated and the coated particles re-suspended in complete growth medium and conditioned at 37ºC for at least one hour prior to use.

3.6.1.3. Cytodex-1 (GE Healthcare, USA) preparation

Cytodex-1 microcarriers were employed as positive control for cell attachment and proliferation studies. Cytodex-1 is a cross-linked dextran based microcarrier with positively charged N, N-diethylaminoethyl groups distributed throughout the microcarrier matrix. The characteristics of these microcarriers as given by the manufacturer are provided in Table 3-5. The preparation of the microcarriers was performed according to manufacturer’s instructions. Briefly, the dry microcarriers were weighed and then swollen in Ca²⁺ and Mg²⁺-free D-PBS for at least three hours at room temperature. The supernatant was then decanted/ aspirated and the microcarriers were washed several times with fresh Ca²⁺ and Mg²⁺-free D-PBS. The hydrated Cytodex-1 microcarriers were sterilised by autoclaving at 121ºC for 15 minutes. Prior to use, the microcarriers were conditioned in complete growth medium at 37ºC for at least one hour.
Table 3-5. Characteristics of Cytodex-1 (GE Healthcare) microcarriers

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density / g/L</td>
<td>1.03</td>
</tr>
<tr>
<td>Size (d_{50}) / µm</td>
<td>190</td>
</tr>
<tr>
<td>Size (d_{5.95}) / µm</td>
<td>147-248</td>
</tr>
<tr>
<td>Approximate area / cm²/g dry weight</td>
<td>4,400</td>
</tr>
<tr>
<td>Approx. number of microcarriers / g dry weight</td>
<td>4.3 x 10⁶</td>
</tr>
<tr>
<td>Swelling factor / mL/ g dry weight</td>
<td>20</td>
</tr>
</tbody>
</table>

3.6.2. Microcarrier general cell culture procedure

Prior to use, all microcarriers utilised in this work were conditioned in complete growth medium for at least one hour at 37°C, 5% CO₂. After conditioning, the used medium is aspirated as much as possible and with care to minimise loss of microcarriers, and replaced with fresh pre-warmed complete growth medium. Microcarriers were first seeded in Ultra Low Attachment 12-well plates (ULA 12) and 2 mL respectively of complete growth medium. Cells were seeded onto the prepared microcarriers at a density of 1 x 10⁵ cells/ well in ULA 12, unless otherwise stated. Complete medium exchange was performed every two days unless otherwise stated. All experiments were done in static conditions.
3.7. Analytic techniques

3.7.1. Cell counting and visualisation

Cell counts were performed using the CompacT SelecT Cedex automated cell counter platform (Tap Biosystems, UK). Viable and non-viable cells are determined using the Trypan Blue dye exclusion method. Live, healthy cells are impermeable to Trypan Blue, while dead or damaged cells easily absorb Trypan Blue, thus becoming stained in a distinct blue colour. Cedex counting method is based on imaging stained cells and picture analysis. The minimum cell density required by the device is $1 \times 10^5$ cells/mL in order to be detected. Cedex provides the user with information regarding viable cell density (cells/mL), total cell density (cells/mL), viability (%), aggregate rate (%), total cell count, average diameter ($\mu$m) and standard deviation (cells/mL). All cell imaging was performed by using an inverted phase contrast microscope (Nikon Ti Eclipse) for tasks such as visualisation of cell attachment, cell morphology and culture confluency on both TCPS and microcarrier cell culture.

3.7.2. Cell viability/cytotoxicity assessment

Cell viability/cytotoxicity was assessed by employing the Live/Dead Viability/Cytotoxicity kit for mammalian cells (Invitrogen, UK). The kit provides a two-colour fluorescence cell viability assay based on simultaneous determination of live and dead cells with two probes that measure recognized parameters of cell viability e.g. intracellular esterase activity and plasma membrane integrity. Live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually non-fluorescent cell permeant calcein AM to the intensely fluorescent calcein. The polyanionic dye calcein is well retained within live cells producing an intense uniform green
fluorescence in live cells (Excitation ~ 495 nm; Emission ~ 515 nm). Ethidium homodimer-1 (EthD-1) enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (Excitation ~ 495 nm; Emission ~ 635 nm). EthD-1 is excluded by the intact plasma membrane of live cells. Backgrounds fluorescence levels are inherently low with this assay because the dyes are virtually non-fluorescent before interacting with cells.

Fresh solutions were prepared before each assessment. 20 µL of the supplied 2 mM EthD-1 stock solution was added to 10 mL of sterile Ca\(^{2+}\) and Mg\(^{2+}\) free D-PBS and vortexed to ensure thorough mixing. This generated a concentration of 4 µM EthD-1. The reagents were then combined by adding 5 µL of the supplied 4 mM calcein-AM stock solution to the 10 mL EthD-1 solution and then vortexed to ensure thorough mixing. The resulting working solution contained 2 µM calcein-AM and 4 µM EthD-1. The working solution was then added directly to the cells and incubated at 37ºC for 40 minutes. After the incubation, the labelled cells were visualized under the fluorescence microscope (Nikon Ti Eclipse).

### 3.7.3. Cell proliferation assessment

Cell proliferation was measured and quantified by using Presto Blue assay (Invitrogen, UK). The Presto Blue® Assay incorporates a fluorometric/colorimetric growth indicator based on detection of metabolic activity. Specifically, the system incorporates an oxidation-reduction (REDOX) indicator that both fluoresces and changes colour in response to chemical reduction of growth medium resulting from cell growth. Presto Blue® is a proven cell viability indicator that uses the natural reducing power of living cells to convert resazurin to the fluorescent molecule, resorufin. The active ingredient (resazurin) is a nontoxic, cell
permeable compound that is blue in colour and virtually non-fluorescent. Upon entering cells, resazurin is reduced to resorufin, which produces very bright red fluorescence (Figure 3-4). Viable cells continuously convert resazurin to resorufin, thereby generating a quantitative measure of viability and cytotoxicity.

Figure 3-4. Presto Blue assay principle: The weakly fluorescent resazurin is reduced when entering the viable cell environment to resorufin, a strongly fluorescent component.

Other assays based on the same principle are commercially available, but the advantage that Presto Blue assay has other them is that it requires lower incubation times, is very sensitive if working with greater than $5 \times 10^3$ cells and relatively non-toxic. On the other hand, the main disadvantage of this assay is that it is not a direct cell counting technique like haemocytometry because the fluorescence or absorbance signal can be affected by both changes in cell number and cell metabolism.

In order to correlate the fluorescence signal with cell number, calibration curves were generated and are provided in Appendix A3. The calibration curves generated were used to quantify the actual number of cells in culture, following the following equation:

$$y = m \times x + c,$$  \[3-4\]
Where:  
\[ y = \text{fluorescence intensity} \]  
\[ m = \text{gradient} \]  
\[ x = \text{cell number} \]  
\[ c = \text{‘y intercept’} \]

In order to minimize the background noise and error, 10% volume Presto Blue in medium solution was used as a negative control and the positive control was the 100% reduced form of Presto Blue obtained by autoclaving the 10% volume solution at 121°C for 15 minutes.

The percentage reduction of Presto Blue for each case was calculated using the equation [3-5]:

\[
\% \text{reduction of PrestoBlue} = \frac{S_x - S_{\text{control}}}{S_{100\% \text{reduced}} - S_{\text{control}}} \tag{3-5}
\]

Where:

- \( S_x \) is the Presto Blue® fluorescence signal of the sample at day \( x \),
- \( S_{100\% \text{reduced}} \) is the signal of the 100% reduced form of Presto Blue;
- \( S_{\text{control}} \) is the signal from the control: the culture medium supplemented with 10 vol. % Presto Blue. The 100% reduced form of Presto Blue was produced by autoclaving the control at 121°C for 15 minutes.

The protocol described here was applied for all experiments employing Presto Blue assay. ATCC 3T3 mouse fibroblastic cells cultured at passage 30 in TCPS 6-well plates were employed for the generation of the Presto Blue calibration curves presented in Appendix 1.A3. Briefly, the consumed media was aspirated, the cells were washed with \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \)-free D-PBS and 1 mL of 10% vol. Presto Blue solution was added per well in TCPS 6-well
plates, followed by incubation at 37°C for 10 minutes and 40 minutes. Three replicates were
done for each seeding density. Following incubation, 100 µL from each well was transferred
to a Falcon 96-well black polystyrene microplate with clear flat bottom (Becton Dickinson,
UK) and replicated three times. Fluorescence intensity (Excitation / Emission: 544 nm / 590 nm) was measured on a BMG Labtech FLUOStar Omega microplate reader and
correlated to cell number based on the calibration curve (Appendix 1.A3). For all other
experiments employing Presto Blue assay, incubation time was 40 minutes (based on
calibration curve).

3.7.4. Determination of metabolite concentrations

Metabolite concentrations in spent cell growth medium at different time points were
measured with the help of the BioProfile FLEX analyser (NOVA Medical). One milliliter of
spent medium was aseptically obtained, transferred to an Eppendorf tube and then run on the
bioanalyzer.

3.8. Statistical analysis

Statistical analysis of the generated data was performed with the SPSS software (IBM, USA).
Statistical significance was determined with mixed analysis of variance (ANOVA) test and
it was considered statistical significant at p-value <0.05.
4. CONTROLLED TEMPERATURE RESPONSIVE SOLID PARTICLE PRODUCTION BY MEMBRANE EMULSIFICATION

4.1. Introduction

Stimuli-responsive polymers have been increasingly used to solve biotechnological problems, from intelligent drug delivery systems (Bawa P. et al., 2009) to the design of artificial tissues and smart culture substrates (Yang L. et al., 2012). Amongst the many stimuli-responsive polymers, pNIPAM has been widely employed for Tissue Engineering applications due to its phase transition temperature being close to the physiological temperature (Heskins M. and Guillet J.E., 1968; Tauer K. et al., 2009). Above this temperature, referred to as LCST for polymer solutions or Volume Phase Transition Temperature (VPTT) for polymer hydrogels (Zhu X. et al., 2012), pNIPAM hydrogels are sufficiently hydrophobic to allow cell adhesion and proliferation. Lowering the temperature below the LCST makes pNIPAM surface more hydrophilic causing cell detachment without the need for enzymes (Kwon O.H. et al., 2000; Liu D. et al., 2012; Tang Z. et al., 2012). Based on this remarkable and reversible phase transition, pNIPAM represents a valid option for achieving a non-destructive and non-harmful cell harvesting by avoiding the use of proteolytic enzymes (e.g. porcine trypsin) that have been shown to have an irreversible effect on cell adhesion capacities (Baumann H. et al., 1979).

To address the need for an up-scaling system capable of not only achieving large number of cells, but also maintaining their properties, the physical properties of pNIPAM were harnessed in a format compatible with large scale manufacturing of stem cells in stirred tank bioreactors. Thus, solid temperature responsive particles were produced in a controlled manner by membrane emulsification technique applied on the Dispersion Cell (refer to section 3.2). The work described in this chapter focuses on the controlled production of
solid pNIPAM particles (section 4.3) by investigating the effect of process parameters, as well as formulation parameters on particle size and size distribution. The generated particles are then characterised in terms of size, size distribution, temperature responsiveness and wettability (section 4.4) in order to assess their potential to be used as scaffolds for cell culture and non-damaging cell harvesting based on external stimuli response rather than substrate disintegration.

4.2. Aims and objectives

The main aim of the work presented in this chapter consisted in demonstrating that controlled production of temperature responsive particles can be achieved when applying membrane emulsification on the Dispersion Cell device described in detail in section 3.2. The achievement of this aim was possible through the realisation of the following objectives:

- Selection of the optimal formulation for the temperature responsive particle production
- Controlled particle production by using the Dispersion Cell with generation of pNIPAM particles with sizes ranging from 35 µm to 1400 µm and size distributions expressed as span values as low as 0.5
- Proof of temperature responsive behaviour
- Evaluation of the generated particles as substrates for cell adhesion and non-enzymatic release
CHAPTER 4. CONTROLLED TEMPERATURE RESPONSIVE SOLID PARTICLE

4.3. Controlled production of pNIPAM solid particles

Particle production was done by employing the metallic membranes described in detail in section 2.4.3.2. and 3.2.2 and the Dispersion Cell device described in section 2.4.3.1. The controlled production of particles was achieved by both varying process parameters (e.g. monomer injection rate; shear stress applied at the membrane’s surface; inter-pore spacing) and formulation parameters, such as surfactant concentration or crosslinker concentration, both having a major influence on particle size.

4.3.1. Effect of crosslinker concentration on particle size

The crosslinker employed in the polymerisation of pNIPAM particles was MBA which has a similar structure to the NIPAM monomer. MBA is the most commonly used crosslinker for the production of pNIPAM hydrogels, but other crosslinkers have been regularly used. The basis for selecting MBA as a crosslinker in this work is described in detail in section 2.2.3.1.1.4. Based on the crosslinking mechanism presented in section 2.2.3.1.1.5 the amount of MBA added to the reaction controls the crosslinking degree of the polymer by the number of bridges created between the linear strings of monomer units. Thus, the effect on particle size of the crosslinking degree given by the concentration of MBA used in the formulation was studied. Figure 4-1 shows the effect of crosslinker concentration on particle size (Figure 4-1A) and particle size distribution (Figure 4-1B) expressed as span values, when the dispersed phase was injected through an array of 20 µm pores and 80 µm pore spacing at a flux of 1.1 mL/min and a maximum shear stress at the membrane’s surface of 8.5 Pa calculated by using equation [2-9] (refer to section 2.4.3.3.). The dispersed phase used was composed of either 5% wt or 10% wt monomer and different concentrations of crosslinker, while the continuous phase used in this study was 2% wt Span 80 in kerosene.
All the experiments were performed by utilising the same amount of APS (10 mg/mL of dispersed phase) and TEMED (0.05 mL/mg of APS). Particle size decreased with increasing crosslinker concentration with median particle sizes as low as 42 µm when using the 5% wt monomer formulation and 37 µm when using the 10% wt monomer formulation. Generally, the particle size appeared to be lower for the formulation containing more monomer, regardless of the crosslinking degree. This can be explained based on the crosslinking mechanism provided in section 4.3.2. Greater monomer and crosslinker availability results in more bridges between the linear strings producing a more compact polymer structure, which limits the final particle size (Figure 4-1A). Figure 4-1B shows the size distribution of the produced particles of different crosslinking degrees. Generally, the span values obtained for the particles containing 10% wt monomer were smaller than the ones comprising of 5% wt monomer. However, all the span values were in the range of 0.6 to 0.9, except for the 5%wt particles with the lowest degree of crosslinking that had a span value of approximately 0.5. For a visual confirmation of this behaviour, microphotographs of the generated solid particles with different degrees of crosslinking were taken and are shown in Figure 4-2.
Figure 4-1. The effect of crosslinker concentration on 5% wt and 10% wt pNIPAM particles A) size and B) size distribution expressed as span values, when the dispersed phase was injected through an array of 20 µm pores and 80 µm pore spacing at a rate of 1.1 mL/min and a shear stress of 8.5 Pa.
Figure 4-2. Micrographs of temperature responsive particles containing a) 5% wt monomer and b) 10% wt monomer and different concentrations of crosslinker, generated at an injection rate of 1.1 mL/min and a shear stress of 8.5 Pa when employing an array of 20 µm pores and 80 µm pore spacing. Scale bars correspond to 100 µm.
The micrographs shown in Figure 4-2 correspond to pNIPAM particles containing 5% wt monomer (Figure 4-2a) and 10% wt monomer (Figure 4-2b) at different crosslinker concentrations. The micrographs were acquired after the generated particles were intensively washed with RO water in order to remove any traces of oil and unreacted reagents.

4.3.2. Effect of surfactant concentration on particle size

Surfactants are compounds that are amphiphilic in nature – part hydrophilic and part lipophilic. The combination of these opposing affinities in the same molecule dictates the surfactant’s ability to reduce surface and interfacial tensions. The surfactant distributes at the surfaces of the suspended material and forms a protective layer around each particle. This ultimately decreases the overall free energy of the system and increases stability of the solution by discouraging separation of the phases. In addition to surfactant effects, the stability of suspensions is related to particle size and density of the suspended material. (Schramm L.L., 2005)

As part of formulation improvement, the effect of surfactant concentration on pNIPAM particle size and size distribution was studied (Figure 4-3). 10 mL of dispersed phase with the formulation consisting of 5% wt or 10% wt monomer, 0.2% wt MBA and 10 mg/mL APS were injected through a 20 µm pores array with 80 µm pore spacing into 100 mL of continuous phase consisting of kerosene with different concentrations of surfactant. pNIPAM particle production was achieved through the production of W/O emulsions which require a surfactant soluble in the oil phase. The selected surfactant fulfilling the requirements was sorbitan monooleate (Span 80) which is a non-ionic surfactant with a
CHAPTER 4. CONTROLLED TEMPERATURE RESPONSIVE SOLID PARTICLE

HLB of 4.3 in the range recommended for water-in-oil emulsifiers (3 to 6) (Becher P., 2001; Tadros T.F., 2009). The dispersed phase containing the initiator was injected at a rate of 1.1 mL/min and a shear stress applied at the membrane’s surface of 8.5 Pa. Once the emulsion was generated, TEMED (0.05 mL/mg of APS) was added in order to accelerate the reaction between the monomer and crosslinker and permit its completion at room temperature. The reaction mechanism is described in detail in section 2.2.3.1.1.5.

Figure 4-3A shows the effect of surfactant concentration in the continuous phase on particle median diameter. It was observed that by increasing the surfactant concentration, the particle size decreased. Particle size decreased as the surfactant concentration increased to 3% wt regardless of the monomer concentration. Surfactant concentrations above 3% wt had no effect on particle size with minimal particle diameters of 99 µm and 65 µm for the 5% wt monomer and 10% wt monomer, respectively. For the 5% wt monomer formulation, particle sizes ranging from 1050 µm to 100 µm could be produced by varying the surfactant concentration. Similarly, particle diameters between 670 µm and 70 µm could be achieved with the 10% wt monomer formulation when varying surfactant concentration. Similar to the results presented in Figure 4-1, the particles produced from 10% wt monomer are smaller in size than those generated with less monomer. This behaviour is consistent with the crosslinking mechanism described in detail in section 2.2.3.1.1.5. The size distribution (expressed as span values) of the generated particles is shown in Figure 4-3B. The highest span values were obtained for the formulation without any surfactant suggesting a high degree of coalescence. For the formulations containing various concentrations of surfactant, the span values ranged from 0.5 to 1. Based on these findings, for further tests, the formulation containing 2% wt surfactant was chosen.
Figure 4-3. The effect of surfactant concentration on 5% wt and 10% wt pNIPAM particle A) size and B) size distribution expressed as span values, when the dispersed phase was injected through an array of 20 µm pores and 80 µm pore spacing at a rate of 1.1 mL/min and a shear stress of 8.5 Pa.
4.3.3. Influence of process parameters

Controlled pNIPAM particle production can be achieved when using the Dispersion Cell device described in detail in section 2.4.3.1 and 3.2 by varying process parameters e.g. injection flux of the dispersed phase, shear stress applied at the membrane’s surface, pore size, inter-pore distance etc. For the work presented in this chapter, two membranes with different inter-pore spacing (described in detail in section 3.2.2) were employed for achieving the objectives of this chapter.

The emulsification formulation used for the production of pNIPAM particles exhibited parameter limitations when using different types of membranes. By using a lower pore spacing membrane, the production of pNIPAM particles was limited to using lower injection rates (e.g. up to 3.3 mL/min) resulting in a wide size distribution expressed as span values varying between 0.499 and 2.5. Using a 200 µm pore spacing membrane allowed higher injection rates ranging between 1.6 mL/min and 17 mL/min, resulting in a narrower particle size distribution (span values between 0.5 and 1.2). The injection rate limitations of the membranes employed could be attributed to the formulations utilised for the production of particles. The low interfacial tension of 2.5 mN/m supports this statement.

For the work presented here, the effect of process parameters on solid particle size was measured and considered. The effect on pNIPAM droplet size could not be measured. The pNIPAM droplets coalesced upon contact with the hydrophilic glass microscope slides, making droplet imaging impossible. Immersing the microscope slides in the oil phase used in this system to provide a hydrophobic coating for droplet retention was ineffective.
4.3.3.1. Influence of injection flux on particle size

When using the Dispersion Cell, two main parameters (paddle stirring speed and injection rate) have a major influence on droplet size and implicit particle size (Dragosavac M.M. et al., 2008; Kostvintsev S.R. et al., 2005; Stillwell M.T. et al., 2007). The dispersed phase injection flux is defined as volume of dispersed phase injected through the uniform pores of the membrane per minute (mL/min). The effect of injection rate on particle size was studied by varying paddle rotation speed. When using an array of 20 µm pores with 80 µm pore spacing, the dispersion phase rate was varied between 0.58 mL/min and 3.3 mL/min. At higher injection rates, solid pNIPAM particles could not be generated which is attributed to a lack of crosslinking caused by incomplete dissolution of APS into the dispersed phase/aqueous phase. Figure 4-4 shows particle volume median diameter and span variation with injection flux for three different rotational speeds when using a 20 µm pore membrane with 80 µm pore spacing. Particle size increased with increasing the injection flux when more volume is forced through the pores of the membrane before droplets detach from the membrane surface. On the other hand, as the rotational speed was increased, volume median particle diameter decreased, which is in agreement with similar observations reported in the literature (Kosvintsev S.R. et al., 2005; Egidi E. et.al, 2008; Dragosavac M.M. et al., 2008; Vladisavljević G.T. & Schubert H., 2003).

A previous study using the Dispersion Cell and a similar membrane with 80 µm pore spacing (Egidi E. et al., 2008) demonstrated the presence of a supplementary force that can occur in such a system, known as the ‘push-off’ force resulting in very monosized distributions. This force can occur when the droplet production rate is high and the droplets from adjoining pores may touch each other, leading to a distortion of the drop shape from spherical to elongated, thus acting as an additional force for droplet detachment from the membrane’s surface. This force is also believed to have a role in assisting droplet growth up
to a similar volume, thus explaining the generation of very monosized distributions. Unlike this study, in the system presented in this chapter, as shown in Figure 4-4, no evidence to suggest the presence of such a force was observed. In general, a distribution is considered to be monosized if it is smaller than unity (Williams R.A. et al., 1998), but the nearer the value of the span to 0, the more monosized the distribution is. The lowest size distribution expressed as span was obtained for one of the lowest injection fluxes (1.1 mL/min) and the value was 0.46. The particle size distribution obtained varied between 0.46 and 2, as shown in Figure 4-4b.
Figure 4-4. a) Variation of particle volume median diameter with dispersed phase injection flux at three different rotational speeds applied. b) Variation of span values with dispersed phase injection rate at three different rotational speeds applied. The membrane employed for this experiment had 20 µm pores and 80 µm pore spacing.
Similar results were obtained when using a membrane with the same pore diameter, but a larger inter-pore spacing of 200 µm. Unlike the previously described membrane (80 µm pore spacing), when employing the larger inter-pore spacing membrane, it was possible to apply higher injection rates for the production of solid particles. The dispersed phase injection flux was varied between 1.6 mL/min and 17 mL/min. The effect of the dispersed phase injection rate on particle volume median diameter when three different rotational speeds were applied is shown in Figure 4-5. Particle median diameter increases with higher injection fluxes as a result of droplet growth and decreases with the increase in the rotational speed applied.

The rotational speed influences directly the shear stress applied at the membrane’s surface and has an effect on droplet detachment time, thus influencing droplet size and then particle size. Droplets produced at high rotation speed (high shear stress) had smaller droplet diameter than the ones produced at low rotation speed (low shear stress) which is in agreement with previous published studies (Nakashima T. et. al., 1992; Dragosavac M.M. et al., 2008; Kosvintsev S.R. et al., 2005). The membrane used had 200 µm inter-pore spacing and therefore it was expected that droplet and particle size respectively would be below 200 µm. For the parameters tested for this experiment, the particle size did not exceed 185 µm. The span values of the particles generated with most sets of the conditions applied in this study were in the range of 0.5 to 0.8, except for two sets of conditions where the spans were slightly above the value of 1.
Figure 4-5. Variation of particle volume median diameter (a) and span values (b) with dispersed phase injection flux at three different rotational speeds applied when employing a membrane with 20 µm pores and 200 µm pore spacing.
4.3.3.2. Influence of the shear stress applied at the membrane’s surface

Compared to microfluidic devices that do not require shear stress to assist with droplet detachment, for emulsification techniques based on the use of membranes, it is widely accepted that shear stress has to be applied at the membrane’s surface to facilitate drop detachment (Peng S.J. and Williams R.A., 1998). The applied shear stress has a major influence on droplet / particle size and size distribution (Holdich R.G. et al., 2010; Dragosavac M.M. et al., 2008; Stillwell M.T. et al., 2007).

The shear stress profile in the Dispersion Cell is not constant over the whole surface of the membrane, and it has been shown to have a maximum value at the transitional radius between the free and the forced vortex (Kostvintsev S.R. et al., 2005) generated by the paddle blade stirrer. Two mathematical models described in detail in section 2.4.3.3 were applied for predicting drop and particle size generated at different shear stress values applied at the membrane surface. Model A is based on maximum shear stress values, while model B refers to average shear stress values at the membrane surface. Examples of mathematical calculations for each model are provided in Table B3 in Appendix B2.

The effect of the shear stress generated at the membrane surface on the resulting particle size is illustrated in Figure 4-6a when using a 20 µm pore membrane with 80 µm pore spacing and Figure 4-6b when using a 200 µm pore spacing membrane. Increasing the paddle rotation speed, the shear stress on the membrane surface increases (calculated with the equations provided in section 2.4.3.3) resulting in a shorter droplet formation time that generates smaller sized droplets and particles. The size dependence to the shear stress applied has been previously reported (Dragosavac M.M. et al, 2008; Holdich R.G. et al, 2010) and is consistent with the results presented in Figure 4-6.
The experimental data obtained by generating temperature responsive particles at different shear stress values was compared to the predicted size models described in detail in section 2.4.3.3 (Figure 4-6). When employing a smaller pore spacing membrane, the experimental data was obtained at an injection rate of 1.1 mL/min (Figure 4-6a), while for higher pore spacing membrane, the data was generated at an injection rate of 3.03 mL/min (Figure 4-6b). Since the shear stress is not constant across the membrane surface regardless of the pore spacing, the average shear stress appears to be more suitable for describing the influence of shear stress on particle size. Thus mathematical model based on average shear stress (Model B) fits the experimental data better than the mathematical model based on maximum shear stress (Model A), regardless of the membrane employed. On the other hand, since the model does not take into consideration the injection rate, it is expected that the model will predict the droplets / particle size generated when applying a low injection rate such as 1.1 mL/min for 80 µm pore spacing membrane and 3.03 mL/min for 200 µm pore spacing membrane. The fit of the experimental data onto Model B was more accurate for the 200 µm pore spacing membrane, as shown in Figure 4-6b.
Figure 4-6. Median particle size dependence on the shear stress applied at the membrane surface when using a 20 µm pore membrane with a) 80 µm and b) 200 µm pore spacing.
4.3.3.3. Reproducibility of the results

Due to the high costs of reagents needed, as well as the high number of experiments required for these studies, all tests are based on single measurements. However, the reproducibility of the collected data was assessed for several sets of process parameters. The reproducibility of the particle size generated by using two membranes with different inter-pore spacing was assessed by performing triplicate experiments when 10 cm$^3$ of dispersed phase was injected at a rate of 1.1 mL/min through the 20 µm pores of the nickel membrane with 80 µm pore spacing (Figure 4-7) and an injection flux of 3.03 mL/min through the 20 µm pores of the nickel membrane with 200 µm pore spacing (Figure 4-8).

The results obtained with the smaller pore spacing membrane were more consistent than when employing the other membrane. Regardless of the membrane used, the level of variation recorded was higher for lower peak shear stress values (0.92 Pa and 4 Pa), while for 8.5 Pa and 14 Pa the results were more consistent. The level of variation recorded regardless of the membrane employed can be attributed to the formulation applied resulting in a very low interfacial tension (2.5 mN/m), as well as to poor control over nitrogen gas purging required during the entire particle production. Thus, the variation could be minimised by ensuring a controlled gas flux during the nitrogen purging.
CHAPTER 4. CONTROLLED TEMPERATURE RESPONSIVE SOLID PARTICLE

Figure 4-7. Median particle diameter obtained from three different sets of experiments when employing a 20 µm pore membrane with an 80 µm inter-pore spacing at different shear stress values and an injection rate of 1.1 mL/min.

Figure 4-8. Median particle diameter obtained from three different sets of experiments when employing a 20 µm pore membrane with a 200 µm inter-pore spacing at different shear stress values and an injection flux of 3.03 mL/min.
4.4. Solid particle characterisation

4.4.1. Particle preparation for analysis and further use

Numerous characterisation techniques, *e.g.* SEM, usually require dry particles. As a result, attempts to dry the generated pNIPAM particles were made. pNIPAM particles were first washed intensively with soapy water and then RO water over a period of several days in order to remove all traces of oil and any unreacted reagents. Several drying techniques were attempted and compared.

4.4.1.1. Particle drying methods

Two drying methods were employed for the drying process of the generated temperature responsive particles: a slow-freezing method using a -20°C freezer followed by freeze drying in a manifold dryer chilled at -55°C and air drying (at room temperature) from ethanol 70% vol. Particle shape was evaluated before drying, after drying and after subsequent rehydration. Figure 4-9 provides photomicrographs of pNIPAM 5% wt low crosslinked particles (0.2% wt crosslinker) before and after freeze drying. The images provided before freeze drying belong to hydrated particles in solution.
Figure 4-9. Photomicrographs of 5% wt low crosslinked thermo-responsive particles before and after freeze drying; the scale bars in the images represent 100 µm. The arrows point out the elongated tail-shaped dried pNIPAM particles.

Fully hydrated pNIPAM particles are spherical. After slow freezing at -20°C, followed by freeze drying, particles became ovoid or elongated with a tip-shaped tail at the end of the microcarrier (Figure 4-9). For a higher resolution imaging, SEM was employed. The recorded SEM images of the freeze dried particles are provided in Figure 4-10.
Figure 4-10. High resolution SEM images of pNIPAM 5% wt low crosslinked freeze dried particles. Scale bars represent: a) 1 mm; b) 100 micrometers; c) 60 micrometers.

The observed elongated morphology of the freeze dried pNIPAM particles was previously reported in literature (Cheng C-J et al., 2008; Lin S-Y et al., 1999). The formation of the tip-shaped tails of the elongated particles was explained by Cheng C-J. et al. (2008). The water within the top of the microsphere network was pressed to flow downwards during the freezing process by the gravitational force. The top of the microcarrier was the first to be frozen and the water streaming from the top was gradually frozen, resulting in the tip-
shaped tail. At a higher magnification (Figure 4-9), it can be observed that the freeze dried temperature responsive particles present a large number of micropores with sizes of several microns. These micropores were formed as a result of the ice crystals formation during the freezing sub-process (Cheng C-J. et al., 2008).

The second type of drying method chosen consisted of particle air drying from 70% vol ethanol. By air drying the particles suspended in ethanol, after three days a jelly-like mass was obtained, compared to the white dry powder formed after freeze drying. By air drying from ethanol, the pNIPAM particles dried as agglomerates of particles rather than separate particles as obtained by slow freezing followed by freeze drying, but without any major change in morphology (Figure 4-11) as exhibited when freeze drying was applied. SEM was used to acquire high resolution images of the air dried pNIPAM particles and assess their structure. The acquired images (Figure 4-12) showed that the generated temperature responsive particles have a solid core making them suitable for our application as they provide a solid base for cell culture.
Figure 4-11. Photomicrographs of temperature responsive particles air dried from ethanol before and after drying. Scale bars represent 100 µm.

Figure 4-12. High resolution SEM images of the air dried temperature responsive 5% wt low crosslinked particles.
4.4.1.2. Rehydration of the dried temperature responsive particles

Freeze-dried pNIPAM particles did not fully rehydrate, with a subset of particles being ovoid rather than spheroids, and some particles retaining their wrinkled dry morphology (Figure 4-13). This is likely to the way in which the sublimation process was controlled during primary drying. It is likely non-hydrating particles have irreversibly collapsed due to sub-optimal freeze drying.

Figure 4-13. Images of re-hydrated freeze dried temperature responsive particles after 24 hours of hydration. Scale bars represent 100 μm. The arrows point to particles that have irreversibly collapsed, thus rendering them incapable of re-swelling.

On the other hand, the air dried pNIPAM particles recovered their initial spherical shape almost immediately (within 5 minutes) after resuspension in RO water (Figure 4-14). The
choice of the drying method to be employed for these temperature responsive particles has a major importance in preserving the properties of the particles. In accordance with previous studies published in literature (Lin S.Y. *et al*., 1999), drying methods can significantly influence the particle size distribution, deswelling/reswelling volume, surface topography and morphology of pNIPAM microgel beads, but do not seem to affect the phase transition of the microgel beads.

Figure 4-14. Microscope images of re-hydrated air dried pNIPAM particles. Scale bars represent 100 µm.

### 4.4.2. FT-NIR spectra collection

Taking into consideration that in the process of particle production, monomer droplets are initially generated and then polymerised and crosslinked, Fourier Transform Near Infrared spectroscopy (FT-NIR) technique was used to confirm the presence of the polymer in the generated solid particles. The near Infrared spectrum of the dried produced pNIPAM 5% wt
low crosslinked particles was acquired and was further compared to a commercially available pNIPAM (Sigma Aldrich, UK), as shown in Figure 4-15.

Figure 4-15. FT-NIR spectrums of generated pNIPAM particles compared to commercially available pNIPAM (Sigma Aldrich, UK) and the NIPAM monomer (Sigma Aldrich, UK).

The NIR spectrum acquired for the dry solid pNIPAM particles had a similar trend compared to the control represented by commercially available pNIPAM. NIR spectroscopy is based on overtones and combinations of bands of OH, NH and CH absorptions found in the Mid-IR. For pNIPAM, the overtone and combination bands of the CH groups would be expected to appear in the regions 6000–5700 cm\(^{-1}\) and 4500–4000 cm\(^{-1}\), while those for the amide groups would be mostly located in the regions 6900–6100 cm\(^{-1}\) and 5000–4500 cm\(^{-1}\) (Sun B. et al., 2007). A very similar absorption trend in NIR region was recorded previously.
in literature (Sun B. et al., 2007). However, the recorded NIR spectra in this study, showed the presence of an additional absorption peak at approximately 5100 cm\(^{-1}\) that could be assigned to the combination between amide overtones and NH groups contribution. The similarities between the spectrum recorded for the in-house produced pNIPAM particles and the commercially available polymer suggests that the polymerisation reaction applied for the production of particles was successful.

### 4.4.3. SEM coupled with EDX analysis

Energy dispersive X-ray spectroscopy (EDX) coupled with Scanning Electron Microscopy (SEM) was utilised to characterise the generated temperature responsive polymer particles by acquiring the elemental spectrum provided in Figure 4-16. The EDX data provided the percentage composition of the elements detected is shown in Table 4-1.

![EDX spectrum](image.png)

Figure 4-16. Elemental spectrum of the generated pNIPAM particles obtained by EDX coupled with SEM.
Table 4-1. Elemental compositions of the pNIPAM particles detected by EDX coupled with SEM

<table>
<thead>
<tr>
<th>Element</th>
<th>Weight %</th>
<th>Atomic %</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (K)</td>
<td>65.93</td>
<td>70.72</td>
</tr>
<tr>
<td>N (K)</td>
<td>16.15</td>
<td>14.85</td>
</tr>
<tr>
<td>O (K)</td>
<td>17.92</td>
<td>14.43</td>
</tr>
</tbody>
</table>

The SEM/EDX technique allows only for a qualitative evaluation of the produced particles providing the elemental composition of the surface analysed. However, the information acquired by this technique is limited to providing the compositions of the elements expected to be present in the chemical structure of pNIPAM (e.g. carbon, oxygen and nitrogen) and does not firmly confirm the presence of pNIPAM in the produced particles. However, this information together with the data generated by the FT-NIR spectroscopy analysis suggests that the polymerisation reaction applied was successful and resulted in the formation of pNIPAM.

4.4.4. Evaluation of the temperature responsiveness of the generated particles

One of the most important characteristics of the temperature responsive polymer ensuring its suitability for cell-based applications is the reversible phase transition from hydrophilic to hydrophobic. Reported studies suggest that the phase transition occurs at around 32ºC for pure pNIPAM (Heskins M. and Guillet J.E., 1968; Tauer K. et al., 2009). The fact that this value is lower than the physiological temperature (37ºC) permits the use of pNIPAM in cell-
based applications. Several studies reported that the phase transition temperature can be easily shifted left or right from the 32°C value by simply functionalizing the polymer through the use of different comonomers or by changing the crosslinker concentration (Kaneko Y. et al., 1995; Varga I. et al., 2001; Zhang X-Z. et al., 2005). In this regard, it is imperative that the phase transition temperature of our generated particles is measured.

The most commonly used experimental methods for determination of the pNIPAM phase separation consist of calorimetric (Constantin M. et al., 2011) or absorbance studies (Constantin M. et al., 2011; Cao Y. et al., 2007). Usually, the 50% point of the generated curve is considered to be the Lower Critical Solution Temperature (LCST) also referred to as ‘cloud point’ that describes the phase transition of the polymer (Constantin M. et al., 2011). As a result, two different techniques were attempted for the measurement of the critical phase transition temperature of the generated temperature responsive particles. These techniques consisted in recording the absorbance changes in response to temperature variation or a more unusual method: refractive index measurements with temperature variation. The refractive index changes were measured with an automatic digital refractometer, while the absorbance changes were measured in the visible domain at 560 nm with a microplate reader as described in detail in sections 3.4.1 and 3.4.2.

4.4.4.1. Cloud point measurements by absorbance variation with temperature

4.4.4.1.1. Cloud point measurements of pNIPAM in solution

The cloud point method was used to evaluate the temperature responsiveness of a commercially available pNIPAM (Sigma Aldrich, UK) in solution. The polymer was dissolved in RO water at room temperature to achieve different concentrations in solution.
which were further used for determination of the cloud point. It is well known that the LCST of temperature responsive polymers is dependent on polymer concentration in solution (Heskins M. and Guillet J.E., 1968). As a result, the cloud point of pNIPAM was assessed at both lower (Figure 4-17) and higher concentrations (Figure 4-18).

For concentrations ranging from 0.05 mg/mL to 10 mg/mL, by increasing the temperature responsive polymer’s concentration in solution the LCST value decreased as shown in Figure 4-17. A similar dependence was previously reported in literature by Constantin M. et al. (2011). At very low concentrations (0.05 mg/mL and 0.1 mg/mL), the changes in absorbance in response to temperature increase were so small that couldn’t be recorded in the temperature range employed. By increasing the polymer concentration in solution (above 0.5 mg/mL), the absorbance changes in response to temperature suggesting polymer phase transition became more obvious. At concentrations of 5 mg/mL and 10 mg/mL, the phase transition became more sensitive to temperature increase and suggested LCST values (50% point of the curve) of 36ºC and 35.5ºC respectively (Figure 4-18). The cloud point method detects the clouding of the polymer solution with temperature increase. The clouding of the polymer solution is caused by the hydrophobic interactions between the polymer chains (Schild H.G., 1992). As a result of an increased polymer concentration in solution, the hydrophobic interactions occur faster and therefore the phase transition appears to be sharper at higher polymer concentrations.
Figure 4-17. Absorbance variation with temperature for pNIPAM (Sigma Aldrich, UK) solutions of low concentrations varying from 0.05 mg/mL to 10 mg/mL.

On the other hand, at very high polymer concentrations in solution ranging from 30 mg/mL up to 100 mg/mL, the trends generated were clearly defining the phase transition of the polymer (Figure 4-18). By increasing the concentration from 30 mg/mL to 100 mg/mL, the LCST values increased, resulting in an LCST of approximately 37°C for the concentration of 100 mg/mL which is the equivalent of a concentration of 10% wt generally used for the production of crosslinked pNIPAM hydrogels with applications in the biomedical field (Ramkissoon-Ganorkar C. et al., 1999).
4.4.4.1.2. Cloud point measurements of pNIPAM crosslinked hydrogels

Attempts to measure the VPTT considered being the same as the LCST value (Constantin M. et al., 2011) of pNIPAM crosslinked hydrogels were made by recording absorbance changes at 560 nm with temperature increase. The absorbance variation with temperature for 10% wt pNIPAM hydrogels with different crosslinking degrees is provided in Figure 4-19. No apparent trend suggesting the cloud point value of the hydrogels was observed (as shown in Figure 4-19). On the other hand, the absorbance values recorded seem to increase with the increase in the crosslinker concentration used for the preparation of the temperature responsive hydrogels (Figure 4-19). By increasing the crosslinker concentration, a denser,
higher crosslinker polymer chain (refer to section 2.2.3.1.1.5) is generated. Greater polymer chain densities produced higher optical densities.

Figure 4-19. Absorbance variation with temperature increase from 27ºC (below Cloud Point) to 42ºC (above Cloud Point), recorded at 560 nm for pNIPAM hydrogels containing 10% wt monomer and with different crosslinking degrees given by the crosslinker concentration used for the preparation of the hydrogels.

This apparently random behaviour compared to the one described in section 4.4.4.1.1 suggested that the approached method is not sensitive enough and not appropriate for hydrogels. The high absorbance values achieved could be attributed to the change in membrane opacity as a result of polymer phase change transition above its LCST. Furthermore, this behaviour could be associated to the volume changes of the hydrogels resulting in their shrinkage above the cloud point value, as shown in Figure 4-20. During the polymer phase transition, the crosslinked hydrogels exhibited a volume shrinkage resulting
in some cases in a rougher surface, thus influencing the reliability of the absorbance based method.

Figure 4-20. a) Schematic diagram of pNIPAM films distribution in the 12-well plate. b) Photographs of 5% wt and 10% wt pNIPAM hydrogels with different crosslinking degrees above and below LCST showing volume changes in response to temperature increase.
4.4.4.2. Refractive index measurements for determination of LCST

As a different approach for measuring the LCST of the pNIPAM crosslinked hydrogels, an unusual method was employed consisting of recording the change in refractive index as temperature varies. Figure 4-21 shows the refractive index measurements for 5% wt low crosslinked pNIPAM hydrogels when temperature was varied between 27ºC and 42ºC. A sharp decrease in refractive index was observed at approximately 33ºC suggesting pNIPAM particles underwent a phase transition. Refractive index measurement was sufficiently sensitive and suitable for recording the phase transition temperature of the crosslinked hydrogels compared to the method described in section 4.4.4.1.2.

Figure 4-21. Refractive index variation with temperature increase for low crosslinked pNIPAM 5% wt hydrogels.
4.4.4.2.1. Effect of polymer crosslinking degree on the LCST measurements

The effect of polymer crosslinking degree given by the concentration of MBA used for the preparation of the hydrogels on the phase transition temperature was studied and is provided in Figure 4-22. Decreasing the degree of crosslinking within the pNIPAM 5% wt hydrogels, increases the phase transition temperature, but still within the range of 30°C to 33°C. The refractive index is an indicative of how the light propagates through the analysed medium. By increasing the degree of hydrogel crosslinking, more covalent bonds are created (section 2.2.3.1.1.5) resulting in a denser network of polymer and a denser hydrogel, therefore the sharp decrease in refractive index suggesting the phase transition temperature of the polymer appears to be lower at higher crosslinking degrees.

Figure 4-22. Phase transition temperatures measurement of pNIPAM 5% wt hydrogels with different crosslinking degrees.
CHAPTER 4. CONTROLLED TEMPERATURE RESPONSIVE SOLID PARTICLE

4.4.5. Changes recorded in response to temperature variation

4.4.5.1. Swelling ratio of gels in different media

pNIPAM hydrogels containing 10% wt monomer were prepared using the recipe described in section 3.6.1.1 employing different crosslinker concentrations to achieve different degrees of hydrogel crosslinking. The swelling behaviour of these pNIPAM hydrogels was studied at two different temperatures (23ºC and 40ºC) (Figure 4-23) in RO water. The pNIPAM hydrogels were left to swell at the studied temperature for 24 hours in order to ensure that the swelling equilibrium was reached. After 24 hours, the swollen hydrogels were weighed after gently blotting the excess liquid and then placed in an oven at 50ºC to dry overnight to ensure that a constant mass was reached. The dried hydrogels were then weighed again. Equilibrium swelling ratio was defined according to equation [4-1]:

\[
ESR = \frac{W_s - W_d}{W_s} \quad [4-1]
\]

Where:  
ESR – equilibrium swelling ratio (g/g)  
Ws – weight of the swollen pNIPAM hydrogels at a particular temperature  
Wd – weight of the dried pNIPAM hydrogels after oven drying overnight

The swelling behaviour of the temperature responsive hydrogels differed below and above the polymer phase transition temperature as shown in Figure 4-23. Thus, when swollen below phase transition temperature at 23ºC, equilibrium swelling ratio (ESR) decreased with increasing the crosslinker concentration, while above LCST at 40ºC, the equilibrium swelling ratio (ESR) increased with increasing the crosslinker concentration (Figure 4-23).
It is well known that there is a relationship between the polymer crosslinking degree and its swelling capacities (Alli A. and Hazer B., 2008; Seddiki N. and Aliouche D., 2013). Degree of crosslinking is dependent on crosslinker concentration. Hydrogels with a greater degree of crosslinking have a lower free volume within the polymer which restricts water accessibility during swelling. Below LCST, the presence of the hydrophilic groups ensures a high degree of swelling, while above LCST, increased system entropy occurs (Hertle Y. et al., 2010) and the swelling decreases due to the hydrophobic interactions of the dominant hydrophobic groups present in the hydrogel (Seddiki N. and Aliouche D., 2013). The maximum swelling occurs below the polymer phase transition, as shown in Figure 4-23.

Figure 4-23. Swelling ratio variation of 10% wt pNIPAM hydrogels with crosslinker concentration in RO water, below and above LCST; Experimental data plotted as mean values of three replicates; Error bars are given by standard deviation; n=3.
4.4.5.2. Shrinkage ratio as a response to temperature changes

An important characteristic of pNIPAM hydrogels is the phase transition temperature (VPTT) reported at around 31°C -32°C for pure pNIPAM (Heskins M. and Guillet J.E., 1968; Tauer K. et al., 2009; Zhu X. et al., 2012). Below this value, pNIPAM hydrogels are hydrophilic and fully swollen owing to the strong interactions between the polymer chains and the water molecules, while above the VPTT the hydrogels become hydrophobic due to the breakage of the hydrogen bonds and deswell with expelling water from their structure. In the light of the above stated pNIPAM behaviour in response to temperature, particle size modifies resulting in volume shrinkage and a lower surface area provided for cell adhesion and proliferation. From this point of view, it is imperative to measure the particle volume shrinkage in order to assess the surface area provided by the particles for cell culture at 37ºC.

The preferred method for measuring particle size is laser diffraction by using the Mastersizer (Malvern Instruments, UK). Unfortunately, this device only allows particle size analysis at room temperature (below the polymer’s VPTT). In order to assess particle size above VPTT, light microscopy and Image J software were employed for measuring particle size. The protocol employed for particle size analysis is described in detail in Appendix 2.B1. At the end of the analysis, the Feret diameter was further used to calculate the volume of the particles below and above the phase transition temperature by using equation [4-2].

\[ V = \frac{\pi D_m^3}{6} \]  

[4-2]

Where: V represents the particle volume (µm³) and \(D_m\) represents the particle Feret mean diameter (µm).
In order to determine how much the particles shrink with temperature increase, the volume ratio of the microgel particles below and above the VPTT was calculated by using equation [4-3] (Tokarova V. et al., 2012).

\[ \text{Shrinkage ratio} = \frac{V_{\text{shrunk}}}{V_{\text{swollen}}} = \left(\frac{D_{\text{shrunk}}}{D_{\text{swollen}}}\right)^3 \]  

[4-3]

Where: \( D_{\text{shrunk}} \) represents the Feret’s diameter of particles in the shrunk state (at 38ºC) and \( D_{\text{swollen}} \) is the Feret diameter of particles in the swollen state (at 23ºC).

Table 4-2. Shrinkage ratio of pNIPAM solid particles calculated using equation [4-3] based on Feret’s diameter values obtained from Image J analysis at a temperature below (23ºC) and above phase transition temperature (38ºC)

<table>
<thead>
<tr>
<th>Shrinkage ratio ((V_{38}/V_{23}))</th>
<th>Crosslinker concentration (%wt)</th>
<th>Swollen (23ºC)</th>
<th>Shrunken (38ºC)</th>
<th>D23 (µm)</th>
<th>Standard deviation (µm)</th>
<th>Number of analysed particles</th>
<th>D38 (µm)</th>
<th>Standard deviation (µm)</th>
<th>Number of analysed particles</th>
<th>D23a (µm) (obtained from lased diffraction analysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.75</td>
<td>0.2</td>
<td>105</td>
<td>14</td>
<td>355</td>
<td>95</td>
<td>15</td>
<td>432</td>
<td>15</td>
<td>432</td>
<td>81</td>
</tr>
<tr>
<td>0.75</td>
<td>0.4</td>
<td>72</td>
<td>15</td>
<td>477</td>
<td>65</td>
<td>12</td>
<td>468</td>
<td>12</td>
<td>468</td>
<td>65</td>
</tr>
<tr>
<td>0.65</td>
<td>0.6</td>
<td>56</td>
<td>12</td>
<td>307</td>
<td>49</td>
<td>11</td>
<td>376</td>
<td>11</td>
<td>376</td>
<td>47</td>
</tr>
<tr>
<td>0.70</td>
<td>0.8</td>
<td>51</td>
<td>10</td>
<td>390</td>
<td>45</td>
<td>11</td>
<td>336</td>
<td>11</td>
<td>336</td>
<td>43</td>
</tr>
<tr>
<td>0.60</td>
<td>1.0</td>
<td>66</td>
<td>16</td>
<td>365</td>
<td>56</td>
<td>15</td>
<td>419</td>
<td>15</td>
<td>419</td>
<td>53</td>
</tr>
</tbody>
</table>

Below the VPTT (23ºC), particles are fully swelled owing to the strong interactions between polymer chains and water molecules (Tokarova V. et al., 2012). As a result, the determined
particle diameter at this temperature was larger than at a value above VPTT (38°C) when the polymer particles are forced to expel the water found within their structure. When fully swollen, two different methods for size measurement were employed and compared. The swelled particle diameters obtained by laser diffraction method were smaller by a percentage ranging from 10% to 22% when compared to the values generated by Image J analysis. This size difference can be attributed to the number of analysed particles during each method and the different analytical techniques used by the separate methods. For the Image J analysis, the number of particles analysed varied between 307 and 477 for each set of conditions.

The data in Table 4-2 shows a decrease of shrinkage ratio with increasing the crosslinker concentration. By increasing the crosslinker concentration, more bridges are created between the polymer linear chains resulting in a more compact structure, thus resulting in smaller sized particles. A denser particle can absorb a limited amount of water when swollen, thus during phase transition when it is forced to expel the water found inside its structure, the change in size becomes smaller, resulting in a smaller shrinkage ratio. This behaviour has been previously reported in other studies involving temperature responsive particles (Tokarova V. et al., 2012; Zhu X. et al., 2012).

4.4.6. Wettability of pNIPAM solid particles in comparison with commercially available temperature responsive surfaces

Cell adhesion to artificial materials is affected by their surface properties such as wettability, roughness, surface charge, and chemical functionalities (Ratner B.D., 2004). With respect to surface wettability of polymeric materials, cells effectively adhere onto polymer surfaces
presenting moderate wettabity with water contact angles ranging between 40° and 70° (Van Wachem P.B. *et al.*, 1985; Tamada Y. and Ikada Y., 1986; Tamada Y. and Ikada Y., 1993; Lee J.H. *et al.*, 1997). For example, Tamada Y. and Ikada Y. (1986) claimed that a polymer surface with a water contact angle of 70° gave the most suitable surface for cell adhesion.

In regards to the pNIPAM synthetic surface, because of its phase transition in response to temperature from hydrophilic to hydrophobic, the water contact angle also exhibits changes in response to temperature increase. In the work presented in this chapter, the static water contact angle (WCA) of the pNIPAM film prepared following the chemistry described in detail in section 2.2.3.1.1.5 was measured at room temperature. In regards to the applicability of the temperature responsive particle for cell culture and release, the WCA value measured was compared to a generally used surface for cell culture (TCPS) and a commercially available temperature responsive surface that was shown to be very efficient at promoting cell adhesion and proliferation (refer to Chapter 6). Commercially available temperature responsive surfaces that support non-enzymatic harvesting of adherent cells for preservation of cell viability and surface proteins are available as two dimensional surfaces. Such a surface is the NUNC UpCell dish (Thermo Scientific), but these are not available as 3D microcarriers. The UpCell consists of covalently grafted pNIPAM to tissue culture polystyrene (TCPS) vessels (*e.g.* Petri dish, multi-well dish etc.) by radical polymerisation initiated with electron-beam irradiation (Yamada N. *et al.*, 1990).

The WCA measurements for the surfaces mentioned above are provided in Figure 4-24. The value obtained for TCPS as a result of nine different measurements was 99.5° which is higher than the reported value of 70° reported by Tamada Y. and Ikada Y. (1986) indicating a more hydrophobic surface. Several studies have previously reported the capability of promoting cell adhesion and proliferation is closely related to surface wettability that
determines protein adsorption. Thus, the optimum protein adsorption and cell adhesion are usually achieved at mild, intermediate values of surface wettability. However, extremely hydrophobic surfaces present cell anti-adhesive properties, despite their ability to allow protein adsorption even in relatively large quantities. However, these proteins become very rigid and reorganisation-resistant, thus blocking the specific amino acid sequences involved in cell adhesion to surfaces (Heitz J. et al., 2003; Bacakova L. et al., 2004; Parizek M. et al., 2009). In regards to the temperature responsive synthetic surfaces, the commercially available surface (NUNC UpCell) exhibited a mean WCA of 56.4º, while the produced 10% wt pNIPAM film exhibited a mean value of 59.9º (Figure 4-24). The values measured for the temperature responsive surfaces are below the hydrophobicity level reported by Tamada Y. and Ikada Y. (1986), but this was expected as it is well known that such temperature responsive surfaces are hydrophilic below their LCST value (e.g. room temperature).

![Figure 4-24. Water Contact Angle images and measurements recorded for pNIPAM 10% wt hydrogel films compared to TCPS and commercially available two dimensional temperature responsive surfaces UpCell (NUNC).](image-url)

WCA = 59.9º ± 2.8º; n=10 (Mean ± SD)

WCA = 99.5º ± 6.6º; n=9 (Mean ± SD)

WCA = 56.4º ± 1.5º; n=5 (Mean ± SD)
4.5. Conclusions

The approach taken in this thesis for producing spherical monodispersed temperature responsive particles consisted of applying a membrane emulsification technique using the stirred Dispersion Cell system. I have successfully produced pNIPAM spherical particles with median sizes controllable within the range of 37 µm to 1050 µm and spans as low as 0.5. The shear at the membrane surface is provided by a simple paddle blade stirrer placed above the membrane in order to assist with droplet detachment from the membrane’s pores.

As a different approach, it was possible to successfully correlate the drop size formed using the system with a published model for membrane emulsification, relating droplet size to shear stress at low dispersed phase injection rates. By controlling the stirring speed and the injection rate of the dispersed phase through the membrane pores, it was possible to achieve precise control over particle size and size distribution. The pNIPAM solid particles generated by this method are in a size range of interest for various biopharmaceutical applications, such as microcarriers for cell culture.

The spherical pNIPAM particles were shown to exhibit a temperature responsive behaviour in a temperature range of 31°C to 33°C depending on the formulation employed for their production. The degree of crosslinking was shown to have a major impact on some of the particle properties (size, swelling degree, shrinking ratio). All the illustrated characteristics (e.g. shape, size, size distribution, temperature responsiveness) demonstrated the potential of these particles to be used as supports for cell culture and as well as potential substrates for non-enzymatic and non-damaging cell harvesting.
5. CONTROLLED CORE-SHELL PARTICLE PRODUCTION BY MEMBRANE EMULSIFICATION

5.1. Introduction

Temperature responsive polymers can be effectively used as scaffolds for thermally regulated cell adhesion / detachment control based on the physical and conformational polymer changes in response to an environmental switch. Previous studies showed that some surfaces based on pNIPAM alone do not support cell adhesion even above its phase transition temperature when the surface is hydrophobic in nature (Akiyama Y. et al. 2004; Yamato M. et al. 2003). These observations are consistent with the results obtained in this work and presented in detail in the following chapter (refer to Chapter 6). Several previous studies using two dimensional surfaces (i.e. not microcarriers) suggested that pNIPAM layer thickness has a major influence on cell adhesion and proliferation. Critical layer thickness for cell adhesion and proliferation was found to be between 20 nm and 30 nm (Yamato M. et al. 2001; Shiroyanagi Y. et al. 2003; Harimoto M. et al. 2002; Nandkumar M.A. et al. 2002). Values greater than 30 nm were inefficient at promoting cell adhesion (Akiyama Y. et al. 2004; Yamato M. et al. 2003).

Several methods have been employed to graft pNIPAM onto different surfaces at thicknesses which allow cell adhesion. This study is focused only on methods that generate pNIPAM coatings with the ability for cell harvesting by employing hydrophobicity/hydrophilicity changes in response to temperature stimuli, rather than disintegrating for cell harvesting. This behaviour ensures that the cells alone are harvested without any unwanted polymer residues. Such methods are plasma polymerisation (Cheng X. et al. 2005; Canavan H.E. et al. 2005; Canavan H.E. et al 2005A; Pan Y.V. et al 2001), electron beam (e-beam) polymerisation (Yamada N. et al., 1990; Ide T. et. al., 2006;
Akiyama Y. et al., 2004; Yamato M. et al., 2003); Ultra Violet (UV) irradiation methods (von Recum H.A. et al., 1998; Chen G. et al., 1998), gamma irradiation methods (Akiyama Y. et al., 2004), Atom transfer radical polymerization technique (ATRP) (Mizutani A. et al., 2008), Reversible addition fragmentation chain transfer polymerization technique (RAFT) (Yang Y. et al., 2012).

Electron beam induced polymerisation is commonly used as it generates thin and even polymer grafting allowing the possibility of a large scale production. However, electron beam polymerisation can result in high production costs. Polymer grafting is performed in two steps: covalently binding the monomer, followed by inducing polymerisation onto the surface by electron beam irradiation. The layer thickness is controlled by the monomer concentration and the energy applied (Akiyama Y. et al., 2004). A different single step method for generating pNIPAM grafted onto surfaces is plasma polymerisation which consists of applying a plasma glow discharge of monomer, thus depositing the polymer onto the required surface. Several studies showed that the layer thickness obtained by this method does not influence cell adhesion or detachment from these surfaces. Unlike the e-beam induced polymerisation, this method can’t be utilised for large scale production (Canavan H.E. et al., 2005). As a different approach, RAFT (Yang Y. et al., 2012) and ATRP (Mizutani A. et al., 2008) methods allow controlled preparation of surfaces with pNIPAM brushes, but a major disadvantage is the use of very toxic chemicals that can affect the cell adhesion capabilities of the surface.

Most of the methods described above are expensive and not easily available to all researchers. As a result, a facile and easy to apply in 3D method to produce the core-shell temperature responsive particles by taking advantage of the electrostatic interactions between the carboxyl groups of the alginate and amino groups of the amine modified
pNIPAM was developed. Core calcium alginate particles were produced by membrane emulsification employing the internal gelation method (solids contained in the dispersed phase) and the temperature responsive monolayer coating was obtained by a chemisorption process of the pNIPAM onto the alginate bead.

Core particle formulation is described in detail in section 5.3. Controlled core particle production is then demonstrated in section 5.4 and successful coating with the temperature responsive polymer is provided in section 5.5 based on the construction of a Langmuir sorption isotherm. The successful generated temperature responsive core-shell particles were then characterised using different techniques to demonstrate temperature responsiveness and applicability as scaffolds for thermally induced cell adhesion (section 5.6).

5.2. Aims and objectives

The main aim of the work presented in this chapter was to demonstrate that controlled production of core-shell temperature responsive particles can be achieved using membrane emulsification applied on the Dispersion Cell device described in detail in section 3.2.1. This was possible through the realisation of the following objectives:

- Selection of the optimal formulation for core-shell particle production.
- Controlled particle production by using the Dispersion Cell with generation of calcium alginate particles with controllable sizes ranging from 55 µm to 690 µm and size distributions expressed as spans as low as 0.2.
- pNIPAM coating of calcium alginate particles with the generation of core-shell
5.3. Formulation

Alginate particle cross-linking can be easily performed by dripping sodium alginate solution into a 10% wt calcium chloride solution (Gombotz W.R. et al., 1998; Gu F. et al., 2004), but this is known to produce very large particles with sizes in the order of millimeters. To produce smaller droplets, the ‘internal gelation’ technique is used; where the crosslinking calcium carbonate is pre-dispersed within the alginate solution before emulsification, with triggered release of the calcium ions after emulsification by the provision of hydrogen ions to the emulsion. This approach is easy to apply when emulsifying by stirring, but presents a challenge when attempting membrane emulsification using microfiltration membranes, which can be blocked with the solid particles.

As a result, several formulations were tried and are provided in Table 5-1. The calcium alginate particles were generated by injecting 10 cm$^3$ of dispersed phase at an injection rate of 0.58 mL/min into 100 cm$^3$ of continuous phase at a stirring speed of 627 rpm. The membrane used for this testing was an array of 20 µm pores with an inter-pore spacing of 200 µm. The formulations tested in this study were adaptations of previously reported formulations applied in emulsification methods (You J.O. et al., 2001; Liu X.D. et al., 2002; Liu X.D. et al., 2003; Ribeiro A.J. et al., 2005; Silva C.M. et al., 2006). However, as the majority of formulations previously mentioned used toxic continuous phases, hence for the work presented in this thesis, oil phases with low toxicity were considered to be suitable given the final application.
Table 5-1. Different formulations (F₁ to F₉) for calcium alginate particle production

<table>
<thead>
<tr>
<th>Nr..</th>
<th>Dispersed Phase</th>
<th>Continuous Phase</th>
<th>Ca²⁺ source</th>
<th>Gelation</th>
</tr>
</thead>
<tbody>
<tr>
<td>F₁</td>
<td>2%wt Na Alginate + 5%wt CaCO₃</td>
<td>Sunflower oil without surfactant</td>
<td>CaCO₃</td>
<td>5% v/v Mini-emulsion of glacial acetic acid in sunflower oil</td>
</tr>
<tr>
<td>F₂</td>
<td>2%wt Na Alginate</td>
<td>1.5%wt Tween80 in sunflower oil</td>
<td>10%wt CaCl₂</td>
<td>10%wt CaCl₂</td>
</tr>
<tr>
<td>F₃</td>
<td>1%wt Na Alginate + 0.5%wt CaCO₃</td>
<td>2%wt Span80 in sunflower oil</td>
<td>CaCO₃</td>
<td>Glacial acetic acid</td>
</tr>
<tr>
<td>F₄</td>
<td>1.5%wt Na Alginate + 1%wt CaCO₃</td>
<td>2%wt Span80 in a 50:50 mixture of kerosene-sunflower oil</td>
<td>CaCO₃</td>
<td>Glacial acetic acid</td>
</tr>
<tr>
<td>F₅</td>
<td>1.5%wt Na Alginate + 1%wt CaCO₃</td>
<td>Sunflower oil without surfactant</td>
<td>CaCO₃</td>
<td>Glacial acetic acid</td>
</tr>
<tr>
<td>F₆</td>
<td>1.5%wt Na Alginate + 0.5%wt CaCO₃</td>
<td>1.5%wt Tween80 in kerosene</td>
<td>CaCO₃</td>
<td>5% v/v Mini-emulsion of glacial acetic acid in kerosene</td>
</tr>
<tr>
<td>F₇</td>
<td>1.5%wt Na Alginate + 0.5%wt CaCO₃</td>
<td>Sunflower oil without surfactant</td>
<td>CaCO₃</td>
<td>Glacial acetic acid</td>
</tr>
<tr>
<td>F₈</td>
<td>1.5%wt Na Alginate + 0.5%wt CaCO₃</td>
<td>1.5%wt Tween80 in sunflower oil</td>
<td>CaCO₃</td>
<td>Glacial acetic acid</td>
</tr>
<tr>
<td>F₉</td>
<td>1.5%wt Na Alginate + 0.5%wt CaCO₃</td>
<td>1%wt Span80 in Miglyol 840</td>
<td>CaCO₃</td>
<td>Glacial acetic acid</td>
</tr>
</tbody>
</table>

Photomicrographs of the obtained particles were taken with an optical microscope (Leitz Ergolux) and are provided in Figure 5-1 below.
Figure 5-1. Optical photomicrographs of produced calcium alginate particles when employing different formulations (F1 to F9) (refer to Table 5-1). Scale bars represent 100 µm.

Ignoring formulations which failed to generate spherical calcium alginate particles, sunflower oil or Miglyol 840 with CaCO3 as a source of Ca^{2+} was most suitable. The release of calcium ions was achieved by the addition of glacial acetic acid to the system. It is well known that an alginate solution has the ability to form gels, often referred to as ‘acid gels’ by lowering the pH to the pKa value of the uronic acid residues contained, in the range of 3 to 4 (Draget K.I. et al., 1994). In the system selected in this thesis, the system pH value was measured to be 4.8, thus favouring the formation of calcium-crosslinked alginate gels over the formation of acid gels. Based on these observations, the formulation most suitable to choose was Formulation F9. This formulation generated spherical shaped micron-sized calcium alginate particles as
shown in Figure 5-2 and uses only food grade and pharmaceutical grade reagents, such as Miglyol 840 which is a propylene glycol diester of saturated plant fatty acids. Figure 5-2a provides photographs of hydrated calcium alginate particles, while Figure 5-2b provides SEM microphotographs of dried particles. For the SEM analysis, alginate particles were air dried from acetone. It is well-known that an alginate particle consists of a large volume of water with a low solid content; a typical particle is over 90% wt water with around 2% wt solids. Hence the shrinkage and deformation which occurred upon drying was substantial (Figure 5-2b).

Figure 5-2. (a) Optical micrographs of monosized spherical calcium alginate particles generated at an injection rate of 0.58 mL/min and a shear stress of 27 Pa (b) SEM photographs of dried calcium alginate particles. Scale bars represent: a) 100 µm; b) 100 µm (left) and 90 µm (right).
5.4. Controlled production of calcium alginate particles

The main metallic membranes employed for the production of calcium alginate particles are described and characterised in section 3.2.2. Tests with 5 µm and 10 µm pore size membranes were also performed, but membrane blockage occurred quickly, even at the lowest injection rate of 0.58 mL/min. There was no evidence of any blockage when using the 20 µm pore size membranes, with either pore spacing (80 µm and 200 µm). The controlled particle production resulting in a controlled particle size and size distribution was achieved by modifying both formulation and membrane emulsification process parameters. Different concentrations of CaCO₃ (section 5.4.1) and surfactant (5.4.2) were investigated, as well as the effect of process parameters, such as shear stress (section 5.4.3.1) and injection flux (section 5.4.3.2) on particle size and size distribution.

5.4.1. The effect of CaCO₃ concentration

As part of the formulation, the effect of CaCO₃ concentration in the dispersed phase was evaluated. The use of calcium carbonate in membrane emulsification has major implications as even finely divided calcium carbonate particles will foul or block the membrane when using the sinter type of ceramic or glass membrane (Williams R.A. et al., 1998). A metal sieve type membrane has been previously used successfully (Liu X.D. et al., 2003), but in order to achieve the dispersion of solid particulate containing aqueous phase through the sieve-type membrane nanoparticles of calcium carbonate (40 nm) had to be employed, and the membranes used were specialist products, of a very limited surface area, from a Chinese nuclear institute with pore sizes between 2.9 and 5.2 µm. As a relatively new approach, compared to the previously reported devices for membrane emulsification, the sieve-type membranes reported in Chapter 3 (used in the work reported in this thesis) can be used as
they have no internal surface or structure on which the particles can deposit and are available at different scales for laboratory and commercial production rates. Furthermore, the approach reported here involves the use of calcium carbonate with a volume median size of 2.3 µm (Figure 5-3). Its full size distribution is provided in Table 5-2 and it is the same size distribution as a commercially available calcite that is GRAS (generally recognized as safe), whereas the previously reported Chinese study required specialised disperse 40 nm calcite particles.

Figure 5-3. Scanning Electron microscopy (SEM) image of calcium carbonate particles used to provide internal gelation of alginate.

The effect of different concentrations of calcium carbonate on the resulting alginate particle size was studied using an array of 20 µm pores with 80 µm pore spacing at an injection rate of 0.58 mL/min and a shear stress of 27 Pa as shown in Figure 5-3. In this work, the maximum shear stress is calculated using equations [2-9] (refer to section 2.2.3.1.1.5), with
$r = r_{\text{trans}}$ for a given rotation speed and continuous phase viscosity. Up to a suspended solids calcium carbonate concentration of 5% wt, there was no evidence of membrane blockage. Particle size decreased with increasing calcium carbonate concentration, consistent with the stronger calcium crosslinking during particle formation (Figure 5-4A). Figure 5-4B shows the variation of span values of the produced particles with the calcium carbonate concentration. The span values increased with the increase in the calcium carbonate concentration, thus ranging from highly monosized (span of 0.1) to a span of 2.6 suggesting a high degree of coalescence taking place.

Table 5-2. Full size distribution of calcium carbonate employed for the internal gelation of alginate particles

<table>
<thead>
<tr>
<th>Undersize (%)</th>
<th>Particle Diameter (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.17</td>
</tr>
<tr>
<td>5</td>
<td>0.60</td>
</tr>
<tr>
<td>10</td>
<td>0.88</td>
</tr>
<tr>
<td>20</td>
<td>1.40</td>
</tr>
<tr>
<td>30</td>
<td>1.72</td>
</tr>
<tr>
<td>40</td>
<td>2.03</td>
</tr>
<tr>
<td>60</td>
<td>2.69</td>
</tr>
<tr>
<td>70</td>
<td>3.09</td>
</tr>
<tr>
<td>80</td>
<td>3.56</td>
</tr>
<tr>
<td>90</td>
<td>4.28</td>
</tr>
<tr>
<td>95</td>
<td>4.90</td>
</tr>
<tr>
<td>100</td>
<td>7.70</td>
</tr>
</tbody>
</table>

There were no noticeable advantages in using a higher concentration of calcium carbonate for further tests. Hence, it was concluded that a 20 µm pore size membrane using a
concentration of 0.5% wt calcium carbonate suspension was appropriate for the production of alginate particles for functionalization and cell growth.

Figure 5-4. The effect of calcium carbonate concentration on A) median calcium alginate particle size and B) size distribution expressed as span values
5.4.2. The effect of surfactant concentration

Ideally, the emulsifier molecules should not electrostatically sorb to the membrane’s surface. Electrostatic interactions could cause alterations to the membrane’s surface making it more hydrophobic (Tong J. et al., 2000). Keeping this in mind for the production of W/O emulsions, a surfactant soluble in the oil phase was used. The surfactant was sorbitan monooleate (Span 80) which is a non-ionic surfactant with a HLB of 4.3 which is in the range recommended for water-in-oil emulsifiers (3 to 6) (Becher P., 2001; Tadros T.F., 2009).

As part of the formulation, the surfactant concentration can have an effect on particle size. By increasing the surfactant concentration the interfacial tension between the two immiscible phase decreases until the critical micelles concentration is reached, generating smaller droplets and therefore smaller particles (Schramm L.L., 2005). Figure 5-5 shows the effect of surfactant concentration on the resulting alginate particle size and size distribution, studied using an array of 20 µm pores with 80 µm pore spacing at an injection rate of 0.58 mL/min and a shear stress of 27 Pa. By varying the surfactant concentration, calcium alginate particles can be generated within the size range of 499 µm to 96 µm (Figure 5-5A) with span values ranging from 0.2 to 1.6, as shown in Figure 5-5B. The less monosized particles were generated for the formulation without any surfactant suggesting a high level of coalescence. For further tests, the formulation containing 1% wt surfactant was employed.
Figure 5-5. Influence of surfactant concentration on particle A) size and B) size distribution expressed as span values.
CHAPTER 5. CONTROLLED CORE-SHELL PARTICLE PRODUCTION

5.4.3. Influence of process parameters

Controlled particle production can be achieved when using the Dispersion Cell device described in detail in section 3.2. The controlled particle size and size distribution are achieved by varying process parameters e.g. injection rate of the dispersed phase, shear stress applied at the membrane’s surface, pore size, inter-pore distance etc.

5.4.3.1. Influence of the shear stress and dispersion flux

In this work, the maximum shear stress is calculated using equations [2-9] (refer to section 2.2.3.1.1.5), with $r = r_{\text{trans}}$ for a given rotation speed and continuous phase viscosity. The dispersion flux was expressed as volume of dispersed phase injected through the pores of the membrane per minute (mL/min).

In Figure 5-6 the effect of shear stress generated at the membrane surface on the resulting particle size is illustrated, together with data on the influence of increasing the flow rate of the injected phase on the particle size and size distribution expressed as span values. As expected, increasing the injection rate results in the formation of larger droplets (Holdich R.G. et al., 2010), and this influence was observed in this study (Figure 5-6a). Increasing the rotation speed increases the shear stress on the membrane surface and the droplet formation time shortens, therefore, the size of the produced droplets and thereby particle size decreases.

For the system employed here (an array of 20 µm pores and 80 µm pore spacing), a shear stress of 6 Pa (equivalent of 402 rpm) generates alginate particles with sizes between 306 µm and 378 µm, while a shear stress of 42 Pa (equivalent of 1087 rpm) generates particles with sizes in the range of 55 µm and 145 µm. The model (refer to section 2.2.3.1.1.5) presented in Figure 5-6a was constructed based on a force balance at one pore of the
membrane when maximum shear stress was applied at the transitional radius. The model fit is good at a low injection rate (0.58 mL/min), while for higher rates the produced particles are larger than predicted. The model does not take into consideration the injection flow rate. Hence, it should be considered only as a model for the minimum particle size produced at the lowest injection rate used. An example of model calculations is provided in Appendix B2.

The size distribution of the generated calcium alginate particles expressed as span values varies between 0.2 and 1.4 as shown in Figure 5-6b.
Figure 5-6. Effect of peak shear stress at the membrane surface on (a) size and (b) size distribution expressed as span values of the generated alginate particles, as a measure of different flow rates expressed as volume of dispersed phase injected through the membrane’s pores per minute (mL/min); the membrane used in this study had an array of 20 μm pores and 80 μm spacing between pores.

5.4.3.2. Influence of the inter-pore spacing

For comparison, when using a 20 μm pore array with a larger inter-pore spacing (200 μm), the same pattern was followed, with only marginal differences in particle size and span values. The generated data is illustrated in Figure 5-7. These tests were used to determine if an additional force involved in droplet detachment known as ‘push-off’ force was occurring (Egidi E. et al, 2008). The ‘push-off’ force involving the ‘push-to-detach’ droplet formation
mechanism (Zhu J. et al., 2005) occurs when most or all of the membrane’s pores become active and droplet formation at one pore is influenced by the presence of other droplets forming at adjacent pores. When the ‘push-off’ force is present, smaller droplets with greater size distribution are expected to form (Egidi E. et al., 2008). In this investigation, particle size and size distributions were measured at a low injection rate (0.58 mL/min) over a wide range of shear stresses (0.95 Pa to 42 Pa) applied on the membrane’s surface (Figure 5-7). There was no significant evidence of the ‘push-off’ effect observed in this study as the particle sizes and spans generated when using the two different membranes were similar in value for the tested conditions.

Figure 5-7. Comparison of alginate particles size and size distribution expressed as span values generated at 0.58 mL/min with 20 µm pore size with different pore spacing.
5.4.3.3. Comparison between disc and annular membrane

It was argued in a previous study that at a certain point on the membrane surface there is a transitional point between the forced and free vortex around the paddle-blade stirrer known as the transitional radius (Kosvintsev S.R. et al., 2005; Stillwell M.T. et al. 2007). At this point, the shear stress has the highest value; hence this region is the most productive in terms of droplets formed through the membrane, as the pressure difference across the membrane is the greatest at this radius. The transitional radius can be calculated by using equation [2-5] described in section 2.2.3.1.1.5.

A special type of metallic membrane was also employed in this study. This type of membrane has a reduced operating area consisting of an annular ring of open pores. The advantage of this membrane is that the transitional radius is within the ringed radial area and several studies performed using this type of membrane showed potential for production of highly monosized emulsions with an improved size distribution compared to a normal disc shaped metallic membrane (Stillwell M.T. et al., 2007). The dispersed phase was injected through the membrane’s pores at rates ranging from 0.3 to 6.1 mL/min and it generated calcium alginate particles with sizes between 140 µm and 490 µm. Size variation was controlled by adjusting the process parameters (e.g. shear stress and injection rate) as shown in Figure 5-8. Alginate particle size increased with increasing injection rate, while increasing the shear stress applied at the membrane surface generated smaller particles, as is the case with the conventional non-ringed membrane. However, the ringed membrane size distribution results were not any better than those for the non-ringed membrane.
Figure 5-8. Controlled alginate particle production when employing an annular ring membrane; the effect of process parameters on median particle size.

The span values of the generated particles varied between 0.6 and 1.0 (Figure 5-9) which was greater than the values obtained when employing 20 µm pore and 80 µm pore spacing membranes, as described in section 5.3.3.1.
Figure 5-9. The effect of process parameters on particle size distribution expressed as span values.

For a more obvious evaluation of the suitability for the production of the alginate particles, a study comparing the annular ring membrane with the disc shaped membrane, both with 20 µm pores and 200 µm pore spacing was performed. The dispersed phase was injected at a rate of 0.58 mL/min with different shear stress values applied on the membrane. Figure 5-10 shows the both membrane types behaved similarly. The particle size decreased with the increased application of shear stress, as expected. Alginate particles with sizes ranging from 115 µm to 520 µm were generated when employing the disc shaped membrane, while particles with sizes ranging from 158 µm to 325 µm were generated when using the ring annular membrane. Particle size distribution span values varied widely from 1.4 to 0.2 when employing the disc membrane compared to a narrower variation between 0.77 and 0.68 when using the annular ringed membrane, as shown in Figure 5-11.
CHAPTER 5. CONTROLLED CORE-SHELL PARTICLE PRODUCTION

Figure 5-10. Comparative study of the shear stress effect on particle size when employed two different types of membranes: disc and annular.

Figure 5-11. Comparative study of the shear stress effect on size distribution when employed two different types of membranes: disc and annular.
In terms of the narrowness of the resulting particle size distribution, the use of the annular ring membrane had no apparent advantages when using the preferred formulation for the production of calcium alginate particles (refer to section 5.3). It is possible to use such a membrane for the production of alginate particles with sizes localised within 150 µm and 300 µm and spans of approximately 0.7. However, better size distributions could be obtained when using the conventional non-ringed membrane.

5.4.3.4. Reproducibility of the results

Due to the high costs of reagents needed, as well as the large number of experiments required for these studies, all tests were based on single measurements. However, the reproducibility of the collected data was assessed for several sets of process parameters. The reproducibility of the particle production results was assessed by performing triplicate experiments where 10 cm$^3$ of dispersed phase was injected at a rate of 0.58 mL/min through the 20 µm pores of the nickel conventional membrane with 80 µm pore spacing (Figure 5-12). Some level of variation was recorded for lower peak shear stress values (6 Pa and 15 Pa), while for 27 Pa and 42 Pa the results were generally consistent.
Figure 5-12. Reproducibility of the results as demonstrated by three sets of experiments performed at different peak shear stress values and an injection rate of 0.58 mL/min when the dispersed phase was passed through the 20 µm pores of a metallic membrane with 80 µm pore spacing.

5.5. Generation of core-shell temperature responsive particles

Alginate forms a good scaffold onto which some form of functionalization can be grafted. One advantage of alginate structures is that they have an appreciable natural internal pore structure (Gombotz W.R. and Wee S.F., 1998), which provides a high surface area for interfacial phenomena to occur. In the case of cell culture a coupling agent that promotes cell attachment, and promotes cell detachment under controllable conditions would be ideal.
Hence, the ability to functionalise the alginate particles formed by membrane emulsification was tested by chemisorption of pNIPAM to form a shell upon the alginate core.

5.5.1. Temperature responsive polymer characterisation

The pNIPAM shell was generated by chemisorption while taking advantage of the existence of carboxylic functional groups present in alginate and employing a modified amine-terminated pNIPAM (Sigma Aldrich, UK) as a source of amine functional groups. One of the main characteristics of temperature responsive polymers (e.g. pNIPAM or modified-pNIPAM) is a phase transition at a specific temperature (LCST) which changes its surface property from hydrophilic to hydrophobic. The LCST usually refers to aqueous polymer solutions of low concentrations (0.5 – 1% w/v) (Constantin M. et al., 2011). Aqueous pNIPAM solutions exhibit a reversible temperature-induced phase separation. Below the cloud point, pNIPAM is soluble in water due to its ability to form hydrogen bonds with the water molecules via the amide groups. However, above the cloud point, the polymer becomes insoluble in water and precipitates generating a two-phase system (Figure 5-13). The polymer’s insolubility in water above the cloud point is determined by the hydrogen bonds created between the adjacent groups of the macromolecule (Ottaviani M.F. et al., 2001). The same behaviour can also be attributed to amine-modified pNIPAM.
Figure 5-13. Phase transition of amine-terminated pNIPAM (20 ppm) below and above LCST.

The generally recognised method for determining the LCST of a temperature responsive polymer is the cloud point method (Ottaviani M.F. et al., 2001; Kawaguchi T. et al., 2008) which exploits the fact that absorbance changes in response to temperature. It is well known that the LCST of temperature responsive polymers is dependent on polymer concentration in solution (Heskins M. and Guillet J.E., 1968). As a result, the cloud point of amine-terminated pNIPAM (Sigma Aldrich, UK) was assessed at different concentrations (Figure 5-14). By increasing the polymer concentration in solution, it was observed that the LCST value decreased as shown in Figure 5-14. The LCST value is considered to be the halfway point of the generated curve. The clouding of the polymer solution is caused by the hydrophobic interactions between the polymer chains (Schild H.G., 1992). As a result of an increased polymer concentration in solution, the hydrophobic interactions occur faster and therefore the phase transition appears to be sharper at higher polymer concentrations.
Temperature induced transition of the amine-terminated pNIP (Sigma Aldrich, UK) in aqueous solution was confirmed at different pH values by measuring absorbance at 560 nm. The amine terminated polymer’s phase transition temperature is shifted towards a lower temperature value for pH 7, while at pH 5 the temperature response is delayed, as shown in Figure 5-14. In acidic conditions, the amino groups of the amine-terminated pNIPAM are protonated and it is expected that intra-molecular electrostatic forces develop. As a result, the temperature induced collapse of the modified polymer chains will occur at a higher temperature which explains the behaviour shown in Figure 5-15.
Figure 5-15. Effect of pH on the temperature-induced transition of amine-terminated pNIPAM, in a 5 mg/mL aqueous solution; values are expressed as mean; the error bars represent the standard deviation obtained from four separate measurements.

5.5.2. Langmuir sorption isotherm

5.5.2.1. Sorption isotherm theory

The most common and preferred equation for the absorption isotherm is a Langmuir type equation. The Langmuir isotherm (Langmuir I., 1916; Langmuir I., 1917) can be derived from a consideration of the monolayer coverage of the solid surface by the adsorbate. This isotherm assumes that the surface consists of sites onto which the adsorbate can adsorb thus forming a monolayer and that each site can accommodate one entity at a time.
Where: \( qe \) [mg/g] is the amount of adsorbate adsorbed at equilibrium; \( q_{\text{max}} \) [mg/g] is the maximum monolayer adsorption capacity of the adsorbent; \( Ce \) [mg/L] is the equilibrium concentrations of the adsorbate and \( Kl \) [L/mg] is a Langmuir adsorption constant and it is related to the energy of sorption which increases as the strength of sorption increases.

In order to determine \( Kl \) and \( q_{\text{max}} \), the Langmuir equation is often linearized [5-2] and by plotting \( Ce/qe \) against \( Ce \) a linear trend is achieved and the two parameters can be then determined from the slope.

\[
\frac{Ce}{qe} = \frac{1}{q_{\text{max}}*Kl} + \frac{Ce}{q_{\text{max}}} \tag{5-2}
\]

The exchange efficiency of the Langmuir isotherm is evaluated by using the following equation to determine Hall’s parameter \( R_l \) (Hall K.L. et al., 1966):

\[
R_l = \frac{1}{1+Kl*Co} \tag{5-3}
\]

Where \( Co \) [mg/L] is the highest initial adsorbate concentration in solution and \( Kl \) [L/mg] is Langmuir adsorption constant. Also when the dimensionless sorption capacity (\( q/qo \)) is plotted against the dimensionless concentration (\( C/Co \)) for different \( RL \) values, the shape of isotherm can be developed and used to predict if the process of sorption is favourable or unfavourable. The effect of \( R_l \) can be interpreted according to the classification in Table 5-3.
Table 5-3. Efficiency of Langmuir isotherm

<table>
<thead>
<tr>
<th>$R_L$</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R_L &gt; 1$</td>
<td>UNFAVOURABLE</td>
</tr>
<tr>
<td>$R_L = 1$</td>
<td>LINEAR</td>
</tr>
<tr>
<td>$0 &lt; R_L &lt; 1$</td>
<td>FAVOURABLE</td>
</tr>
<tr>
<td>$R_L = 0$</td>
<td>IRREVERSIBLE</td>
</tr>
</tbody>
</table>

5.5.2.2. Construction of the Langmuir sorption isotherm of pNIPAM chemisorbed on the calcium alginate particles

In order to quantify the amount of temperature responsive polymer adsorbed onto the alginate particles, a Langmuir isotherm was generated. The experimental methods normally employed for the construction of adsorption isotherms can be direct or indirect as described by Tadros T.F. et al., 2009. Indirect methods usually involve spectroscopic techniques such as ultraviolet-visible (UV), nuclear magnetic resonance (NMR), infrared (FT-IR), refractive index recordings or fluorescence spectroscopy techniques to measure the concentration of polymer in the solvent before and after adsorption to some surface. Direct methods involve techniques such as Infrared methods if there is specific interaction between the polymer segments and the surface, electron spin resonance (ESR) which requires molecule labelling or NMR pulse gradient to measure the concentration of polymer at the surface.

Even though direct methods are more reliable, indirect methods of measurement based on calibration curves of the residual solute in solution were used because they are easier to perform and more accessible. Briefly, 0.5 g of alginate particles were suspended in 20 ml of
CHAPTER 5. CONTROLLED CORE-SHELL PARTICLE PRODUCTION

pNIPAM amine-terminated solutions of different solute concentrations and stirred at room temperature for 24 h. After contact time, the core-shell particles were retrieved by filtration and the recovered solution filtered through a 0.2 µm filter to remove any impurities for further analysis. The measurement of the amount of polymer remaining in solution was based on a generated calibration curve of different concentrations of amine-terminated pNIPAM.

5.5.2.2.1. UV/VIS measurement method

The initial method employed for these measurements consisted of using a UV/VIS spectrophotometer by measuring the absorbance at 300 nm. This wavelength was selected based on a spectrum scan of a 10 mg/mL pNIPAM solution (Sigma Aldrich, UK) (Figure 5-16). This method was a failure, and did not provide data for the generation of the Langmuir isotherm, as final values suggested that there was more polymer adsorbed on the alginate particles than there was in the initial solution. This can be explained by the fact that sodium alginate is still present and possibly entrapped inside the generated calcium alginate particles and it is interfering with absorbance measurements. To confirm this assumption, a wavelength scan ranging from 190 nm to 600 nm was performed which is shown in Figure 5-17. Deionized water was used as a negative control for calibration. For the coating procedure, the negative control was obtained by re-suspending the calcium alginate beads in deionized water (0 ppm).
Figure 5-16. Spectrum scans of a 10 mg/mL pNIPAM solution (Sigma Aldrich, UK).

Figure 5-17. Wavelengths scan of pNIPAM aqueous solution before and after contact with calcium alginate particles.
By comparing the UV/VIS scans of the controls used and the 1000 ppm initial solution with the 1000 ppm residual polymer solution, it was observed that an additional peak was obtained at approximately 270 nm which explains the obtained abnormal values of absorbance for the residual polymer solutions. To further confirm this hypothesis, SEM coupled with EDX was performed on the pNIPAM-coated calcium alginate particles obtained with different polymer concentrations. By analysing the EDX spectrum, a sodium peak is recorded in the calcium alginate particles (Figure 5-18) used as negative control in the coating procedure, a peak that wasn’t found to be present inside the pNIPAM-coated calcium alginate particles (Figure 5-19). This confirms the presence of non-crosslinked sodium alginate inside the calcium alginate particles, which interfered with the UV/VIS measurements of pNIPAM concentration in solution.

<table>
<thead>
<tr>
<th>0 ppm</th>
<th>Weight%</th>
<th>Atomic%</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>44.25</td>
<td>55.59</td>
</tr>
<tr>
<td>O</td>
<td>40.41</td>
<td>38.11</td>
</tr>
<tr>
<td>Na</td>
<td>1.87</td>
<td>1.23</td>
</tr>
<tr>
<td>Ca</td>
<td>13.48</td>
<td>5.07</td>
</tr>
</tbody>
</table>

Figure 5-18. SEM coupled with EDX analysis of uncoated calcium alginate particles.
5.5.2.2.2. Turbidimetry measurement method

The second method employed consisted of turbidity measurements of the polymer solutions when the temperature is above the phase transition temperature (40°C). Ten measurements were performed for each sample and average values were considered. Figure 5-20 shows the calibration curve generated based on turbidity values for different concentrations of amine-terminated pNIPAM. The polymer solutions of different concentrations were kept at constant temperature (40°C) for at least 10 minutes prior to measurement.

Figure 5-19. SEM coupled with EDX analysis of pNIPAM-coated calcium alginate particles.
Figure 5-20. Calibration curve based on turbidity measurements of amine terminated pNIPAM solutions of different concentrations.

For the quantification of the amount of amine-terminated pNIPAM adsorbed onto the calcium alginate particles, a Langmuir isotherm was employed (described in detail in section 5.4.2.1). Figure 5-21 shows the Langmuir isotherm and its fit to the experimental data obtained from turbidity measurements for the system employed here.
Figure 5-21. Mass balance Langmuir type adsorption isotherm of amine terminated pNIPAM onto calcium alginate particles.

In order to evaluate the efficiency of the Langmuir isotherm, Hall’s parameter was calculated and the results are shown in Table 5-4, confirming the applicability of the Langmuir isotherm for the system employed here.
5.6. Core-shell particle characterisation

5.6.1. Visual confirmation of a successful coating

In order to visually confirm the successful sorption of the temperature responsive polymer onto the alginate scaffold, photographs of the both flat membranes and spherical micro-particles were taken at room temperature, followed by increasing the temperature to 40°C as shown in Figure 5-22. A change in opacity was observed when increasing the temperature, from transparent and near colourless to opaque (milky white), confirming the phase transition characteristic of the temperature responsive polymer suggesting its presence on the produced alginate membranes and particles.
CHAPTER 5. CONTROLLED CORE-SHELL PARTICLE PRODUCTION

Figure 5-22. Photographs of pNIPAM-coated alginate A) membranes; B) particles, below and above the phase transition temperature.
5.6.2. SEM coupled with EDX analysis

To further confirm the successful sorption of the polymer, elemental analysis was performed by SEM coupled with EDX and spectra were collected. The elemental analysis is provided in Table 5-5. The EDX data provides the percentage composition of the elements: carbon, oxygen, nitrogen, calcium and sodium measured at the surface of the particle under investigation. In the case of the alginate beads, there is no nitrogen present, as alginate does not contain this element. However, in the case of the pNIPAM coated alginate beads the average weight per cent of nitrogen detected over many tests (at different positions on the surface) is 14%, with a standard deviation of 2.3%. This nitrogen comes from the pNIPAM coating on the alginate beads, which have received repeated washing in water and acetone prior to the EDX tests and is, therefore, firmly attached (probably by chemisorption) onto the alginate bead surface. The significant level of washing can be demonstrated by reference to the sodium content in the two different beads: it is notable that the alginate particles still demonstrate the presence of sodium, which clearly hasn’t been washed out of the beads whereas the pNIPAM coated beads have no measurable sodium present – where the washing must have been more effective.
Table 5-5. Alginate particles and pNIPAM-coated particles analysed by SEM coupled with EDX spectra elemental analysis demonstrating a nitrogen peak present only in the latter material

<table>
<thead>
<tr>
<th>Description</th>
<th>C</th>
<th>O</th>
<th>N</th>
<th>Ca</th>
<th>Na</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alginate beads</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Average weight %</strong></td>
<td>39.6</td>
<td>33.2</td>
<td>0</td>
<td>25.1</td>
<td>1.96</td>
</tr>
<tr>
<td><strong>Standard deviation</strong></td>
<td>5.35</td>
<td>6.95</td>
<td>0</td>
<td>12.3</td>
<td>0.54</td>
</tr>
<tr>
<td><strong>pNIPAM-coated Alginate beads</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Average weight %</strong></td>
<td>55.3</td>
<td>27.0</td>
<td>14.3</td>
<td>3.40</td>
<td>0</td>
</tr>
<tr>
<td><strong>Standard deviation</strong></td>
<td>2.27</td>
<td>1.12</td>
<td>2.33</td>
<td>1.33</td>
<td>0</td>
</tr>
</tbody>
</table>

5.6.3. FT-NIR spectrums collection

In order to further investigate the chemisorption of pNIPAM onto calcium alginate particles, FT-NIR spectra were collected and compared to the individual components of the core-shell structure (e.g. calcium alginate and pNIPAM), as shown in Figure 5-23. For pNIPAM, the overtone and combination bands of the CH groups would be expected to appear in the regions 6000–5700 cm⁻¹ and 4500–4000 cm⁻¹, while those for the amide groups would be mostly located in the regions 6900–6100 cm⁻¹ and 5000–4500 cm⁻¹ (Sun B. et al., 2007). The spectra obtained for the pNIPAM-coated alginate particles records the presence of additional bands in the region of 6000 – 5500 cm⁻¹ compared to calcium alginate particles. These additional bands indicate that structural and conformational changes incurred in the analysed sample post coating and could be attributed to the combination bands of CH groups and amide groups that are only present in the pNIPAM structure. Thus, the FT-NIR spectra acquired could be an indication of the successful pNIPAM coating of the alginate
core beads. This technique alone is not sufficient to firmly conclude that the successful coating has been achieved, but combined with the data obtained by SEM coupled with EDX (section 5.6.2) and visual confirmation of structural changes in response to temperature (section 5.6.1), a strong indication of a successful coating was implied.

Figure 5-23. FT-NIR spectrums of pNIPAM-coated Alginate particles compared to alginate and pNIPAM particles.

5.6.4. Particle shrinkage ratio as a response to temperature variation

An important characteristic of pNIPAM is its phase transition with temperature increase. Below its transition temperature, pNIPAM is hydrophilic and the temperature responsive hydrogels are fully swollen, owing to the strong interactions between the polymer chains and the water molecules, while above the phase transition temperature, it becomes hydrophobic and the hydrogels shrink by expelling the water from their structure. This
behaviour has an effect on particle size by inducing particle shrinkage with implications in the surface area provided for cell adhesion and proliferation.

The method used for measuring particle size employs laser diffraction when using the Mastersizer (Malvern Instruments, UK). Unfortunately, this device only allows particle size analysis at room temperature (below LCST of pNIPAM). In order to assess particle size above LCST, ImageJ software was employed for image analysis described in detail in Appendix 2. B1. Briefly, phase contrast images were acquired with an inverted microscope (Nikon Eclipse Ti) when using a Nikon heating stage (Linkam DC60 Control Unit) and further analysed with ImageJ software. At the end of the analysis, the mean diameter is further used to calculate the volume of the particles below and above the transition temperature by using equation [5-4].

\[
V = \frac{\pi D_m^3}{6} \quad [5-4]
\]

Where: \(V\) represents the particle volume (\(\mu m^3\)) and \(D_m\) represents the particles Feret diameter (\(\mu m\)).

In order to determine how much the particles shrink with temperature increase, the volume ratio of the particles below and above the phase transition temperature was calculated [5-5].

\[
Shrinkage\ ratio = \frac{V_{\text{shrunken}}}{V_{\text{swollen}}} = \left(\frac{D_{\text{shrunken}}}{D_{\text{swollen}}}\right)^3 \quad [5-5]
\]

Where: \(D_{\text{shrunken}}\) represents the Feret’s diameter of particles in the shrunken state (at 38°C) and \(D_{\text{swollen}}\) is the Feret diameter of particles in the swollen state (at 23°C).
Following Image J analysis, for 60 particles, the Feret’s diameter was measured below and above the phase transition temperature and the mean diameter values are provided in Table 5-6.

Table 5-6. Shrinkage ratio of pNIPAM-coated alginate particles calculated based on Feret’s diameter obtained from Image J analysis of 60 particles at a temperature below (23°C) and above LCST (38°C)

<table>
<thead>
<tr>
<th></th>
<th>Below LCST</th>
<th>Above LCST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Feret’s diameter / µm</td>
<td>209</td>
<td>202</td>
</tr>
<tr>
<td>Standard deviation / µm</td>
<td>51</td>
<td>47</td>
</tr>
<tr>
<td>Volume / µm³</td>
<td>4.78 x 10⁶</td>
<td>4.31 x 10⁶</td>
</tr>
<tr>
<td>Shrinkage ratio</td>
<td></td>
<td>0.90</td>
</tr>
</tbody>
</table>

Based on the Image J analysis, the pNIPAM-coated Alginate particles experience volume shrinkage of 10% when the temperature is set above the polymer’s phase transition value. This behaviour was expected and it can be explained by the changes occurred during the pNIPAM phase transition from the coil to the globule transition.

To compare with the Image J method, particle size was also measured below phase transition temperature by employing laser diffraction and the results are compared and shown in Table 5-7. A small particle size difference was observed when comparing the two methods. This can be explained based on the number of particles analysed for each method and the different analytical techniques used by the separate methods.
Table 5-7. Particle size comparison between laser diffraction method and Image J picture analysis. The measurements for both methods were performed at room temperature (23°C) below polymer’s phase transition temperature

<table>
<thead>
<tr>
<th>Method</th>
<th>D (0.1) / µm</th>
<th>D (0.5) / µm</th>
<th>D (0.9) / µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laser diffraction method</td>
<td>121</td>
<td>217</td>
<td>397</td>
</tr>
<tr>
<td>Image J analysis</td>
<td>121</td>
<td>209</td>
<td>334</td>
</tr>
</tbody>
</table>

5.6.5. Wettability of pNIPAM-coated Alginate

Surface wettability studies usually involve the measurement of contact angles as the primary data, which indicates the degree of wetting when a solid and liquid interact. Small contact angles (< 90°) correspond to high wettability, while large contact angles (> 90°) correspond to low wettability (Yuan Y. and Lee T.R., 2013). In respect to cell adhesion to artificial substrates, Tamada Y. et al. (1986) found that surfaces with water contact angles of 70° gave the most suitable surface for cell adhesion. In regards with the pNIPAM synthetic surface, because of its temperature dependency, its water contact angle changes in response to increasing temperature. In this work, the static water contact angle (WCA) of the pNIPAM-coated alginate film was measured only at room temperature. The static WCA value for the calcium alginate film could not be measured accurately as no settling drop was obtained as shown in Figure 5-24. Referring to the specific applicability of these core-shell temperature responsive particles for cell culture and release, WCA of the pNIPAM-coated
Alginate surface was compared to the values recorded for regular TCPS. The contact angle of TCPS is known to facilitate cell adhesion and proliferation and it was employed in this study as a positive control. The value obtained for TCPS as a result of 9 different measurements was 99.52° which is higher than the reported optimal value for cell adhesion of 70° (Tamada Y. et al., 1986). However, TCPS was found to be effective for 3T3 cell culture (section 3.5.1). As a different perspective, the core-shell surfaces have temperature responsive properties due to pNIPAM as shown in section 5.6. Commercially available temperature responsive surfaces that support non-enzymatic harvesting of adherent cells for preservation of cell viability and surface proteins are available as two dimensional surfaces under the name of UpCell dish (NUNC, Thermo Scientific).

Figure 5-24. Water Contact Angle images recorded for calcium alginate films compared to Tissue Culture Treated Plastic and commercially available temperature responsive surface UpCell (NUNC). Data expressed as mean ± SD.
5.7. Conclusions

Spherical monodisperse alginate particles with median sizes controllable within the range of 55 to 650 µm and spans as low as 0.2, were successfully produced by membrane emulsification using the stirred cell system. In this system a simple paddle stirrer provides the shear at the surface of the membrane to detach the drops which are then crosslinked to form particles. Several formulations have been previously reported, but they all involved the use of toxic chemicals that can have a harmful effect on cultured cells. As an improvement, the formulation used in this work for the production of alginate particles avoided non-GRAS chemicals by only using food grade and pharmaceutical grade reagents.

As a different approach, it was possible to successfully correlate the drop size formed using the system with a published model for membrane emulsification, relating drop size to shear stress at low dispersed phase injection rates. A key feature of the emulsification process employed is the membrane pore structure; which is an array of highly uniform pores that pass straight through the membrane with no internal tortuosity. Hence, it was possible to pass suspended solids through the membrane with minimal risk of blockage, as would be expected when using microfiltration style membranes for membrane emulsification. The preferred formulation facilitated the possibility of using the internal gelation method in membrane emulsification processes in a process that can scale to levels of production that are of commercial interest.

Inevitably, there has to be a balance between pore size, concentration of solids in the injected phase and the injection rate; as a poor choice can lead to membrane blockage of even the sieve-type of membrane used. Tests with membrane pore sizes of 5 and 10 µm were unsuccessful at any injection rate whilst using a calcium carbonate (median size 2.3 µm) concentration suitable for internal gelation (0.5% wt or more). However, when using a 20 µm pore size membrane no blockage was noticed at any of the operating conditions of
injection rate or dispersed phase concentration. By controlling the stirring speed and the injection rate of the dispersed phase through the membrane pores, it was possible to achieve precise control over particle size and size distribution. The alginate beads generated by this form of membrane emulsification are in a size range of interest as scaffolds for various biopharmaceutical applications, including microcarriers for cell culture, where some form of functionalising or encapsulating medium can be grafted onto the surface of the alginate. The presence of naturally occurring carboxylic acid groups at the alginate surface was exploited to provide chemisorption sites onto which cationic groups can be attached. This was demonstrated by the successful chemisorption of modified pNIPAM where the amine groups provide the link to the carboxylic acid groups to form a coating of pNIPAM around the alginate particle. The amounts of polymer adsorbed onto the calcium alginate beads were measured successfully and it was demonstrated to fit a Langmuir type isotherm. The successful coating of this temperature responsive polymer and the maintenance of its temperature responsive properties was demonstrated by visual assessment of the particle surface at temperatures above and below the critical transition temperature, as well as SEM coupled with EDX analysis of the coated particle surface and wettability and volume shrinkage studies, all demonstrating the potential of these particles to be used as supports for non-enzymatic harvesting of adherent cells.
6. EVALUATION OF PRODUCED PARTICLES FOR CELL ATTACHMENT AND PROLIFERATION CAPABILITIES

6.1. Introduction

There is an increased demand in biotechnology-related fields to find materials that not only are compatible with cells, but also allow control over cell adhesion on the provided substrates. Cell adhesion is determined by the interaction between cell membrane-embedded proteins and the underlying substrate. Different cells prefer different surfaces depending on which adhesion proteins are expressed at the outer layer of the membrane envelope. However, there will be a general purpose surface suitable for most commonly cultured cell types. Several material characteristics have been identified as being critical for cell adhesion and proliferation, for example, texture, water content, rigidity, hydrophobicity, layer thickness etc. (Ratner B.D. et al, 2004A; DaSilva R.M.P et al, 2007; Biazar E. et al, 2013).

It is well known that cell adhesion defines cell proliferation on the provided substrates (Grinnell F., 1980). The study reported here will refer to the use of a temperature responsive material, pNIPAM with remarkable potential to be used as a substrate for cell culture and as a major advantage, to be used for non-invasive and non-destructive temperature-induced cell harvest (DaSilva R.M.P et al, 2007; Schmidt S. et al, 2010; Tang Z. et al, 2012). Several studies showed that some surfaces based on pNIPAM alone do not support cell adhesion even above its phase transition temperature (Akiyama Y. et al., 2004; Yamato M. et al., 2003) when the surface is hydrophobic in nature. Critical layer thickness for cell adhesion was found to be between 20 nm and 30 nm (Yamato M. et al., 2001; Shiroyanagi Y. et al., 2003; Harimoto M. et al., 2002; Nandkumar M.A. et al., 2002). Values higher than 30 nm were found to be inefficient in promoting cell adhesion (Akiyama Y. et al. 2004; Yamato M. et al., 2003).
This chapter will determine whether the in-house produced temperature-responsive particles allow cell adhesion and proliferation during routine culture at 37°C. The morphology of the cells adhered on these substrates was assessed, as well as cell growth and viability defined as membrane integrity. Cell adhesion and growth on the assessed substrates was improved by employing proteins known to promote cell adhesion and proliferation.

6.2. Aims and objectives

The main aim of this chapter was to evaluate the suitability of the in-house produced temperature responsive particles as microcarriers with emphasis on the ability of cells to adhere and proliferate on their surface when using commercially available microcarriers (e.g. GE Healthcare Cytodex-1) as positive controls. This was done by completing the following objectives:

- Evaluation of the suitability of pNIPAM particles of different chemical compositions to be used as cell culture substrates by monitoring cell morphology, viability and growth on the provided surfaces
- Qualitative and quantitative evaluation of cell growth on the provided temperature responsive substrates
- Improving cell adhesion and growth by employing and comparing different protein coatings
6.3. Experimental procedure

3T3 fibroblasts at passage 18 were seeded onto provided microcarriers at an initial cell seeding density of 0.5x10^5 cells/well. Triplicate wells were seeded for each type of microcarrier provided. Several wells were sacrificed at different time points for analysis. Complete growth medium changes were performed every three days. Prior to cell seeding, the provided sterile microcarriers were initially seeded in Ultra Low Attachment 12 well-plates (ULA 12) and microcarrier cell culture was carried out following the procedure described in section 3.6.2.

Due to the severe deformation and structure collapse encountered during particle drying procedures (refer to section 4.4.1.1. for pNIPAM solid particles and Figure 5-1 in section 5.3. for pNIPAM-coated Alginate particles), the specific surface area of either type of particles could not be determined. In respect to this, the generated particles were only partially dried and then re-suspended in complete growth medium to achieve a concentration of 30% wt. One mL of 30% wt microcarrier suspension was then seeded per well in the ULA plates. The volume per well was then adjusted to a total volume of 2 mL by adding 1 mL of complete growth medium. The seeded particles were conditioned for at least 1 hour prior to cell culture in an incubator at 37ºC and 5% CO₂. After the conditioning step, the plates were tilted and particles were let to settle for 2 minutes, after which the medium was aspirated with increased attention to minimise loss of microcarriers. A negative control consisting of wells not containing any microcarriers was present on each of the ULA plates set up for the experiments. The purpose of the negative control was to ensure that the ULA plates were efficient at inhibiting unwanted cell attachment on the well surface, thus allowing only cell attachment to the provided substrates (i.e. microcarriers). The ULA plates are specifically designed for this purpose by being coated with a hydrogel.
The microcarrier analysis focused mainly on cell proliferation and was determined by both qualitative and quantitative methods. The qualitative analysis involved acquiring representative phase-contrast images at different time points. Several wells were sacrificed at different time points for a second qualitative analysis method based on fluorescent staining of cells (Live/Dead Viability / Cytotoxicity kit; Invitrogen, UK). This method in conjunction with phase-contrast images provided an insight into cell morphology and proliferation. On a different approach, quantitative data offering an insight into the extent of cell proliferation on the provided substrates was collected based on the Presto Blue cell viability assay (Life Technologies, UK) which provides a measure of the metabolic activity of the viable cells. This specific analytical technique is the most prone to error as it can be easily influenced by background fluorescence. To address this possible issue, background fluorescence was minimised by utilising black well plates with a clear bottom for the fluorescence measurements performed with a microplate reader. It was expected that the combination of these techniques, qualitative and quantitative, would corroborate each other and provide valuable information on the extent of cell proliferation on the provided substrates compared to commercially available substrates already proven to have great cell proliferation promoting abilities.

6.4. Evaluation of cell proliferation on pNIPAM solid microcarriers

Even though over the last decade an increased interest was shown to pNIPAM hydrogels due to their remarkable potential in cell sheet engineering promising a simple and attractive method of cell harvesting, unfortunately several studies showed that bulk pNIPAM coatings display resistivity to cell adhesion even above LCST when they are hydrophobic in nature, allowing only a small number of cells to adhere and proliferate (Akiyama Y. et al, 2004;
Yamato M. *et al.*, 2003; Nash M.E. *et al.*, 2012; Matsuda N. *et al.*, 2007). The same behaviour is expected on this type of in-house produced microcarriers (refer to Chapter 4) due to previous studies reporting the critical importance of the pNIPAM coating thickness (Yamato M. *et al.*, 2001; Shiroyanagi Y. *et al.*, 2003; Harimoto M. *et al.*, 2002; Nandkumar M.A. *et al.*, 2002). This section of the chapter was therefore focused on evaluating cell adhesion and proliferation on the in-house produced pNIPAM solid particles. The investigation consisted initially in identifying which of the particle chemical compositions would best facilitate cell proliferation by employing both qualitative and quantitative analytical techniques. Following this study, a selection was made. All experiments preceding this study were performed by using the selected composition.

In this study, cell expansion has been expressed as fold-increase defined as [eq. 6-1]:

\[
\text{Fold Increase} = \frac{C_x(f)}{C_x(0)},
\]

Where \(C_x(f)\) is the final viable cell number at the end of the passage and \(C_x(0)\) is the initial viable cell number.

### 6.4.1. Comparison of different chemical composition particles

3T3 fibroblasts were seeded onto various pNIPAM microcarriers or Cytodex-1 microcarriers within ULA plates and cultured over 216 hours (see Chapter 4 for synthesis). Table 6-1 provides the main characteristics of the pNIPAM solid particles employed in this study. As a positive control for cell proliferation, Cytodex-1 (GE Healthcare) was employed. Cytodex-1 microcarriers provide a positively charged dextran solid base and they have been
widely used for the successful expansion of different types of cells, such as chondrocytes (Cetinkaya M. et al., 2011), mesenchymal stem cells (Schop D. et al., 2010; Weber C. et al., 2007) or even human embryonic stem cells (Chen A. K-L. et al., 2011). Therefore, cell adhesion and proliferation in the culture conditions presented here was expected on Cytodex-1 with its characteristics detailed in section 3.6.1.3.

Table 6-1. Detailed characteristics of the utilised pNIPAM solid particles

<table>
<thead>
<tr>
<th>Nr.crt.</th>
<th>Notation</th>
<th>Monomer (NIPAM) concentration / % wt</th>
<th>Crosslinker (MBA) concentration / % wt</th>
<th>Median Particle size / µm</th>
<th>Size distribution expressed as SPAN</th>
<th>Volume Shrinkage Ratio (refer to section 4.4.5.2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5-0.2</td>
<td>5</td>
<td>0.2</td>
<td>81</td>
<td>0.46</td>
<td>0.75</td>
</tr>
<tr>
<td>2</td>
<td>5-0.4</td>
<td>5</td>
<td>0.4</td>
<td>65</td>
<td>0.99</td>
<td>0.75</td>
</tr>
<tr>
<td>3</td>
<td>5-0.8</td>
<td>5</td>
<td>0.8</td>
<td>43</td>
<td>0.81</td>
<td>0.7</td>
</tr>
<tr>
<td>4</td>
<td>5-1%</td>
<td>5</td>
<td>1</td>
<td>53</td>
<td>0.74</td>
<td>0.6</td>
</tr>
<tr>
<td>5</td>
<td>10-1%</td>
<td>10</td>
<td>1</td>
<td>52</td>
<td>0.65</td>
<td>N/A</td>
</tr>
</tbody>
</table>

In regards to which of the different chemical composition particles best facilitated cell expansion, viable cell number at different time points (e.g. 24, 96, 168 and 216 hours in culture) was obtained from the Presto Blue assay by measuring ATP-dependent metabolic activity of the cells. The data generated by the assay was then normalised to give a viable cell density using a pre-constructed calibration curve generated with adherent 3T3s (refer to Appendix 1.A3) and analysed for statistical significance by using a mixed-ANOVA method. Figure 6-1 provides the viable cell number generated in static culture on the pNIPAM particles.
particles at different time points. When evaluating the cell growth performance, a significant
difference (p=0.0005<0.05) was determined between the different substrates. As expected,
Cytodex-1 microcarriers generated the largest number of viable cells with a continuous
growth over 216 hours of static culture. In regards to the studied temperature responsive
microcarriers, cell growth was recorded up to 96 hours in culture, followed by a decrease in
cell number.

This decrease in viable cell number could be an indicator of possible cell death occurring
after 96 hours in culture. In this study, the method employed only permits determination of
viable cell number as it provides a measure of the metabolic activity of live and healthy cells.

Previous studies have demonstrated that the NIPAM monomer exhibits cytotoxicity, but
regarding the polymerised form of NIPAM (pNIPAM) there are conflicting opinions
(Cooperstein M.A. et al, 2013). To address this issue, Cooperstein M.A. et al (2013) have
studied the cytotoxicity of pNIPAM produced by different polymerisation (e.g. free radical
polymerisation, plasma polymerisation) and deposition methods (e.g. spin coating, plasma
polymerisation) when employing several different cell lines, including 3T3 fibroblasts. Even
though in their study, it was found that the 3T3 fibroblast cell line was the most robust and
less sensitive to surface chemistry, the author did underline that cell viability decreased after
48 hours when exposed or in direct contact to the polymer at 37ºC and it could be a
consequence of the polymer’s long term cytotoxicity effect possibly achieved through
polymer fragments leaching out of the substrate into the cell culture medium (Cooperstein
CHAPTER 6. EVALUATION OF PRODUCED PARTICLES FOR CELL ATTACHMENT AND PROLIFERATION CAPABILITIES

Figure 6-1. Viable cell number of 3T3 fibroblasts at passage 18 seeded on pNIPAM solid particles with different chemical compositions and kept in static culture up to 216 hours (equivalent of 9 days) (Mean value ± SD; n=3). Statistical analysis performed by mixed-ANOVA (Within groups, p=0.002<0.05; Between groups, p=0.0005<0.05).

In respect to cell expansion, Cytodex-1 microcarriers had the best performance compared to the provided in-house temperature responsive particles with viable cell numbers reaching up to 2.89 x10^5 cells at 216 hours, the equivalent of a 5.8 fold increase. In general, the cell expansion data obtained was reproducible indicated by the error bars. Table 6-2 provides cell expansion data at 96 hours and 216 hours in culture compared to Cytodex-1, marking the time point when polymer’s cytotoxicity determined a decrease in viable cell numbers. Up to 96 hours in culture, all in-house pNIPAM microcarriers except 5-0.2 and 5-0.4 achieved cell expansions expressed as fold increase ranging from 3.1 to 4.1 which is similar to Cytodex-1 (~ 3.3 fold) compared to 216 hours in culture when the fold increase ranged
CHAPTER 6. EVALUATION OF PRODUCED PARTICLES FOR CELL ATTACHMENT AND PROLIFERATION CAPABILITIES

from 1.8 to 2.2 compared to 5.8 fold achieved by Cytodex-1. In accordance with the cytotoxicity study performed by Cooperstein M.A. et al (2013), this decrease in cell expansion could be attributed to cytotoxicity increase due to possible polymer and even monomer leaching from the in-house produced particles into the growth medium.

Table 6-2. Expansion data expressed as fold increase at 96 hours and 216 hours in culture for in-house produced pNIPAM particles of different chemical compositions compared to commercially available Cytodex-1

<table>
<thead>
<tr>
<th>Microcarriers</th>
<th>Fold increase (Mean ± SD; n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>96 hours</td>
</tr>
<tr>
<td>In-house produced pNIPAM</td>
<td></td>
</tr>
<tr>
<td>5-0.2</td>
<td>3.1 ± 0.4</td>
</tr>
<tr>
<td>5-0.4</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>5-0.8</td>
<td>3.7 ± 2.5</td>
</tr>
<tr>
<td>5-1</td>
<td>4.1 ± 0.6</td>
</tr>
<tr>
<td>10-1</td>
<td>3.1 ± 0.9</td>
</tr>
<tr>
<td>Commercially available</td>
<td>Cytodex-1</td>
</tr>
<tr>
<td></td>
<td>3.3 ± 1.2</td>
</tr>
</tbody>
</table>

The negative control used in this study and for all experiments preceding this study consisted of a well in the ULA plate with no microcarriers and cells seeded. The ULA plate was specifically designed to not allow cell attachment by being coated with a hydrogel (as according to manufacturer). In regards to this, no or little cell attachment was expected, as shown in Figure 6-1. The lack of cell attachment to the wells of the ULA plate and the cell proliferation on the provided substrates was corroborated by phase contrast images acquired after 96 hours in culture provided in Figure 6-2.
Figure 6-2. Phase contrast images of 3T3 cells on in-house produced pNIPAM solid particles of different chemical compositions after 96 hours in culture: A) 5-0.2; B) 5-0.4; C) 5-0.8; D) 5-1; E) 10-1; F) Cytodex-1; G) Negative control. Scale bars represent 100 µm (G - 500 µm).
CHAPTER 6. EVALUATION OF PRODUCED PARTICLES FOR CELL ATTACHMENT AND PROLIFERATION CAPABILITIES

The viable cell numbers obtained for the negative control (as shown in Figure 6-1) have set the benchmark for poor cell proliferation. Similar low values were obtained after 96 hours in culture by the 5-0.4 microcarriers with values below the initial cell seeding density (at 0 hours). In regards to this, 5-0.4 particles were not considered for further investigations. Of the remaining microcarriers, the best viable cell numbers maintained after this time point were attributed to the 5-0.8, 5-1 and 10-1 microcarriers. Referring to Figure 6-1, it can be observed that the highest variability obtained was for cell growth on 5-0.8 particles at 96 hours in culture. Taking this into consideration, the best cell expansion performance was achieved when employing the 5-1 and 10-1 temperature responsive particles and these were selected for further studies.

To support these observations, fluorescence images of the cell-microcarrier constructs stained with Live/Dead staining kit were acquired at 216 hours in culture and are provided in Figure 6-3. Live and healthy cells emitted green fluorescence, while dead or damaged cells emitted red fluorescence. The morphology of cells cultured on either Cytodex-1 microcarriers or pNIPAM microcarriers were typical of 3T3 fibroblasts cultured on TCPS plates in 2D culture. As expected in static culture conditions, during cell expansion, bridges between microcarriers were created (Boris M.C. and Papoutsakis E.T., 1992) resulting in different sized agglomerates of cell-microcarrier constructs, as shown in Figure 6-3. This phenomenon is expected to be minimised with appropriate agitation in a stirred tank bioreactor environment.
### Figure 6-3. Live/Dead staining of 3T3 fibroblasts after 216 hours of culture on:

- **A)** 5-0.8; **B)** 5-1; **C)** 10-1; **D)** Cytodex-1 microcarriers; Scale bars represent 100 μm.

<table>
<thead>
<tr>
<th>Dead</th>
<th>Live</th>
<th>Merged</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="A" alt="Image" /></td>
<td><img src="B" alt="Image" /></td>
<td><img src="C" alt="Image" /></td>
</tr>
<tr>
<td><img src="D" alt="Image" /></td>
<td><img src="D" alt="Image" /></td>
<td><img src="D" alt="Image" /></td>
</tr>
</tbody>
</table>

Figure 6-3. Live/Dead staining of 3T3 fibroblasts after 216 hours of culture on: A) 5-0.8; B) 5-1; C) 10-1; D) Cytodex-1 microcarriers; Scale bars represent 100 μm.
6.4.2. Improvements in cell adhesion and growth

Ideally cell culture substrates (e.g. scaffolds, microcarriers, membranes etc.) should be biocompatible, non-toxic and allow and promote cell adhesion and growth. Often the achievement of all these characteristics can be challenging. In regards to section 6.4.1., I have shown that some pNIPAM particles permit cell adhesion and proliferation over 96 hours without compromising cell integrity. Their performance was dependent on chemical composition, but compared to a Cytodex-1, their cell expansion performance was inferior by 216 hours in static culture.

Different approaches for cell adhesion and proliferation enhancement were explored previously (Moran M.T. et al, 2007; Moran M.T. et al., 2007A; Hatakeyama H. et al, 2006). A common technique consists in pre-coating biomaterials with extracellular matrix (ECM) proteins (e.g. fibronectin, collagen, laminin) or ECM-derived adhesion molecules (e.g. gelatine, adhesion peptides etc.) (Moran M.T. et al, 2007; Moran M.T. et al., 2007A; Hatakeyama H. et al, 2006). For example, Moran M.T. et al. (2007 and 2007A) demonstrated enhanced cell attachment and growth of fibroblasts and epithelial cells on protein-coated pNIPAM copolymer films almost comparable to generally used TCPS. The presence of the proteins did not render nor affect cell detachment as intact cell sheets. For future reference, the method employed by Hatakeyama H. et al (2006) involving the use of cell adhesive peptides to modulate cell interactions might be more suitable if clinical applications for the produced microcarriers are considered. By employing synthetic peptides, exposure to animal-derived pathogens that might be present in ECM proteins could be avoided.

Based on the assumption that cell growth enhancement could be achieved by employing ECM products, the in-house produced temperature responsive particles (5-1 and 10-1)
selected (see section 6.4.1) were coated with different cell adhesion promoters such as porcine gelatine (Sigma Aldrich, UK) and Matrigel basement membrane matrix (BD Biosciences, UK). The selection of the adhesion promoters was done based on their performance and taking into considerations cell culture costs. Matrigel is a solubilised basement membrane extracted from Engelbreth-Holm-Swarm mouse sarcoma, a tumour rich in extracellular matrix proteins. Its major component is laminin, followed by collagen IV, entactin and heparan sulfate proteoglycan (Kleinman H.K. et al, 1982). Gelatine is produced by partial hydrolysis of type I collagen extracted from connective tissues. Compared to Matrigel which is expensive and tricky to use due to its temperature sensitivity, gelatine is readily available, inexpensive and easy to use. In regards to performance, Matrigel allows a more even cell distribution upon initial seeding with enhanced cell growth compared to gelatine (Danoviz M.E. et al, 2012). The adhesion promoters were physically bound to the surface of the investigated substrates by following the protocol described in section 3.6.1.1.

Based on previous studies (Moran M.T. et al, 2007; Moran M.T. et al., 2007A), it was expected that enhanced cell proliferation would be achieved. Table 6-3 provides cell expansion data expressed as fold increase at two different time points in static culture. The performance of the microcarriers coated with the selected adhesion promoters was evaluated and compared to uncoated. The commercially available Cytodex-1 regardless of the coating generally achieved the best cell expansion at both time points in culture up with a maximum of 9.9-fold increase after 96 hours using Matrigel-coated Cytodex-1. Of the pNIPAM microcarriers tested, the 10-1 variant was the best performing, supporting a 5.2-fold expansion of 3T3 fibroblasts after 96 hours when coated with Matrigel (compared with 4.4-fold and 3.1-fold for gelatin-coated and uncoated microcarriers respectively). On the other hand, at 216 hours in culture, a maximum 4.6-fold was achieved when gelatine-coated compared to 4.5-fold when coated with Matrigel or 2.2-fold when uncoated, as shown in
Table 6-3. To corroborate these results, Figure 6-4 provides viable cells numbers obtained at 96 and 216 hours in static culture when employing different cell adhesion promoters. Statistical significance was determined by mixed-ANOVA method. At 96 hours in culture, a significant difference in viable cell numbers obtained on the different substrates tested was determined ($p=0.001<0.05$) with Cytodex-1 performing the best. When different protein coatings were employed, a significant difference ($p=0.0005<0.05$) in viable cell number was also recorded suggesting that an improved cell growth was achieved when employing protein coatings (Figure 6-4A). However, later in the culture, at 216 hours, no significant difference ($p=0.792>0.05$) in viable cell numbers was recorded on the different substrates, but a significantly improved cell growth was still achieved when employing different protein coatings ($p=0.004<0.05$), as shown in Figure 4-6B. The obtained data was relatively reproducible given the tight error bars as shown in Figure 6-4.
Table 6-3. Expansion data expressed as fold increase at 96 hours and 216 hours in culture for the prior selected in-house produced pNIPAM particles compared to commercially available Cytodex-1 microcarriers when employing different cell adhesion promoters (e.g. Matrigel, gelatine)

<table>
<thead>
<tr>
<th>Microcarriers</th>
<th>Time points</th>
<th>Fold increase (Mean ± SD; n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Uncoated</td>
</tr>
<tr>
<td>In-house produced pNIPAM</td>
<td>5-1%</td>
<td>96 h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>216 h</td>
</tr>
<tr>
<td></td>
<td>10-1%</td>
<td>96 h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>216 h</td>
</tr>
<tr>
<td>Commercially available</td>
<td>Cytodex-1</td>
<td>96 h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>216 h</td>
</tr>
</tbody>
</table>

Live/Dead staining was also performed on the microcarriers when cell adhesion promoters were employed to confirm that the cells adhered to the coated microcarriers are physically intact, as shown in Figure 6-5. Similar to uncoated microcarriers, at 216 hours in culture fibroblasts showed a tendency to aggregate with adjacent cells thus forming ‘bridges’ between the provided surfaces (Figure 6-5).
Figure 6-4. Viable cell number of 3T3 fibroblasts at passage 18 seeded onto pNIPAM solid particles coated with different cell adhesion promoters and kept in static culture for: A) 96 hours; B) 216 hours (Mean value ± SD; n=3). Statistical significance was assessed by mixed-ANOVA.
Figure 6-5. Live /Dead staining of 3T3 fibroblasts after 216 hours of culture on: A) 5-0.1; B) 10-1; C) Cytodex-1 microcarriers coated with different cell adhesion promoters. Only merged images of the two fluorescence channels are depicted, where live and healthy cells expressed green fluorescence, while dead or damaged cells expressed red fluorescence. Scale bars represent 100 µm.
6.5. Evaluation of cell attachment and proliferation on core-shell temperature responsive microcarriers

Previous studies using 2D surfaces (*i.e.* not microcarriers) suggested that pNIPAM layer thickness plays a major role on cell adhesion and proliferation (Yamato M. *et al.* 2001; Shiroyanagi Y. *et al.* 2003; Harimoto M. *et al.* 2002; Nandkumar M.A. *et al.* 2002). In respect to this finding, the second type of in-house produced temperature responsive microcarriers (refer to Chapter 5) were generated with a core-shell structure. Calcium alginate was used as a core, while the pNIPAM shell was physically deposited by simply taking advantage of the electrostatic interactions between carboxylic groups of alginate and amino groups of the temperature responsive polymer. This reproducible and simple method ensured the generation of a pNIPAM monolayer coating. In the light of the assumption that the electrostatic deposition produced a pNIPAM layer within the 20-30 nm range, cell adhesion and proliferation promoting abilities were investigated and evaluated in comparison with the commercially available microcarriers (Cytodex-1). The ability of coatings (*e.g.* gelatine) to enhance cell growth potential of core-shell pNIPAM microcarriers was also investigated.

6.5.1. Qualitative and quantitative evaluation of cell growth

The focus of this study was to investigate and assess cell adhesion and growth on the in-house produced pNIPAM-coated Alginate (p/Alg) microcarriers (refer to Chapter 5). Qualitative analysis was performed by acquiring representative phase contrast images at different time points. Quantitative analysis involved the measurement of the viable cell number based on a pre-constructed Presto Blue calibration curve (refer to Appendix 1.A3),
as well as metabolic activity measurement. The Presto Blue data was employed for quantifying cell adhesion and evaluating growth enhancement when employing protein coatings (refer to section 6.5.2). With respect to metabolic activity measurements, glucose and lactate concentrations were measured at different time points in static culture. The generated data would provide an insight in cell proliferation on the provided surfaces given that cell growth is closely related to nutrient consumption (e.g. glucose) and metabolite production (e.g. lactate) (Tsao Y-S. et al, 2005). Greater consumption of glucose and greater production of lactate is an indicator of cell proliferation on the provided substrates. As previously shown by Tsao Y-S et al (2005), periodic medium changes are of great importance for cell growth as it provides the cells with replenished nutrients, while avoiding the accumulation of toxic metabolites in culture.

In regards to this study, in general the glucose concentration was lower and the lactate concentration was greater when measured at different time points compared to the negative control sample represented by fresh complete growth medium that hasn’t been in contact with any cells, as shown in Figure 6-6.
CHAPTER 6. EVALUATION OF PRODUCED PARTICLES FOR CELL ATTACHMENT AND PROLIFERATION CAPABILITIES

Figure 6-6. Glucose (A) and Lactate (B) concentrations in consumed growth medium when 3T3 fibroblasts were kept in static culture for up to 168 hours. Presented data points represent the mean metabolite concentration ± standard deviation (n=3). Statistical significance was assessed by mixed-ANOVA method.
Figure 6-6 provides the recorded glucose (Figure 6-6A) and lactate (Figure 6-6B) concentrations at different time points in culture compared to negative and positive controls. The generated data showed low variance as given by the tight error bars (Figure 6-6). The glucose concentration decreased over time, while the lactate concentration in the consumed medium exhibited an increase over longer culture periods. For the glucose concentration measurements, it was found the interactions between substrates and the different time points was not significant (p>0.05) suggesting that any substrate effect on glucose concentration is the same regardless of the time point. However, when lactate concentration was measured, the substrate effect was different at the time points selected (p=0.005<0.05). In comparison with Cytodex-1, the 3T3s cultured on the core-shell microcarriers showed significantly lower glucose consumption (p=0.004<0.05) and significantly lower lactate production (p=0.0005<0.05). When compared to the negative control, higher glucose consumption and higher lactate production was recorded. These results suggested that cell growth was achieved on the core-shell microcarriers, but it was significantly lower compared to the commercially available substrate.

To support these findings, phase contrast images of the 3T3 cells seeded on pNIPAM-coated Alginate microcarriers were acquired at different time points in static culture (Figure 6-7). At 48 hours in culture, 3T3 cells exhibited a spread and flattened morphology typical to adhered cells (Reiter T. *et al.*, 1985). By 72 hours in culture, cell growth was observed and by 120 hours in culture, confluency was achieved on some of the microcarriers. Cells were not evenly distributed across the microcarriers, which was attributed to a lack of mixing. Adhered cells were stained using a Live/Dead staining kit (Invitrogen, UK) to confirm that adhered cells retained their membrane integrity (Figure 6-8). Cell viability was high as proof the majority of cells expressed bright green fluorescence, as shown in Figure 6-8. Only a
few dead cells (bright red) were observed and they were exhibiting a spherical morphology
typical to cells in suspension (Reiter T. et al, 1985).

Figure 6-7. Phase contrast images acquired at different time points in static culture of 3T3
fibroblasts adhered and grown on A) Cytodex-1 at 120 h or on core-shell microcarriers at B)
48 h; C) 72 h and D) 120 h in culture. Scale bars represent 100 µm.
CHAPTER 6. EVALUATION OF PRODUCED PARTICLES FOR CELL ATTACHMENT AND PROLIFERATION CAPABILITIES

Figure 6-8. Live/Dead staining of 3T3 fibroblasts grown on: A) Cytodex-1; B) pNIPAM-coated Alginate microcarriers at 120 hours in static culture. Scale bars represent 100 µm.
6.5.2. Cell adhesion and growth enhancement

This section was focused on investigating the possibility of cell adhesion and growth enhancement by pre-coating the provided substrates with adhesion promoters such as gelatine (Moran M.T. *et al.*, 2007; Moran M.T. *et al.*, 2007A; Hatakeyama H. *et al.*, 2006). Cell expansion performance was assessed for uncoated and coated core-shell microcarriers and compared to Cytodex-1. Cell expansion data expressed as fold increase at 72 hours and 120 hours in culture is provided in Table 6-4. Gelatine coating improved the cell expansion potential of all microcarriers tested. However, the core-shell microcarriers did not outperform Cytodex-1 microcarriers in terms of cell expansion. After 120 hours in culture, 3T3s cultured on Cytodex-1 generated an 11.6 fold increase, while cells cultured on gelatine-coated core-shell microcarriers 8.6-fold increase, compared to a 7.2-fold increase when cultured on uncoated core-shell, as shown in Table 6-4.

Cell adhesion on a provided substrate is a complex process influenced by numerous aspects, such as cell metabolic processes, material characteristics and environmental factors. In regards to the substrate, the main characteristics that have a great impact on cell attachment comprise of hydrophobicity, surface charge, roughness and chemical composition (Chang H-I. *et al.*, 2011; Nilsson K., 1989). Substrate hydrophobicity can be quantified by measuring the static water contact angles. Maximal fibroblast adhesion was found to be on substrates that have contact angles in the range of 60° to 80° (Tamada Y. and Ikada Q., 1993). To this end, the static water contact angles were measured for the hydrogels corresponding to the in-house produced microcarriers utilising the same chemistries (refer to section 4.4.6. and 5.6.5.). The contact angle could only be measured at room temperature; where the pNIPAM was hydrophilic and therefore unsuitable for cell adhesion. Cell proliferation could also be influenced by the surface charge. Thus the greater cell proliferation on Cytodex-1 could be
CHAPTER 6. EVALUATION OF PRODUCED PARTICLES FOR CELL ATTACHMENT AND PROLIFERATION CAPABILITIES

explained by its positively charged surface given by the presence of the tertiary amino groups in its structure.

Table 6-4. Expansion data expressed as fold increase at 72 hours and 120 hours in culture for the core-shell microcarriers uncoated and coated with gelatine 0.1% wt compared to commercially available Cytodex-1 microcarriers

<table>
<thead>
<tr>
<th>Microcarriers</th>
<th>Time points</th>
<th>Fold increase (Mean ± SD; n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>In-house core-shell microcarriers</td>
<td>Uncoated</td>
<td>72 h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120 h</td>
</tr>
<tr>
<td></td>
<td>Gelatine coated</td>
<td>72 h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120 h</td>
</tr>
<tr>
<td>Commercially available</td>
<td>Cytodex-1</td>
<td>72 h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120 h</td>
</tr>
</tbody>
</table>

To corroborate these findings, viable cell numbers were determined by Presto Blue assay at different time points during a 120 hours culture period. Statistical significance was determined by the mixed-ANOVA method. Cell proliferation expressed as viable cell numbers over a period of 120 hours in culture increased and was significantly influenced by the substrate tested (p=0.0005<0.05). As expected, the commercially available substrate tested had a significantly better performance throughout the culture period (p=0.017<0.05), as shown in Figure 6-9.
CHAPTER 6. EVALUATION OF PRODUCED PARTICLES FOR CELL ATTACHMENT AND PROLIFERATION CAPABILITIES

Figure 6-9. Cell expansion over a period of 120 hours in culture for uncoated and gelatine-coated in-house produced pNIPAM-coated Alginate microcarriers compared to commercially available microcarriers. Viable cell numbers are plotted as mean values (n=3). Error bars are standard deviations. Statistical significance was assessed by mixed-ANOVA method.

Cell viability when cultured on the gelatine-coated core-shell particles was assessed by Live/Dead staining and the acquired images are provided in Figure 6-10. Cell viability (green fluorescence) on the coated and uncoated core-shell particles was comparable to Cytodex-1. Only a few dead cells (red fluorescence) were observed and exhibited a rounded morphology suggesting that the cells were floating.
Figure 6-10. Cell viability by Live/Dead staining of gelatine-coated in-house produced pNIPAM-coated Alginate microcarriers compared to commercially available microcarriers. Images were acquired at 120 hours in culture. Scale bars represent 100 µm.

6.6. Conclusions

Owing to the large surface area-to-volume ratio provided, the use of microcarriers overcomes surface limitations of the traditional 2D methods, thus achieving higher cell densities in a space-saving and cost-effective manner (Martin Y. et al., 2011). Despite these advantages, current cell harvesting methods from microcarriers require the use of harsh treatments (e.g. proteolytic enzymes) that can result in reduced cell viability and reattachment efficiency (Baumann H. et al., 1979; Huang H.L. et al., 2010; Heng B.C. et al.,...
In regards to overcoming this issue, an advantageous but challenging approach could involve the use of temperature responsive polymers such as pNIPAM. Cell harvesting could then be achieved in a non-invasive and non-destructive manner by altering the temperature moving away from the use of harsh enzymes (DaSilva R.M.P. et al, 2007; Kim M.R. et al, 2002; Okano T. et al, 1993). Despite its promising potential, several studies showed that pNIPAM alone does not support cell adhesion even above its phase transition temperature (Akiyama Y. et al., 2004; Yamato M. et al., 2003) and cell adhesion is strongly dependent on the polymer layer thickness (Yamato M. et al., 2001; Shiroyanagi Y. et al., 2003; Harimoto M. et al., 2002; Nandkumar M.A. et al., 2002). Numerous cell adhesion studies were done on pNIPAM 2D surfaces (e.g. films, membranes etc.) (Yamato M. et al, 2007; Akiyama Y. et al, 2004; Yamada N. et al, 1990; Chen G. et al, 1998; Zhao T. et al, 2011; Biazar E. et al, 2013), however only a few studies were performed on pNIPAM under the form of microcarriers that have been successfully demonstrated to promote cell adhesion and proliferation (Tamura A. et al, 2012; Tamura A. et al, 2012A; Yang H.S. et al., 2010). However these temperature responsive microcarriers comprised of a commercially available core onto which pNIPAM was grafted to form a thin layer.

In the light of the aforementioned reasons, this chapter was dedicated to investigate cell adhesion and proliferation abilities of both solid pNIPAM (see Chapter 4) and core-shell microcarriers (see Chapter 5). Cell adhesion and growth were achieved on both types of the temperature responsive microcarriers. 3T3 fibroblasts maintained membrane integrity and increased in cell numbers when cultured on the temperature-responsive microcarriers. These achievements make pNIPAM microcarriers fit for cell culture of adherent cells. However, there may be deficiencies when compared to commercially available microcarriers. Improvements in cell adhesion and growth were achieved by employing ECM derived components (e.g. gelatine, Matrigel). However, the ability of cells to adhere and proliferate
while still maintaining cell viability and function could be improved even further by modifying and optimising pNIPAM surface characteristics.

In conclusion, the current study investigated and successfully demonstrated cell adhesion and proliferation abilities of 3T3 fibroblasts when cultured on the temperature responsive microcarriers compared to a commercially available microcarrier proven to possess great cell adhesion and growth capabilities (e.g. Cytodex-1). Cell growth enhancement was achieved when employing cell adhesion promoters (e.g. gelatine, Matrigel) and 3T3 cells maintained their membrane integrity when cultured on the provided substrates. However, Cytodex-1 had the best performance in terms of cell expansion with a maximum of 11.6-fold increase after 120 hours in static culture, being approximately 2 times greater than the cell growth achieved on uncoated pNIPAM microcarriers and approximately 1.5 times greater than cell growth on gelatine-coated ones. In this regard, improvement options are available and needed. Further studies based on these initial findings are required to identify the optimal microcarrier chemical composition and coating to achieve greater cell expansion, while still maintaining cell characteristics and function.
CHAPTER 7. NON-INVASIVE THERMALLY INDUCED CELL DETACHMENT

7. NON-INVASIVE THERMALLY INDUCED CELL DETACHMENT

7.1. Introduction

Traditional cell detachment methods used for adherent cell harvesting include mechanical methods (e.g. cell scraping) (Heng B.C. et al., 2007; Batista U. et al., 2010); enzymatic methods (Heng B.C. et al., 2009; Bajpai R. et al., 2007; Heng B.C. et al., 2007; Hybbinette S. et al., 1999; Batista U. et al., 2010); non-enzymatic methods using chelating agents (Heng B.C. et al., 2009; Batista U. et al., 2010) or external stimuli such as temperature (Canavan H.E. et al., 2005; Canavan H.E. et al., 2005A) and ultrasound (Junge L. et al., 2003). Mechanical cell harvesting method is labour intensive and inefficient with limited reproducibility and low cell recovery due to induced physical damage (Canavan H.E. et al., 2005; Heng B.C. et al., 2007), making it incompatible with large-scale culture systems required for clinical use of stem cells. As an alternative, enzyme treatments employing proteolytic enzymes have been successfully used for adherent cell harvesting with high cell recovery yields (Heng B.C. et al., 2009; Bajpai R. et al., 2007; Heng B.C. et al., 2007; Hybbinette S. et al., 1999; Batista U. et al., 2010). Proteolytic enzymes cleave cell-cell and cell-surface peptide bonds necessary for cell adhesion and proliferation. However, during this process, essential membrane proteins might be damaged resulting in diminished adhesion capacity over time (Baumann H. et al., 1979; Huang H.L. et al., 2010). As a different approach to cell harvesting, non-enzymatic methods present great potential. Increasing attention is being given to the use of chelating agents that have a great affinity for divalent ions (e.g. Ca$^{2+}$, Mg$^{2+}$) needed for cell adhesion. This method can be suitable for non-damaging cell dissociation, but it usually affords lower recovery yields when compared to enzymatic treatments (Heng B.C. et al., 2009; Batista U. et al., 2010). Another non-enzymatic method with great potential and of high interest for this project is the use of
temperature stimuli as a non-destructive cell harvest alternative. By culturing cells on
temperature-responsive surfaces, it is possible to rapidly recover intact cell sheets by simply
using a lower temperature as the sole stimulant for cell detachment (DaSilva R.M.P. et al,

This chapter demonstrates the suitability of the temperature responsive particles generated
in this work (refer to Chapter 4 and Chapter 5) for non-destructive cell harvesting (refer to
section 7.4). Such temperature responsive surfaces are readily commercially available, but
under the form of plane two-dimensional surfaces (e.g. Thermo Scientific NUNC UpCell),
not as 3D microcarriers. This chapter also evaluated this type of surface (section 7.3) and
compared it to the in-house produced temperature responsive carriers in regards to necessary
time for cell harvesting under different temperature conditions.

### 7.2. Aims and Objectives

The main aim of this chapter is to demonstrate the non-destructive harvesting potential of
cells cultured on the in-house produced temperature responsive microcarriers. Following the
proof of cell adhesion promoting capabilities of the generated particles (refer to Chapter 6),
the cell harvesting ability was demonstrated by simply reducing the temperature from 37°C
(physiological temperature) to room temperature or lower. To assist with the non-destructive
cell harvesting and to study the influence on necessary time for detachment, the cell-
microcarrier systems were exposed to low temperature treatment. The cell harvesting
ability of the produced microcarriers was compared to that of an existing commercially
available temperature responsive 2D surface (Thermo Scientific NUNC UpCell multidish).
7.3. Evaluating the commercially available 2D surface for cell detachment abilities without the use of proteolytic enzymes

The commercially available 2D temperature responsive surface (NUNC UpCell) has been successfully employed in cell sheet engineering applications for various types of cells, such as MDCK cells (Kushida A. et al, 2005), microglia (Nakajima K. et al, 2001), human corneal endothelial cells (Sumide T. et al., 2006), lung cells (Nadkumar M.A. et al, 2002), aortic endothelial cells (Kushida A. et al., 1999) etc. The UpCell consists of covalently grafted pNIPAM to tissue culture polystyrene (TCP S) vessels (e.g. Petri dish, multi-well dish etc.) by radical polymerisation initiated with electron-beam irradiation (Yamada N. et al, 1990). Thus UpCell exhibits a temperature responsive behaviour being slightly hydrophobic under cell culture conditions at 37°C and changing reversibly to hydrophilic below the polymer’s phase transition temperature. Due to this remarkable property, cells can be easily detached as intact cell sheets that can then be transferred to different culture vessels to which they can readily adhere because the extracellular matrix deposited during the initial cell culture remains intact on the basal surfaces of the cell sheets (Kushida A. et al., 1999).

The commercial pNIPAM-grafted surface was evaluated for suitability with 3T3 fibroblasts. This is necessary as 3T3 cells might have been sensitive to pNIPAM surfaces of different chemistries as demonstrated in Chapter 6. This section of the work explores the temperature responsive behaviour and cell culture and harvesting potential of an independently manufactured product allowing a comparison with the in-house produced temperature responsive microcarriers.
7.3.1. Experimental procedure

3T3 fibroblastic cells at passage 17 were seeded into NUNC UpCell 12-well plates (Thermo Scientific, Germany) with a surface area of 3.5 cm$^2$/well and a 2 mL/well of working volume for growth medium (for composition refer to section 3.5.2). Two different seeding densities were used, one referred to as Low (0.5x10$^5$ cells/well equivalent of 1.4x10$^4$ cells/cm$^2$) and the other at higher than the manufacturer’s recommendations referred to as High (5x10$^5$ cells/well equivalent of 14x10$^4$ cells/cm$^2$). The plates were then placed in a humidified incubator at 37ºC with 5% CO$_2$ environment and cultured for 48 hours prior to cell detachment experiments. After culture, a complete media change with fresh pre-warmed complete growth medium was performed before cell detachment studies. Proceeding the media change, the plates containing proliferated fibroblasts were transferred to ambient temperature (approximately 23ºC) or lower (4ºC). At different time points, phase contrast images were acquired. Necessary time for cell detachment was recorded, as well as changes in cell morphology at different stages of detachment. Cell viability during detachment was also assessed by Live/Dead staining performed according to the protocol described in section 3.7.2. The harvested cells in suspension were collected and centrifuged at 250 g for 5 minutes to obtain a cell pellet. The cell pellet was then re-suspended in pre-warmed complete growth medium and reseeded into a new TCPS (6 well plate). Cell adhesion and proliferation abilities of the temperature-induced harvested cells were monitored over the course of five days with phase contrast images taken at different time points.
7.3.2. Study of cell seeding density effect on cell detachment

Numerous studies have successfully used pNIPAM surfaces for cell harvesting of different types of cells and reported the ability of harvesting cells in a non-destructive way as intact cell sheets (Kushida A. et al., 1999; Kushida A. et al., 2005; Sumide T. et al., 2006; Nadkumar M.A. et al., 2002; Yamato M. et al., 2007). To harvest cells from a 2D surface as an intact cell sheet with undamaged cell-cell adhesions, the cells must be grown to confluence. To date, studies involving 2D temperature responsive surfaces have only reported their use as intact cell sheet harvesting substrates and not for single cells or small aggregate harvesting which is generally required for analytical techniques such as flow cytometry. It has been proven that cell harvesting by trypsinization determines the internalisation of some membrane proteins (Chiarugi P. et al., 2002), thus interfering with different sensitive analytical techniques (e.g. flow cytometry). In regards to this approach, two different initial cell seeding densities (referred to as Low and High – section 7.3.1) were employed in order to reach different degrees of cell confluency and assess cell detachment behaviour in these conditions. Figure 7-1 depicts a comparison between the generally used cell culture surface (TCPS) and the commercially available temperature responsive surface (NUNC UpCell) when utilising two different initial cell seeding densities. When employing a lower initial cell seeding density, cell confluency was not reached during the 48 h of culture before cell harvesting. During thermal lift-off by applying a lower temperature (e.g. 23°C), 3T3 cells were forced to detach as either single cells or small aggregates by the gradual rehydration of the grafted polymer (Figure 7-1). When cell confluence was achieved, a cell monolayer started to roll up at the margins of the cell culture dish and gradually detached as an intact cell sheet from the margins towards the centre of the dish. The cell sheet appeared as wrinkled and folded (Figure 7-1) after detachment due to the contracting force generated when released from the substrate.
Figure 7-1. Phase contrast imaging of cell harvesting from normal TCPS and UpCell surfaces at two different cell seeding densities, Low and High. Scale bars represent 500 µm.
When employing a higher initial cell seeding density, cell sheet detachment starts from the periphery of the vessel and gradually towards the centre following the hydration pattern of pNIPAM, as shown in Figure 7-2.

Figure 7-2. 3T3 cell sheet thermal-induced detachment starting from the periphery of the dish towards the centre; Scale bar 100 µm.

TCPS vessels were employed as positive controls for cell adhesion and proliferation and negative controls for cell detachment. As shown in Figure 7-1, cell adhesion and proliferation was achieved on these surfaces, but no cell detachment was seen regardless of seeding density. In regards to time necessary for complete cell detachment from the surface, it took 45 minutes for low seeded cells to detach as clumps or singlets compared with 55 minutes for high seeded cells which detached as a sheet (Figure 7-1). The increased detachment time of the cell sheet could be caused by a delay in detachment kinetics due to
the high density of cell-cell junctions established between confluent cells (Tamura A. et al., 2012; Tamura A. et al., 2012A).

In regards to cell morphology during the detachment step, 3T3 cells exhibited a normal flattened and spread morphology when attached to the UpCell surface, comparable to the cell morphology observed when culturing on general TCPS. However, at the end of the detachment process, the cells gained a spherical morphology characteristic to cells in suspension (Reiter T. et al., 1985). Once the temperature-induced detachment mechanism was initiated by lowering the temperature, the cells gradually lost their flattened and spread shape to a spherical one, as shown in Figure 7-2 and Figure 7-3. This phenomenon could be attributed to the polymer’s gradual phase transition from hydrophobic to hydrophilic (Heskins M. and Guillet J.E., 1968; Tauer K. et al., 2009). Figure 7-2 depicts several cell morphologies varying from flattened to spherical during cell sheet detachment due to the different stages of polymer hydration resulting in different stages of cell detachment within the cell monolayer.

Figure 7-3. A close-up representation of cell morphology changes experienced during temperature-induced cell harvesting on the UpCell surface. Scale bars represent 100 µm.
Cell viability during detachment was assessed by fluorescent staining employing the Live/Dead viability assay (Invitrogen, UK) and the acquired images are presented in Figure 7-4. A majority of cells maintained their membrane integrity during thermal induced detachment. A small number of dead or damaged cells (red fluorescence) were dispersed between the small clumps of cells (low initial cell seeding density) and between the intact cells sheet (high initial cell seeding density). Referring to the cell sheet, the dead cells were localised in the peripheral area of the sheet (as shown in Figure 7-4) and this could be caused by the overgrowth of the marginal cells due to achieving a high level of confluency, therefore resulting in their loss of anchorage dependency. Thus, the acquired images confirm that cell viability during the thermal-induced detachment was not compromised.
Figure 7-4. Live/Dead staining of 3T3 fibroblastic cells during thermal-induced detachment at two different initial cell seeding densities; live and healthy cells are stained in green, while dead or damaged cells in red. Scale bars represent 100 µm.
7.3.3. Cell detachment recording for different conditions

Detachment times for cell and cell sheet harvesting were recorded and compared when employing two different incubation temperatures, room temperature (approximately 23ºC) and 4ºC for each of the two different initial cell seeding densities (Table 7-1).

Table 7-1. Cell detachment time comparison for different sets of conditions

<table>
<thead>
<tr>
<th>Cell detachment time / min</th>
<th>Initial cell seeding density</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Incubation temperature</strong></td>
<td>Low</td>
</tr>
<tr>
<td>23ºC</td>
<td>45 min</td>
</tr>
<tr>
<td>4ºC</td>
<td>30 min</td>
</tr>
</tbody>
</table>

Table 7-1 presents the recorded detachment times for the conditions employed. The recorded values suggested less time was required when using lower incubation temperatures. Thus, regardless of the initial cell seeding density, the recorded detachment time was 30 minutes which is within the detachment time range obtained in other studies (Okano T. *et al*, 1995; Wang T. *et al*, 2011). Phase contrast images acquired during cell detachment in growth medium at 4ºC when using two different initial cell seeding densities are provided in Figure 7-5. Similarly to detachment at ambient temperature, 3T3 cells were harvested as either small aggregates or singlets or intact cell sheets.
Figure 7-5. Phase contrast images acquired during 3T3 fibroblasts cell detachment in growth medium at 4°C when using two different initial cell seeding densities. Scale bars represent 500 µm.
CHAPTER 7. NON-INVASIVE THERMALLY INDUCED CELL DETACHMENT

7.3.4. Cell recovery after external stimuli-induced detachment

After cell harvesting as either single cells or cell sheets, the recovered cells were reseeded onto new TCPS vessels in fresh complete growth medium. The adhesion and proliferation abilities of the harvested cells were monitored in culture over a period of six days with complete medium changes every three days. Phase contrast images were acquired at different time points (Figure 7-6).

Thermally-induced harvested cells recovered well when reseeded, showing good proliferation when in culture, as shown in Figure 7-6. Although the harvested cells needed a recovery period of two days when their growth was slower, by day six they have reached confluency as shown in Figure 7-6. When mammalian cells are exposed to sub-physiological temperatures (<37°C), cell growth is stopped in the G1 cell cycle (Kaufmann H. et al., 1999). In agreement with this, Underhill M.F. et al. (2007) demonstrated that cell viability was influenced by the low temperature value employed. Thus, when incubating cells at 32°C, cell viability dropped by approximately 25% compared and using incubation at 10°C the cell viability was reduced by 50%. Moreover, Underhill M.F. et al. (2007) discovered that the sudden culture temperature shifting from physiological to a low value (10°C) resulted in an immediate and drastic reduction in cell viability when compared to a higher temperature incubation (Underhill M.F. et al., 2007). In regards to cell harvesting, the incubation at 4°C resulted in a more rapid cell detachment at the expense of cell viability. Thus, the images in Figure 7-6 show that regardless of the initial cell seeding density, fewer cells adhered and proliferated on TCPS after the thermo-responsive detachment at 4°C when compared with detachment performed at ambient temperature. This reduction in cell number is corroborated by Underhill M.F. et al. (2007).
CHAPTER 7. NON-INVASIVE THERMALLY INDUCED CELL DETACHMENT

Figure 7-6. Six days in culture monitoring of cell adhesion and proliferation abilities of 3T3 harvested cells in different conditions based on a temperature induced method. Scale bars represent 500 µm.
CHAPTER 7. NON-INVASIVE THERMALLY INDUCED CELL DETACHMENT

When reseeding harvested cell sheets, an interesting behaviour was observed (Figure 7-6). During cell detachment, because of contracting forces generated by releasing from the substrate, the detached cell sheet contracts and folds. When reseeded to a new surface, the folded cell sheet attaches on the margins and after a few days in culture, it shrinks in size due to cell migration to the new provided substrate, as shown in Figure 7-6.

7.4. Temperature-induced cell harvesting capabilities of produced temperature responsive particles used as cell microcarriers

To date, only a few recent studies have been reported on thermally-induced cell harvesting from microcarriers (Tamura A. et al, 2012; Tamura A. et al, 2012A; Yang H.S. et al., 2010). In order to harvest cells from a 2D surface under the form of an intact cell sheet with undamaged cell-cell adhesions, the cells grown on the provided surface have to reach confluency. In regards to 3D surfaces such as microcarriers, cell detaching as an intact cell sheet from a confluent microcarrier can be problematic resulting in reduced cell harvesting efficiency (Tamura A. et al., 2012). When cells reach confluency on the bead surface, the number of cell-cell junction increases causing a retardation of cell detachment kinetics and possibly even restriction of cell detachment (Tamura A. et al., 2012).

7.4.1. Experimental procedure

3T3 fibroblast cells at passage 19 were seeded onto in-house produced sterile core-shell microcarriers (refer to Chapter 5) in Ultra Low Attachment 12-well plates with 2 mL/well of pre-warmed fresh complete growth medium (for composition refer to section 3.5.2). The
Ultra Low Cell Attachment (ULA) plates were employed in order to avoid unwanted cell detachment to plate walls instead of provided surfaces of the microcarriers. After seeding, the plates were placed in a humidified incubator at 37°C with 5% CO₂ controlled environment and cultured in static conditions for 72 hours prior to cell detachment experiments. After 72 h in culture, a complete medium change with fresh growth medium was performed.

Preceding the media change, the dishes containing the adhered fibroblasts onto temperature responsive core-shell microcarriers in fresh growth medium were removed from the incubator and placed on the bench at room temperature (approximately 23°C) or refrigerated at 4°C. At different time points, phase contrast images were acquired. The time necessary for cell detachment was recorded, as well as changes in cell morphology at different time points. The harvested cells were recovered and reseeded on new TCPS dishes. Briefly, the suspension containing the harvested cells and microcarriers was recovered and initially passed through 40 µm sterile cell strainers (BD Biosciences, UK) in order to recover the microcarriers. Single cells and small clumps of cells passed through the cell strainer and were collected in a centrifuge tube. The recovered microcarriers were then re-suspended in fresh pre-warmed complete growth medium and passed again through a 70 µm sterile cell strainer, in order to allow bigger cell clumps to be collected. Several washes with fresh pre-warmed complete growth medium were performed on the recovered microcarriers to ensure maximum cell recovery and collection. The collected cell suspension was then centrifuged at 250 g for 5 minutes and the cell pellet was re-suspended in fresh pre-warmed complete growth medium and seeded onto TCPS. The reseeded cells were monitored over a period of several days to assess if they have maintained the adhesion and proliferation abilities. Phase contrast imaging was performed at different time points.
CHAPTER 7. NON-INVASIVE THERMALLY INDUCED CELL DETACHMENT

7.4.2. Cell harvesting from pNIPAM solid particles

Cell attachment and proliferation on uncoated and protein-coated solid pNIPAM particles generated by our method (refer to Chapter 4) was demonstrated in section 6.4. For evaluating the cell harvesting capabilities, only the hard crosslinked pNIPAM particles containing 10%wt monomer were employed for this study. Although cell adhesion and proliferation was possible on these particles, as shown in section 6.4, cell detachment as a result of temperature change was not observed, not even after 240 minutes of low temperature treatment, either at 23°C or 4°C, as shown in Figure 7-7. This behaviour could be attributed to the rehydration behaviour of bulk pNIPAM (refer to section 2.2.3.1.1). This contrasts with the release behaviour exhibited on the in-house core-shell particles described in the next section.

Figure 7-7. Phase contrast images acquired at 0 min and 240 min after temperature was lowered to 23°C in order to initialise pNIPAM phase transition and force attached cells to lift off the microcarrier surface. Scale bars represent 100 µm.
7.4.3. Cell harvesting from core-shell thermo-responsive particles

Based on previous studies (Yamato M. et al., 2001; Shiroyanagi Y. et al., 2003; Harimoto M. et al., 2002; Nandkumar M.A. et al., 2002), cell adhesion and proliferation were expected to be successful on pNIPAM monolayer coatings and it was successfully demonstrated in section 6.5. By simply applying a low temperature treatment on the culture vessels containing the cells-microcarrier assemblies, thermally-induced harvesting was expected, but not found to be so simple for the solid pNIPAM particles described in previous section. This chapter section is dedicated to investigate the suitability of the core-shell microcarriers (refer to Chapter 5) used for non-invasive cell harvesting. Regardless of the harvesting method employed, cells are expected to round up upon detachment from a surface as they lose their focal adhesions and the cytoskeleton disassembles (Reiter T. et al., 1985). 3T3 fibroblasts morphology when attached to the temperature responsive in-house generated particles was assessed and compared to the morphology shown on general TCPS and on commercially available microcarriers (GE Healthcare- Cytodex 1) (Figure 7-7). Cytodex 1 microcarriers were used in this study as a positive control for cell adhesion and proliferation and as negative controls for thermal-induced cell detachment (Weber C. et al, 2007).
Figure 7-8. 3T3 cell morphology assessment when cultured on: A) TCPS; B) and D) commercially available Cytodex-1 microcarriers (GE Healthcare); C) and E) core-shell microcarriers. Phase contrast images are shown in A), B) and C); while Live/Dead staining images are shown in D) and E). Live and healthy cells emitted green fluorescence, while dead or damaged cells emitted red fluorescence. Scale bars represent 100 µm.

As shown in Figure 7-8, cell morphology when attached to the core-shell microcarriers was similar and consistent with previous studies reported in literature (Reiter T. et al., 1985). For a better visualisation of cell morphology together with cell viability evaluation when attached, Live/Dead staining assay was employed as a qualitative method and acquired images are provided in Figure 7-8 (D and E). Cells adhered to the core-shell microcarriers and were detached by low temperature shift. Cell detachment was assessed at ambient temperature (approximately 23°C) and when refrigerated at 4°C.
CHAPTER 7. NON-INVASIVE THERMALLY INDUCED CELL DETACHMENT

When incubated at 23ºC, the first signs of cell detachment were observed after approximately 20 minutes when cells started to retract from the surface and gained a more rounded shape, as shown in Figure 7-9. Complete cell detachment occurred within 200 minutes of incubation finalising with the generation of single cells and clumps of cells of different sizes. The generation of the first single cells in suspension was observed within 20 minutes of incubation. Small patches of cells responded faster to temperature stimuli by detaching as small clumps in a shorter time. On the other hand, in the case of fully confluent and 70% confluent microcarriers, cell detachment was delayed and in some cases didn’t occur not even after 160 minutes at lower temperature incubation, as shown in Figure 7-10. It is believed that this delay in cell detachment was determined by the increased number of cells present on the microcarrier surface resulting in the formation of a barrier rendering polymer hydration (Tamura A. et al, 2012).
Figure 7-9. Thermal-induced cell harvesting evaluation at 23°C temperature treatment of A) pNIPAM-coated Alginate particles; B) commercially available Cytodex-1 microcarriers (GE Healthcare); Scale bars represent 100 µm.
CHAPTER 7. NON-INVASIVE THERMALLY INDUCED CELL DETACHMENT

Figure 7-10. Image acquired after 160 minutes of incubation at 23°C depicting a 70% confluent microcarrier that barely initiated cell detachment process. Arrows point out the cell detachment pattern starting from the margins of the cell blanket. Scale bar represents 100 µm.

Cell detachment on these microcarriers was initially seen within 50 minutes of incubation and started at the free margins of the cell ‘blanket’, as shown in Figure 7-11. This cell detachment behaviour is consistent with gradual hydration of the pNIPAM starting at the periphery and advancing towards the centre (Lai J.Y. et al., 2006). This is also comparable to the cell detachment pattern exhibited during cell sheet detachment on flat pNIPAM surfaces (e.g. NUNC UpCell) (refer to section 7.3.3). When incubated at 4°C, cells detached more rapidly with complete detachment of single cells and cell clumps being achieved within 175 minutes, (Figure 7-12). Faster cell detachment after incubation at lower temperature can be attributed to the faster polymer rehydration as a result of faster cooling.
Figure 7-11. Cell detachment as cell sheets from core-shell microcarriers started at the periphery of the cell sheet where cells exhibited a rounded morphology indicating detachment from the surface. Arrows point out the cell detachment pattern starting from the margins of the cell blanket. Scale bar represents 100 µm.

Figure 7-12. Thermally-induced cell harvesting when culture on A) pNIPAM-coated Alginate microcarriers; B) Cytodex-1, after incubation at 4°C; Scale bars represent 100 µm.
7.5. Discussion and conclusions

In this study, the in-house produced temperature responsive microcarriers were demonstrated to promote cell adhesion and proliferation, as well as thermally-induced cell detachment, thus avoiding the use of harsh methods (e.g. cell scraping, proteolytic enzymes) fulfilling the detachment in a non-invasive and non-destructive way. One of the current issues reported for thermal induced cell harvesting is the long incubation period at a temperature lower than physiological value for complete cell sheet detachment affecting directly the viability of the recovered cells, as well as their biological functions. Adherent-dependent cells can only survive in suspension for a period of time after which cell death occurs (Reiter T. et al., 1985). Cell detachment rate from thermo-responsive surfaces has been shown to depend on the temperature, cell-type, polymer composition and the method used to prepare the surface (Nash M.E. et al., 2012). For example, Okano T. et al. (1995) have found that for hepatocytes grown on pNIPAM-grafted surfaces the necessary detachment time was 35 minutes. Another study employing L929 mouse fibroblasts cultured on copolymers of pNIPAM with a more hydrophilic polymer, N-tert-butyl acrylamide, showed that the cell detachment time varied between 15 minutes to 180 minutes depending on the copolymerising ratio (Selezneva I.I. et al., 2006).

Several recent studies demonstrated that by including an appropriate hydrophilic component into the structure of the generated temperature responsive surfaces, the detachment time could be considerably shortened. Thus, Wang T. et al. (2011) reported that the time consumption for the cell sheet detachment of L929 mouse fibroblasts was greatly reduced from 30 minutes to 15 minutes when a small amount (0.2% w/v) of alginate was added into the pNIPAM nanocomposite gel to form a semi-interpenetrating network. A similar result was obtained by Liu D. et al. (2012) by incorporating polyethylene glycol (PEG) into the
structure of their nanocomposite temperature responsive gel resulting in L929 fibroblasts cell sheet detachment times lower than 20 minutes.

Cell harvesting abilities of the core-shell temperature responsive particles were assessed and successfully demonstrated in this chapter. Compared to previous studies and to the 2D commercially available temperature responsive surface, the necessary time of complete cell detachment recorded was of an order higher, approximately 200 minutes compared to previously reported 20-30 minutes (Wang T. et al., 2011; Liu D. et al., 2012; Okano T. et al., 1995). From the polymer chemistry point of view, harvesting time could be improved by introducing a hydrophilic copolymer into the temperature responsive coating, as previous studies demonstrated (Selezneva I.I. et al., 2006; Wang T. et al., 2011). Cell confluency has an important influence on the detachment pattern resulting in either single cells/small clumps or cell sheets. When cultured on microcarriers, cells would be evenly distributed on microcarriers during seeding. In this work, microcarriers were seeded in static vessels, which would contribute to uneven cell seeding and overly confluent microcarriers which do not easily detach. To avoid this issue, thermally-induced cell detachment, as well as cell seeding could be performed with gentle stirring, improving seeding uniformity and potentially the detachment time. To ensure maximum cell recovery and viability, very low incubation temperatures (e.g. 4°C) should be avoided, resuming at room temperature values or higher.

In conclusion, the present study demonstrates that in-house produced temperature responsive particles have the ability to harvest cells in a non-enzymatic, non-invasive and non-destructive way by simply incubating the cell-microcarrier assemblies at a temperature below the physiological value, when using the core-shell particles (core of alginate and shell of pNIPAM). Cells maintained their viability during detachment and their adhesion and
proliferation abilities after reseeding onto new substrates. The harvesting time at hypothermic temperatures was longer than other examples of temperature responsive surfaces in the literature. This work demonstrates proof of concept of novel thermo-responsive microcarriers produced cost-effectively using an operating scale-friendly manufacturing technology. A better understanding of the temperature responsive properties of pNIPAM surfaces when used as microcarriers, will guide improvements to the surface chemistry and allow fine-tuning of the final microcarrier properties. In contrast, the solid pNIPAM microcarriers produced in-house did not release the cells by temperature responsive means. Thus it is necessary to use the core-shell particles, but the method of attachment of the pNIPAM shell to the alginate core is facile, based on simple chemisorption in a stirred beaker.
CHAPTER 8. SUMMARY, CONCLUSIONS AND FUTURE WORK

8. SUMMARY, CONCLUSIONS AND FUTURE WORK

8.1. Summary

There is a growing demand for development of regenerative medicine therapies that employ stem cell-based products for treatment of numerous health conditions with the ultimate goal of improving the quality of life for many. Successful cell therapies ultimately require a reliable and robust system that is not only capable of mass producing stem cells, but still maintains high stem cell viability and intact stem cell potency. As such, one of the weakest points of current technologies is the lack of a robust large scale stem cell manufacturing system that allows a reproducible expansion and harvest. Most anchorage-dependent stem cells have been shown difficult to expand in culture. Typical expansion methods employ two dimensional flat surfaces (e.g TCPS) which have major limitations including limited surface area and expensive, labour intensive protocols. These limitations could be overcome by employing microcarriers which owing to the large surface area-to-volume ratio provided, higher cell densities could be achieved in a space-saving and cost-effective manner (Martin Y. et al., 2011). Microcarriers in conjunction with bioreactors have been previously employed successfully for the production of large numbers of cells (Chen A.K-L. et al., 2011; Rafiq Q. et al., 2013). However, a number of barriers have been identified in these systems, particularly concerning the final step of cell harvesting performed by either employing harsh enzymatic treatments frequently resulting in loss of potency or even cell death (Huang H.L. et al., 2010; Heng B.C. et al., 2009) or by destroying the microcarrier support leaving an impure product. In this regard, the work presented in this doctoral thesis seeks to explore alternative solutions to enzymatic cell harvest from 3D culture systems.

The overall vision of this work consisted in designing a new type of microcarrier capable of not only promoting cell adhesion and proliferation, but also offering the possibility of a non-
destructive and non-enzymatic cell harvesting method by simply applying an external
temperature stimulus. The strategy for achieving this goal required a multidisciplinary
approach based on the understanding of the underpinning biological, chemical and
engineering aspects involved in the successful design of a novel microcarrier type. The
approach undertaken for the production of microcarriers consisted in utilising a platform
technology already demonstrated to be suitable for the controlled production of uniformly
sized particles (Gasparini G. et al., 2008; Gasparini G. et al., 2010; Dragosavac M.M. et al.,
2012A). This technology was developed based on a membrane emulsification approach and
made use of a simple designed stirred cell commercially available as the ‘Dispersion Cell’
utilising disc-shaped metallic membranes with uniformly distributed arrays of micropores.
A precise control over process parameters (e.g. shear stress, dispersed phase injection rate,
surfactant concentration, inter-pore spacing etc.) allowed control over particle size and size
distribution. As a different approach, it was possible to successfully correlate the particle
size formed using the aforementioned system with a published model (Kosvintsev S.R. et
al., 2005; Stillwell M.T. et al., 2007) for membrane emulsification, relating particle size to
shear stress at low dispersed phase injection rates. In the light of the final application of
particles to be used as microcarriers for cell expansion, a specific size range and size
distribution is required to provide a sufficient surface area for cell growth. Typical
commercially available microcarriers are in the size range of 100 µm to 200 µm. Size
distribution of microcarriers has a major contribution towards the homogeneity of the
environment and more specifically of cell growth. Thus, the narrower the size distribution,
the more homogeneous the culture system is.

In the light of the need for a non-damaging cell harvesting method, an advantageous, but
challenging approach could involve the use of temperature responsive polymers such as
pNIPAM. Temperature responsive surfaces are already commercially available, but under
the form of flat 2D surfaces (i.e. not microcarriers) exhibiting surface limitations that need to be overcome. In this regard, the need for developing a novel temperature responsive microcarrier has arisen.

Initially, solid non-porous pNIPAM microcarriers with median sizes controllable within the range of 37 µm to 1050 µm and size distribution expressed as span values as low as 0.5 were produced by starting from the monomer and applying a free radical polymerisation method. Thus generated pNIPAM particles were shown to exhibit a temperature responsive behaviour in the range of 31°C to 33°C depending on the formulation employed. The crosslinking degree of the particles given by the concentration of crosslinker used had an impact on some of the particle properties (e.g. size; LCST). For example, by increasing the crosslinker concentration, the particle size decreased resulting in a more compact structure due to the formation of an increased number of bridges between the polymer linear chains.

All the illustrated characteristics (e.g. shape, size, size distribution, temperature responsiveness) demonstrated the potential of these particles to be used as supports for cell culture and potential substrates for non-enzymatic and non-damaging cell harvesting. Cell growth promoting abilities of the pNIPAM microcarriers were evaluated. It was found that 3T3 fibroblasts grown on pNIPAM solid microcarriers maintained membrane integrity and increased in cell numbers. However, there were deficiencies compared to a commercially available microcarrier (e.g. Cytodex-1) recognised as very good at promoting cell adhesion and proliferation. Improvements in cell adhesion and growth were achieved by employing ECM derived components (e.g. gelatine, Matrigel). However, from a clinical point of view, animal derived products should be avoided. As such, the ability of cells to adhere and proliferate could be improved even further by modifying and optimising pNIPAM surface characteristics. Despite the optimistic findings, cell detachment from the solid pNIPAM microcarriers was not successful. It is recognised that cell detachment from thermo-
responsive surfaces is dependent on temperature, cell-type, polymer composition and the method used to prepare the surface (Nash M.E. et al., 2012).

It was identified in literature that cell adhesion on temperature responsive surfaces is strongly dependent on the pNIPAM layer thickness (Yamato M. et al., 2001; Shiroyanagi Y. et al., 2003; Harimoto M. et al., 2002; Nandkumar M.A. et al., 2002). In this regard, a different approach towards the production of a novel thermo-responsive microcarrier was taken. As such, a core-shell type particle was developed consisting of an alginate core and a pNIPAM shell. Initially, the alginate core was generated in a controlled manner by employing membrane emulsification. Spherical alginate particles with median sizes controllable within the range of 55 to 650 µm and spans as low as 0.2 (highly monosized), were successfully produced. Several formulations have been previously reported, but they all involved the use of toxic chemicals that can result in cell toxicity (Liu X.D. et al., 2002; Liu X.D. et al., 2003; Ribeiro A.J. et al., 2005; Silva C.M. et al., 2006). As an improvement, the formulation used in this work for the production of alginate particles avoided non-GRAS chemicals by only using food grade and pharmaceutical grade reagents. A key feature of the membranes employed is the lack of internal tortuosity which allowed suspended solids to be passed through the membrane pores, thus facilitating the use of internal gelation method for production of alginate particles. The presence of naturally occurring carboxylic acid groups at the alginate surface could be used to provide chemisorption sites onto which cationic groups (e.g. amine) could be attached. Based on this principle, the pNIPAM shell was generated. The successful chemisorption of modified pNIPAM was demonstrated and quantified by measuring the amounts of polymer adsorbed onto the calcium alginate core and found to fit a Langmuir type isotherm. The pNIPAM successful coating and the maintenance of its temperature responsive properties was demonstrated, thus underlying the potential of these particles to be used as cell culture and harvesting substrates. 3T3
fibroblastic cells were seeded onto the core-shell type microcarriers and cell adhesion and proliferation on the provided surfaces were evaluated both qualitatively and quantitatively. Cells remained viable and cell growth was recorded. Cell harvesting abilities of the core-shell temperature responsive particles were assessed and successfully demonstrated. Compared to previous studies, as well as 2D commercially available temperature responsive surfaces, the necessary time for complete cell detachment recorded was of an order higher, approximately 200 minutes compared to previously reported 20-30 minutes (Wang T. et al, 2011; Liu D. et al, 2012; Okano T. et al, 1995). From the polymer chemistry point of view, harvesting time could be improved by introducing a hydrophilic copolymer into the temperature responsive coating, as it was demonstrated in previously (Selezneva I.I. et al., 2006; Wang T. et al, 2011). It was found that cell confluency had an important influence on the detachment pattern resulting in single cells /small clumps or intact cell sheets. All experiments presented in this work were performed in static conditions. As a result, uneven cell seeding was produced, contributing to generation of overly confluent microcarriers that had limited or no cell harvesting performance. To avoid this issue, thermally-induced cell detachment, as well as cell seeding could be performed by applying gentle stirring, thus improving seeding uniformity and potentially the cell harvesting time.
8.2. Concluding remarks

This doctoral thesis seeks to address the current need for a non-damaging cell harvesting method by designing a novel type of microcarrier. To address this need, four research questions (refer to section 1.3) were answered to the best of knowledge.

- To address the first research question referred to the possibility of producing temperature responsive microcarriers in a controlled and reproducible manner. The address this question, this doctoral thesis described a straightforward and reproducible method for producing uniformly sized temperature responsive microcarriers. Membrane emulsification was applied on the Dispersion Cell device which allowed precise control over process parameters. Particle size and size distribution were possible to control by varying either formulation parameters (e.g. surfactant concentration, crosslinker concentration) or process parameters (e.g. shear stress, injection rate, membrane pore size, inter-pore distance). As an addition to this system, it was possible to correlate the particle size generated with a published mathematical model relating particle size to shear stress at low dispersed phase injection rates (Kosvintsev S.R. et al., 2005; Stillwell M.T. et al., 2007). pNIPAM particle size was possible to be controlled within the range of 37 to 1050 µm. With regards to the final application to be used as substrates for cell culture, the range of interest (100 to 200 µm) was possible to achieve by selecting the appropriate conditions included in the studied parameter ranges. Moreover, the size distribution of produced microcarriers reached a low value of 0.5. The generated pNIPAM particles exhibited a specific behaviour with phase transition taking place at a temperature close to the physiological temperature in the range of 31ºC to 33ºC, making them suitable to be used for cell-based applications.
• Based on the decision of developing a second type of temperature responsive microcarrier, a new question arised. Can alginate particles of appropriate sizes be generated for use as microcarrier cores? The basis of developing a core-shell type microcarrier was the importance of pNIPAM layer thickness on cell adhesion and proliferation and possibly even cell harvesting. Alginate was selected for the production of the microcarrier core based on its natural derived origins, its biocompatibility and its chemical structure. The presence of naturally occurring carboxylic acid groups at the alginate surface made it an excellent scaffold for functionalization. Thus, the provided chemisorption sites allowed the formation of electrostatic interactions with the amine groups provided by the modified pNIPAM resulting in a theoretical monolayer coating. In the light of the aforementioned reasons, alginate was suitable to be used as a core. The emulsification process described in this thesis made possible the production of highly monosized calcium alginate cores with spans as low as 0.2 and median sizes within the range of 55 to 650 µm. The generated core-shell microcarriers maintained the pNIPAM specific phase transition behaviour at a temperature close to the physiological temperature at approximately 32°C.

• Once the temperature responsive particles were produced in a controlled manner and characterised, a new research question was raised. Are the developed temperature responsive microcarriers capable of allowing cell attachment and growth? Numerous previous studies have implied that pNIPAM alone does not support cell adhesion and proliferation (Akiyama Y. et al. 2004; Yamato M. et al. 2003). Moreover, successful cell adhesion and growth were shown to be highly dependent on the pNIPAM layer thickness (Yamato M. et al., 2001; Shiroyanagi Y. et al., 2003; Harimoto M. et al., 2002; Nandkumar M.A. et al., 2002). However, the work presented in this thesis
showed the ability of both types of temperature responsive microcarriers to support both cell adhesion and proliferation. Moreover, cells were viable and maintained their typical morphology. However, Cytodex-1 had the best performance in terms of cell expansion with a maximum of 11.6-fold increase after 120 hours in static culture, being approximately 2 times greater than the cell growth achieved on the core-shell type temperature responsive microcarriers. Attempts of improving cell adhesion and growth were made and employed ECM-derived components (e.g. gelatine, Matrigel). However, the use of animal-derived products is not ideal and it should be avoided if possible in order to move towards clinical applications. In this regard, other cell adhesion and growth improvement options are available and could be utilised.

- Already commercially available temperature responsive 2D surfaces (NUNC UpCell) have been successful for thermal-induced cell harvesting. The question relevant to this research then becomes: can thermally induced cell harvesting from microcarriers be achieved? To answer this question, cell detachment studies were performed. The pNIPAM solid microcarriers were found to be unsuccessful in cell harvesting. However, the core-shell type microcarrier allowed thermal-induced cell harvesting. Moreover, cells remained viable during detachment and maintained the adhesion and proliferation ability after reseeding onto new substrates. The harvesting time at hypothermic temperatures was longer than other examples of temperature responsive surfaces in the literature. However, improvements are already available and could be easily applied.

- There are a number of research groups working on other polymer microcarrier systems with possible applications in cell therapies. However, the microcarrier production methods used usually involve the use of toxic reagents and expensive and
complicated methodologies. To the best of knowledge, no core-shell type temperature responsive microcarrier systems with applications in cell expansion and harvesting and produced in a controlled manner, have been previously reported in literature. Therefore, numerous research groups and even possibly industry representative could benefit from the microcarrier production procedures provided in this doctoral thesis, possibly including the use of a core-shell type of particle, where the core is an inert and benign material such as alginate with just the shell formed using the expensive and possibly complex polymer material.

8.3. Future work

This thesis demonstrated proof of concept of a novel designed thermo-responsive microcarrier produced cost-effectively using a scale-friendly manufacturing platform technology, capable of allowing cell adhesion and proliferation and more importantly providing a non-damaging and non-enzymatic cell harvesting method. However, the novel core-shell type microcarrier presented in this work is still at early stages of development which means that more challenges are to be addressed.

- The further optimisation of the developed core-shell type microcarriers is necessary. This could be achieved by improving the surface chemistry employed and mechanical properties, thus allowing fine-tuning of the final microcarrier properties with the goal of achieving greater cell expansion and rapid cell harvesting. For the work presented in this doctoral thesis, the core material selected was alginate, but other core materials could be employed with the possibility of improving the mechanical properties of the final microcarriers. The approach undertaken in this
thesis consisted in taking advantage of the electrostatic interactions between the free carboxyl groups present in alginate and the free amine groups present in the modified pNIPAM. However, other approaches could be considered such as using a core with free amine groups (e.g. chitosan) and modified pNIPAM with free carboxyl groups. The chitosan core could exhibit improved mechanical properties and better surface wettability with possible effects on improved protein adsorption and cell adhesion. Improved mechanical properties could allow the use of other, possibly more efficient methods of sterilisation (e.g. autoclaving), as well as possible determination of the surface area provided per gram of microcarriers, resulting in a more controlled microcarrier cell culture process with controlled and consistent cell seeding density allowing for a better comparison between different conditions. Numerous previous studies have shown that cell seeding density had a significant impact on cell growth on surfaces. Thus, a known surface area could allow for a better control over microcarrier culture, as well as consistency and reproducibility.

- In addition to the optimisation of this novel type of core-shell microcarrier, the potential to become a platform technology for cell expansion and non-enzymatic cell harvesting could be exploited. This could be achieved by testing different types of cells, including stem cells with potential to be used in regenerative medicine applications. Current cell expansion methods for stem cells of interest such as mesenchymal or even embryonic stem cells, involve the use of commercially available microcarriers, such as Cytodex-1 (GE Healthcare) or Plastic (SoloHill). The main disadvantage of these substrates is that they require the use of proteolytic enzymes. However, by employing the core-shell type of temperature-responsive microcarriers described in this thesis, the need for enzymatic treatments for cell harvesting can be overcome. In addition, by using temperature-responsive surfaces,
the ECM secreted by the cells during growth is maintained intact during harvesting. Current methods for embryonic stem cells expansion require numerous sources of variability (i.e. serum, Matrigel coating). To the best of knowledge, previous reported studies have only employed commercially available microcarriers coated with Matrigel for embryonic stem cell expansion. An interesting approach could be the use of a microcarrier type that allows embryonic stem cell attachment and proliferation by avoiding the use of protein coatings. The potential of the core-shell type microcarriers to be used as such substrates is worth investigating. If successful, this approach could be a ground breaker with tremendous potential as a platform technology for cell expansion.

- For a better evaluation of the core-shell microcarriers reported in this thesis to be used as cell expansion substrates, their potential in a stirred environment has to be evaluated. All the cell expansion and harvest experiments presented in this work were performed only in static conditions and at a small scale. However, for a true evaluation and development of a platform for cell expansion, stirred conditions investigations are required. This could be achieved by employing either spinner flasks or stirred tank bioreactors which allow a great control over process parameters. However, other stirred systems are currently commercially available. One such is a novel automated micro-bioreactor system AMBR (TAP Biosystems) that can allow for high-throughput screening and optimisation of different process parameters. The advantages of such a system involve control and extended monitoring of process parameters, the use of lower volumes of media resulting in lower costs, as well as minimising the variation determined by the human error.
Finally, it is worth noting that typical microcarriers have sizes within the range of 100 µm to 200 µm. To the best of knowledge, no research has been done on the effect of different sized microcarriers (less than 100 µm and larger than 200 µm) on cell adhesion. With focus on the novel developed core-shell microcarrier described in this thesis, the effect of different sized particles on cell expansion and temperature-induced harvest needs to be investigated both in static and stirred environment. Microcarrier size could have a major impact on both cell expansion and cell harvesting and possibly implications in maintaining cell quality over culture. From a manufacturing point of view, the use of smaller sized microcarriers could provide higher surface areas for cell growth.
CHAPTER 9. REFERENCES

9. REFERENCES


CHAPTER 9. REFERENCES


CHAPTER 9. REFERENCES


CHAPTER 9. REFERENCES


Hua S., Ma H., Li X., Yang H., Wang A. (2010) pH-sensitive sodium alginate/polyvinyl alcohol hydrogel beads prepared by combined calcium crosslinking and freeze-


CHAPTER 9. REFERENCES


Tamura A., Kobayashi J., Yamato M., Okano T. (2012A) Thermally responsive microcarriers with optimal poly(N-isopropylacrylamide) grafted density for


10. APPENDICES

A. APPENDIX 1

A1. List of equipment and consumables utilised

- Dispersion Cell (MicroPore Technologies, UK)
- Disc-shaped metallic membranes (MicroPore Technologies, UK)
- Syringe pump (Harvard Apparatus, pump 11)
- Syringe pump (KD Scientific KDS100)
- Peristaltic pump (Watson Marlow 101U)
- Mechanical stirrer driven by a 24 DC motor (Instek PR-3060)
- Optical microscope (Leitz Ergolux)
- Centrifuge (Sigma 3-16PK)
- Water bath (Grant SUB Aqua 26Plus)
- Incubator (Sanyo Safe Cell)
- Inverted phase contrast microscope (Nikon Ti Eclipse)
- Microplate reader (BMG Labtech Omega FluoroStar)
- UV/VIS spectrophotometer (Perkin Elmer Lambda 35)
- Heating stage (Linkam DC60)
- Fluorescence microscope (Nikon Ti Eclipse)
- FT-NIR analyser (Thermo Scientific Antaris II)
- Particle size analyser (Malvern Instruments Mastersizer 2000)
- Portable turbidimeter (AFScientific Inc. MicroTPW)
- Drop shape analysis system (Kruss DSA4)
- Electronic tensiometer (White Electronic Instruments DB 2KS)
- Bioanalyser (Nova Medical BioProfile FLEX)
• Automatic digital refractometer (Rudolph Research J357)
• Class II Biosafety Cabinet (Thermo Scientific Herasafe KS)
• Ultra Low Attachment cluster plates with lid and flat bottom (Corning CoStar Ultra)
• 96-well plates, black with clear bottom (BD Falcon)
• Thermo-responsive multidish (Thermo Scientific NUNC UpCell)

A2. Motor and pump calibrations

The Dispersion Cell utilises a mechanical stirrer driven by a DC motor to provide the shear stress at the membrane’s surface. It is possible to change the stirrer rotation velocity by varying the voltage of the motor which requires a calibration to link the voltage to rpm. The calibration is typical for each motor. For motor calibration, the velocity was set to six different voltage values and the revolutions per minute were measured with a digital Infrared tachometer. The measurements were repeated three times and the average values were considered. The trend line generated links the voltage to stirrer rotation velocity expressed as rpm. Figure A1 provides the calibration curve for the motor used.
Figure A1. Calibration curve for the motor used for the particle production experiments (refer to Chapters 4 and 5). Data points represent means of triplicate measurements. Error bars represent standard deviation (n=3).

In the experiments presented in this work, the dispersed phase (aqueous phase) was injected at the base of the Dispersion Cell by using either a peristaltic pump or a syringe pump. For the work presented here, one peristaltic pump (Watson Marlow 101U) and two syringe pumps (Harvard Apparatus pump 11 and KD Scientific KDS100) were employed. The calibration of a syringe pump is straightforward and it only depends on the type and diameter of the syringe used. When employing a syringe pump, there is the possibility to set the injection rate desired directly in mL/min. On the other hand, the calibration of a peristaltic pump is required in order to link the injection rate desired to the pump capacity and it depends on the specific pump, the liquid to be used and the tubing employed. In this
case, the calibrations of the peristaltic pumps were performed by measuring the deionized water volume in millilitres that was pumped through for a fixed period of time at different pump capacities. The calibration curve for the peristaltic pump used is shown in Figure A2. Although the pump was used for the injection of the dispersed phase, the calibrations were performed by using reverse osmosis water. We assumed that the error is small and it can be ignored. For all calibration curves, triplicate measurements were done and average values were used for plotting. Error bars represent standard deviation.

![Watson Marlow 101U pump calibration](image)

Figure A2. Calibration curve for the peristaltic pump used for all particle production experiments. Data points represent means of triplicate measurements. Error bars represent standard deviation (n=3).
A3. Presto Blue assay calibration curves

Presto Blue assay (Invitrogen, UK) was employed for cell proliferation tests in order to assess the cell culture capabilities of generated temperature responsive particles. The assay methodology is described in detail in section 3.7.3. Cell growth curves were generated by using this assay every day and measuring the fluorescence signal daily following the same protocol described in detail in section 3.7.3. To ensure that the output of the assay remains in a linear range, different cell seeding densities were used. Triplicate experiments were performed for each set of conditions.

Figure A3. Presto Blue calibration curves for 3T3 fibroblastic cells at passage 30 at two different incubation times. Fluorescent intensity values are plotted as mean values of three separate samples. Error bars are given by standard deviation (n=3).
CHAPTER 10. APPENDICES

Calibration curves for the cell type employed were generated for different incubation times (Figure A3). Briefly, the consumed media was aspirated, the cells were washed with Ca\(^{2+}\) and Mg\(^{2+}\) free D-PBS and 1 mL of 10% vol. Presto Blue solution was added per well in 6-well plates, followed by incubation at 37\(^\circ\)C for 10 minutes and 40 minutes. Three replicates were done for each seeding density. Following incubation, 100 µL from each well was transferred to a Falcon 96-well black polystyrene microplate with clear flat bottom (Becton Dickinson, UK) and replicated three times. Fluorescence intensity (Excitation / Emission: 544 nm / 590 nm) was measured on a BMG Labtech FLUOStar Omega microplate reader.

B. APPENDIX 2

B1. Image J analysis

The shrinkage ratio of the produced pNIPAM particles was measured by recording the differences in diameter of the particles below and above the phase transition temperature. The diameter was measured by taking phase contrast pictures with a Nikon Ti Eclipse inverted microscope, followed by size analysis when employing Image J software, a public Java-based image processing program http://rsb.info.nih.gov/ij/. Microscope calibrations were performed with the help of a graticule.

The acquired micrographs (Figure B1A) were then imported into Image J software and the scale was set based on the microscope calibrations. Background was then subtracted and the contrast of the image was adjusted followed by conversion into a binary image and threshold adjustments. Once the image was converted into black and white, all the circles were filled with black. In some cases, particles were touching each other making it necessary to apply the watershed function in order to automatically separate the borders of
the particles to be analysed (Figure B1B). The software also allows manual corrections in case that the automatic function of watershed has not been satisfactory. The particles touching the edges of the image were excluded from the analysis. Image J software numbers each droplet in order to be able to link it with the calculated size generated in the worksheet (Figure B1C). The measured diameter is Feret’s Diameter which is the longest distance between any two points along the selection boundary, also known as maximum calliper (Figure B1D). The generated data can then be exported in an Excel spread sheet for further analysis.

Figure B1. Image J analysis A) Original phase contrast picture taken with a Nikon Eclipse inverted microscope. Scale bar represents 100 µm. B) 8-bit processed image with particles coloured in black and separated by watershed function. C) Analysed image representing the final outlines of the particles to be counted and measured. The particles touching the edges of the image were excluded from the analysis. D) Feret’s diameter distribution.
B2. Modelling calculations

For the system employed in this work (Dispersion Cell), the general parameter values used are displayed in Table B1. These parameter values were applied for all calculations of predicted droplet size, regardless of the formulation employed. Based on the equations described above, two different calculations were performed based on two formulations used for particle production. An example of each calculation performed is provided in Table B3 and Table B4. The parameters that differ for each formulation and their values are presented in Table B2.

Table B1. General process parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter / m</td>
<td>D</td>
<td>0.031</td>
</tr>
<tr>
<td>Glass column height / m</td>
<td>H</td>
<td>0.160</td>
</tr>
<tr>
<td>Stirred cell diameter / m</td>
<td>T</td>
<td>0.035</td>
</tr>
<tr>
<td>Number of blades</td>
<td>Nb</td>
<td>2.000</td>
</tr>
<tr>
<td>Stirring blade height / m</td>
<td>b</td>
<td>0.011</td>
</tr>
<tr>
<td>Pore size / m</td>
<td>dₚ</td>
<td>0.00002</td>
</tr>
</tbody>
</table>
Table B2. Formulation specific parameters; F1 represents the formulation employed for solid pNIPAM particle production, while F2 is the formulation used for the production of core-shell pNIPAM-coated Alginate particles.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol</th>
<th>Values / F1</th>
<th>Values / F2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Continuous phase viscosity / Pa s</td>
<td>η</td>
<td>0.00164</td>
<td>0.012</td>
</tr>
<tr>
<td>Continuous phase density / kg/m³</td>
<td>ρ</td>
<td>800</td>
<td>920</td>
</tr>
<tr>
<td>Interfacial tension / mN/m</td>
<td>γ</td>
<td>2.5</td>
<td>32</td>
</tr>
</tbody>
</table>

Table B3. Example of droplet size prediction calculations for the formulation F1 employed for the production of solid pNIPAM particles

<table>
<thead>
<tr>
<th>Volts</th>
<th>rpm</th>
<th>ω (rev/s)</th>
<th>ω (1/s)</th>
<th>Re</th>
<th>δ (m)</th>
<th>R_{max} (m)</th>
<th>τ_{max} (Pa)</th>
<th>τ_{ave} (Pa)</th>
<th>Model A x (µm)</th>
<th>Model B x (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>173</td>
<td>18.12</td>
<td>113.8</td>
<td>1353</td>
<td>0.0001342</td>
<td>0.000804</td>
<td>0.922</td>
<td>157</td>
<td>0.22</td>
<td>284</td>
</tr>
<tr>
<td>3</td>
<td>287</td>
<td>30.09</td>
<td>188.9</td>
<td>2246</td>
<td>0.0001042</td>
<td>0.00093</td>
<td>2.28</td>
<td>103</td>
<td>0.59</td>
<td>175</td>
</tr>
<tr>
<td>4</td>
<td>401</td>
<td>42.05</td>
<td>264.09</td>
<td>3139</td>
<td>8.811E-05</td>
<td>0.000997</td>
<td>4.045</td>
<td>79.7</td>
<td>1.1</td>
<td>130</td>
</tr>
<tr>
<td>5</td>
<td>516</td>
<td>54.01</td>
<td>339.2</td>
<td>4032</td>
<td>7.774E-05</td>
<td>0.001039</td>
<td>6.13</td>
<td>67.1</td>
<td>1.7</td>
<td>106</td>
</tr>
<tr>
<td>6</td>
<td>630</td>
<td>65.97</td>
<td>414.3</td>
<td>4925</td>
<td>7.034E-05</td>
<td>0.001068</td>
<td>8.51</td>
<td>59.4</td>
<td>2.4</td>
<td>90.6</td>
</tr>
<tr>
<td>7</td>
<td>744</td>
<td>77.94</td>
<td>489.4</td>
<td>5818</td>
<td>6.472E-05</td>
<td>0.001089</td>
<td>11.1</td>
<td>54.3</td>
<td>3.2</td>
<td>80.1</td>
</tr>
<tr>
<td>8</td>
<td>859</td>
<td>89.9</td>
<td>564.5</td>
<td>6710</td>
<td>6.026E-05</td>
<td>0.001104</td>
<td>14.00</td>
<td>50.7</td>
<td>4.04</td>
<td>72.6</td>
</tr>
<tr>
<td>9</td>
<td>973</td>
<td>101.8</td>
<td>639.7</td>
<td>7603</td>
<td>5.661E-05</td>
<td>0.001117</td>
<td>17.07</td>
<td>48.2</td>
<td>4.9</td>
<td>66.8</td>
</tr>
<tr>
<td>10</td>
<td>1087</td>
<td>113.8</td>
<td>714.8</td>
<td>8496</td>
<td>5.355E-05</td>
<td>0.001127</td>
<td>20.35</td>
<td>46.4</td>
<td>5.9</td>
<td>62.4</td>
</tr>
<tr>
<td>11</td>
<td>1202</td>
<td>125.7</td>
<td>789.9</td>
<td>9389</td>
<td>5.094E-05</td>
<td>0.001135</td>
<td>23.81</td>
<td>45.04</td>
<td>7.0</td>
<td>58.8</td>
</tr>
<tr>
<td>12</td>
<td>1316</td>
<td>137.7</td>
<td>865.07</td>
<td>10282</td>
<td>4.868E-05</td>
<td>0.001142</td>
<td>27.45</td>
<td>44.02</td>
<td>8.08</td>
<td>56.02</td>
</tr>
</tbody>
</table>
Table B4. Example of droplet size prediction calculations for the formulation F\textsubscript{2} employed for the production of core-shell pNIPAM-coated alginate particles

<table>
<thead>
<tr>
<th>Volts</th>
<th>rpm</th>
<th>( \omega ) (rev/s)</th>
<th>( \omega ) (1/s)</th>
<th>Re</th>
<th>( \delta ) (m)</th>
<th>( R_{\text{trans}} ) (m)</th>
<th>( \tau_{\text{max}} ) (Pa)</th>
<th>Model A x (( \mu )m)</th>
<th>( \tau_{\text{ave}} ) (Pa)</th>
<th>Model B X (( \mu )m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>173</td>
<td>18.12</td>
<td>113.8</td>
<td>213</td>
<td>0.000338</td>
<td>0.000284</td>
<td>0.947</td>
<td>549</td>
<td>0.102</td>
<td>1668</td>
</tr>
<tr>
<td>3</td>
<td>287</td>
<td>30.09</td>
<td>188.9</td>
<td>353</td>
<td>0.000263</td>
<td>0.000409</td>
<td>2.91</td>
<td>314</td>
<td>0.39</td>
<td>854</td>
</tr>
<tr>
<td>4</td>
<td>401</td>
<td>42.05</td>
<td>264.09</td>
<td>493</td>
<td>0.000222</td>
<td>0.000504</td>
<td>5.93</td>
<td>220</td>
<td>0.901</td>
<td>562</td>
</tr>
<tr>
<td>5</td>
<td>516</td>
<td>54.01</td>
<td>339.2</td>
<td>634</td>
<td>0.000196</td>
<td>0.000579</td>
<td>9.93</td>
<td>171</td>
<td>1.63</td>
<td>418</td>
</tr>
<tr>
<td>6</td>
<td>630</td>
<td>65.97</td>
<td>414.3</td>
<td>774</td>
<td>0.000177</td>
<td>0.000640</td>
<td>14.8</td>
<td>141</td>
<td>2.59</td>
<td>332</td>
</tr>
<tr>
<td>7</td>
<td>744</td>
<td>77.94</td>
<td>489.4</td>
<td>914</td>
<td>0.000163</td>
<td>0.000691</td>
<td>20.5</td>
<td>121</td>
<td>3.75</td>
<td>277</td>
</tr>
<tr>
<td>8</td>
<td>859</td>
<td>89.9</td>
<td>564.5</td>
<td>1055</td>
<td>0.000152</td>
<td>0.000733</td>
<td>26.9</td>
<td>106</td>
<td>5.108</td>
<td>237</td>
</tr>
<tr>
<td>9</td>
<td>973</td>
<td>101.8</td>
<td>639.7</td>
<td>1195</td>
<td>0.000143</td>
<td>0.000769</td>
<td>34.1</td>
<td>95.7</td>
<td>6.65</td>
<td>209</td>
</tr>
<tr>
<td>10</td>
<td>1087</td>
<td>113.8</td>
<td>714.8</td>
<td>1335</td>
<td>0.000135</td>
<td>0.000800</td>
<td>41.9</td>
<td>87.3</td>
<td>8.36</td>
<td>186</td>
</tr>
<tr>
<td>11</td>
<td>1202</td>
<td>125.7</td>
<td>789.9</td>
<td>1476</td>
<td>0.000128</td>
<td>0.000827</td>
<td>50.3</td>
<td>80.65</td>
<td>10.24</td>
<td>169</td>
</tr>
<tr>
<td>12</td>
<td>1316</td>
<td>137.7</td>
<td>865.07</td>
<td>1616</td>
<td>0.000123</td>
<td>0.000851</td>
<td>59.3</td>
<td>75.2</td>
<td>12.2</td>
<td>155</td>
</tr>
</tbody>
</table>