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<th>Title</th>
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Integrating the Old & New:

Smart Thermoresponsive Surfaces &

3D Fabrication Technologies for

Tissue Engineering

By

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A thesis submitted for the degree of

Doctor of Philosophy

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1. GENERAL INTRODUCTION .................................................. 1
1.1. Evolution of Tissue Engineering and Regenerative Medicine .. 1
1.2. Smart Thermoresponsive Polymers for Cell Sheet Fabrication . 5
  1.2.1. Definition, Principle of Action and Quality Control of
         Thermoresponsive Cell Culture Dishes ....................................... 5
  1.2.2. Thermoresponsive Film Fabrication Methods ......................... 8
        1.2.2.1. Electron Beam Polymerization ..................................... 8
        1.2.2.2. Plasma Polymerization .............................................. 9
        1.2.2.3. Physical Adsorption .................................................. 9
        1.2.2.4. Solvent Casting ......................................................... 10
        1.2.2.5. Spin-Coating ............................................................ 10
        1.2.2.6. Typical Problems that can be Encountered during the
                 Spin-Coating of Substrates .............................................. 14
        1.2.2.7. Advantages and Disadvantages of Common Polymer
                 Film Preparation Methods .............................................. 18
  1.2.3. Cell Adhesion ............................................................................ 21
  1.2.4. Biophysical Factors Influencing the Cell Adhesion and
        Detachment from Thin Thermoresponsive Films ....................... 23
        1.2.4.1. Surface Chemistry and Electrical Charge ....................... 23
        1.2.4.2. Surface Wettability and Protein Adsorption ................... 25
        1.2.4.3. Surface Roughness and Topography ............................. 28
        1.2.4.4. Polymer Film Thickness ............................................. 29
1.2.5. Mechanisms of Cell Detachment from the Thermoresponsive Cell Culture Dishes................................................................. 31
1.3. Cell Sheet Engineering.............................................................. 32
1.3.1. Advantages of Cell Sheet-based Therapy ............................ 32
1.3.2. Biomedical Applications of Engineered Cell Sheets and Results of Preclinical Studies .......................................................... 34
1.3.3. Cell Sheet Manipulation Methods ......................................... 40
1.4. Scaffold-based Tissue Engineering Technologies .................. 42
1.4.1. Classification of Biomaterials Used in Scaffold-based Biomedical Research ................................................................. 42
1.4.2. 3D Scaffold Fabrication Methods ....................................... 46
1.4.3. Biomedical Applications of 3D Scaffolds ......................... 50
    1.4.3.1. Decellularized 3D Scaffolds in Regenerative Medicine ................................................................. 51
    1.4.3.2. 3D Polymer Scaffolds in Regenerative Medicine ...... 53
1.4.4. Integration of Scaffold-based and Scaffold-free Approaches 56
1.4.5. Comparative Analysis. Advantages and Challenges, Risks and Future Directions ................................................................. 58
1.5. Main Aims/Objectiveness.......................................................... 60
1.6. References............................................................................. 62
2. MATERIALS AND METHODS ............................................. 89
2.1. Materials ............................................................................. 89
2.2. Methods................................................................................ 90
2.2.1. Thermoresponsive Poly (N-isopropylacrylamide) Film Preparation Using Spin-Coating Technique ........................................ 90
    2.2.1.1. Solution Preparation................................................. 90
    2.2.1.2. Film Fabrication .................................................... 90
    2.2.1.3. Drying Process ..................................................... 92
    2.2.1.4. UV Sterilization...................................................... 92
2.2.2. Fabrication of Three-dimensional Star-shape Polylactide Scaffolds by Two-photon Fabrication Technique .......................... 92
2.2.2.1. Synthesis of Star-shaped Poly (D, L-lactide) .......... 92
2.2.2.2. Fabrication of 2PP scaffolds........................................ 93
2.2.2.3. UV Sterilization.............................................................. 95

2.2.3. Fabrication of Three-Dimensional Star-shape Polylactide
Scaffolds by Surface-selective Laser Sintering Method ................. 95
   2.2.3.1. Surface-Selective Laser Sintering of Poly (D, L-
lactide) Polymer Particles .......................................................... 95
   2.2.3.2. UV Sterilization.......................................................... 96

2.2.4. Fabrication of Three-dimensional Decellularized Pericardial
Scaffolds......................................................................................... 97
   2.2.4.1. Decellularization of Bovine Pericardial Tissue......... 97
   2.2.4.2. UV Sterilization.......................................................... 97

2.2.5. Physical Characterization of Biomaterials ......................... 97
   2.2.5.1. Fourier Transform Infrared (FTIR) Spectroscopy.. 97
   2.2.5.2. pNIPAm Film Thickness Assessment ......................... 97
       2.2.5.2.1. Laser Ablation ...................................................... 99
       2.2.5.2.2. pNIPAm Film Thickness Measurements Using
                   Optical Profilometry ......................................................... 99
   2.2.5.3. pNIPAm Film Roughness Assessment by Atomic
            Force Microscopy ................................................................... 101
   2.2.5.4. Hitachi Scanning Electron Microscope with Energy-
dispersive X-ray Analysis System ............................................. 103
   2.2.5.5. Contact Angle Measurements ................................. 106
   2.2.5.6. X-ray Powder Diffractometry ...................................... 109

2.2.6. Cell Culture Techniques ...................................................... 109
   2.2.6.1. Thawing Cryopreserved Cells................................. 109
   2.2.6.2. Cryopreservation of Cells.......................................... 110
   2.2.6.3. Subculture of Adherent Cells................................... 110
   2.2.6.4. Phase Contrast Microscopy ...................................... 111
   2.2.6.5. Cell Seeding on pNIPAm Films............................... 111
   2.2.6.6. Temperature-induced Cell Detachment from
            pNIPAm Films....................................................................... 112
2.2.6.7. Static Cell Seeding on Acellular Pericardial Scaffolds 112

2.2.6.8. Static Cell Seeding on 3D Polymer Scaffolds .......... 112

2.2.7. Assessment of Cell Proliferation, Viability and Metabolic Activity ................................................................. 113

2.2.7.1. Quant-iT™ PicoGreen® Assay ............................... 113

2.2.7.2. AlamarBlue™ Assay............................................. 115

2.2.7.3. Trypan Blue Viability Assay ................................. 117

2.2.7.4. Live/Dead Cell Viability Assay ............................... 118

2.2.8. Fluorescence Microscopy and Imaging Analysis ........ 119

2.2.8.1. Confocal Microscopy ............................................. 119

2.2.8.2. Imaging Analysis...................................................... 121

2.2.9. ECM Deposition Analysis of Cell Sheets .................... 122

2.2.9.1. Short-term Decellularization of Cell Sheets ....... 122

2.2.9.2. Extraction, Isolation and Densitometric Analysis of Type I Collagen ............................................................. 122

2.2.10. Histological Analysis .................................................. 123

2.2.10.1. Hematoxylin and Eosin Staining ............................ 123

2.3. Statistical Analysis ............................................................. 124

2.4. References ......................................................................... 126

3. FILM THICKNESS DETERMINES CELL GROWTH and CELL SHEET DETACHMENT from SPIN-COATED POLY (N-ISOPROPYLACRYLAMIDE) SUBSTRATES ......................... 131

3.1. Introduction ..................................................................... 132

3.2. Results and Discussion ...................................................... 136

3.2.1. Physical Characterization of pNIPAm Films ............ 136

3.2.1.1. pNIPAm Film Thickness Measurements ............ 136

3.2.1.2. Contact Angle Measurements of pNIPAm Films ... 137

3.2.1.3. Surface Roughness Evaluation of pNIPAm Films .. 140

3.2.2. Cell Cultures on pNIPAm Films of Different Thicknesses 141

3.2.2.1. Cell Adhesion, Growth and Morphology Analysis 142

3.2.2.2. Cell Viability and Metabolic Activity Analysis ...... 146
3.2.2.3. Actin Cytoskeleton Organization and Focal Adhesion Assessment ............................................................................................................ 148

3.2.3. Cell Detachment Analysis ................................................................. 153

3.3. Conclusions ........................................................................................................ 156

3.4. References ........................................................................................................ 158

4. FABRICATION of FUNCTIONAL HIGH-DENSITY CELL SHEETS for TISSUE ENGINEERING USING SPIN-COATED POLY (N-ISOPROPYLACRYLAMIDE) THIN FILMS ........................................ 165

4.1. Introduction ........................................................................................................ 165

4.2. Results and Discussion ....................................................................................... 167

4.2.1. Generation and Morphology of MS-5 and HCEC Lifted Cell Sheets ................................................................. 167

4.2.2. Viability and Metabolic Activity Evaluation of MS-5 and HCEC Sheets ........................................................................................................ 170

4.2.3. Cytoskeleton Reorganization Analysis during Cell Sheet Detachment ........................................................................................................ 173

4.2.4. ECM Deposition Assessment of MS-5 and HCEC Sheets ................................................................................................. 178

4.3. Conclusions ........................................................................................................ 182

4.4. References ........................................................................................................ 183

5. TISSUE ENGINEERING: DELIVERY OF LAYERED CELL SHEETS USING THREE-DIMENSIONAL SCAFFOLDS ........................................................................................................ 188

5.1. Introduction ........................................................................................................ 188

5.2. Results and Discussion ....................................................................................... 191

5.2.1. Cell Sheet-seeding and Biological Analysis of Scaffolds Fabricated by Two-photon Polymerization Technique ........................................ 191

5.2.1.1. Structural Analysis of 2PP scaffolds ........................................ 191

5.2.1.2. Cytotoxicity Evaluation of Microstructured 2PP Scaffolds ................................................................. 195

5.2.1.3. Structural and Functional Analysis of Integrated System for 3D Assembly of Cell Sheets and 2PP scaffolds .... 197

5.2.2. Cell Sheet-seeding and Biological Analysis of Scaffolds Fabricated by Surface-selective Laser Sintering Method ........................................ 200

5.2.2.1. Structural Analysis of SSLS Scaffolds ................................ 200
5.2.2.2. Cytotoxicity Evaluation of Microstructured SSLS scaffolds 204

5.2.2.3. Structural and Functional Analysis of Integrated System for 3D Assembly of Cell Sheets and SSLS scaffolds . 207

5.2.3. Cell Sheet-seeding and Biological Analysis of Acellular Pericardial Scaffolds ................................................................. 209

5.2.3.1. Microscopy Analysis of Acellular Pericardial Scaffolds.............................................................................210

5.2.3.2. Cytotoxicity Evaluation of Acellular Pericardial Scaffolds.............................................................................214

5.2.3.3. Structural and Functional Analysis of Integrated System for 3D Assembly of Cell Sheets and Acellular Pericardial Scaffolds ........................................................................... 216

5.3. Conclusions ............................................................................. 220

5.4. References ............................................................................... 222

6. GENERAL CONCLUSIONS ................................................ 227

7. Appendix I ............................................................................... 231

8. Appendix II ............................................................................... 239
LIST of FIGURES

Figure 1.1. The healing of Justinian by Saint Cosmas and Saint Damian [by Fra Angelico, 1438].

Figure 1.2. Dr. Faustus and his Homunculus.

Figure 1.3. Chemical structure of pNIPAm.

Figure 1.4. Schematic illustrating the basic stages of the dynamic spin-coating method.

Figure 1.5. Schematic illustration of the edge bead effect of spin-coating.

Figure 1.6. Illustration of the multicomponent protein layer adsorbed from serum-containing cell culture media on hydrophilic and hydrophobic surfaces of pNIPAm films with increased thickness and hydrophobicity. (A) Albumin adsorbs to the hydrophilic surface along with adhesive proteins allowing the latter to expose their cell-binding sites. The adherent cell has a flattened shape. (B) Albumin rapidly and firmly adsorbs to the hydrophobic surface, spreads and affects the adsorption of adhesive proteins in their active conformation. The cells adhere and spread over more hydrophilic surface while loosely attaching on more hydrophobic pNIPAm surface. The non-adherent cell has a spheroid shape. Albumin is represented in red and adhesive proteins are in green. [24]

Figure 2.1. Illustration of the spin-coating technique. (A) Laurell Technologies WS-400B-6NPP/LITE spin coater. (B) Spin coater deposit a very thin layer of liquid onto the surface of the spinning substrate.

Figure 2.2. Shimadzu FTIR 830 Fourier transform infrared spectrometer. (NCBES, NUIG)

Figure 2.3. Light interference microscope ZygoNew View 100 (National Centre for Laser Applications, School of Physics, NUI Galway).
Figure 2.4. The Dimension 3100 Atomic Force Microscope (NCBES, NUIG).

Figure 2.5. Schematic illustration of interaction volume of the electron beam with a sample.

Figure 2.6. Hitachi S-4700 Scanning Electron Microscope (SEM).

Figure 2.7. Schematic illustration of a liquid drop on a solid surface with energy vectors and contact angle (θ) as described by the equation.

Figure 2.8. The scheme of the reaction mechanism between alamarBlue reagent and viable cells.

Figure 2.9. Hemocytometer gridlines.

Figure 3.1. Polymer film thickness as a function of polymer concentration.

Figure 3.2. The dependence of contact angle on pNIPAm film thickness before and after thermal annealing at 120 °C under vaccum.

Figure 3.3. Contact angles measured on (A) Thermanox control, (B) 50 nm, (C) 80 nm, (D) 120 nm, (E) 300 nm and (F) 900 nm pNIPAm films at 40 °C.

Figure 3.4. Bright field microscopy images of MS-5 cells cultured on (A) Thermanox control, (B) 50 nm, (C) 80 nm, (D) 120 nm, (E) 300 nm and (F) 900 nm pNIPAm films. Initial cell-seeding density 40000 cells/cm², incubation time 48 h, scale bar 100 μm.

Figure 3.5. Adhesion and proliferation of MS-5 grown on pNIPAm films of different thicknesses (50-900 nm). Initial cell-seeding density 40000 cells/cm², incubation time 12 h, 24 h and 48 h. Data is mean ± standard error of mean (SEM), * p<0.05 compared with control. Cell numbers were calculated based on a calibration curve of DNA amount vs. known cell numbers.
Figure 3.6. Cell metabolic activity of MS-5 grown on control slides and pNIPAm films with different thickness. AlamarBlue assay test results. Initial cell-seeding density 40000 cells/cm$^2$, incubation time 48 h. Data is mean ± standard error of mean (SEM), no significant difference compared with control.

Figure 3.7. Live/dead assay images representing cell viability of MS-5 cells on (A) uncoated control glass slides (alive cells), (B) uncoated control glass slides after treatment with DMSO (dead cells), (C) 50 nm, (D) 80 nm, (E) 120 nm, (F) 300 nm and (G) 900 nm pNIPAm films. Initial cell-seeding density 40000 cells/cm$^2$, incubation time 48 h, scale bar 100 μm.

Figure 3.8. Immunofluorescence microscopy images of paxillin (green) and actin (red) staining of MS-5 cells cultured on (A) control slides, (B) 50 nm, (C) 80 nm, (D) 120 nm, (E) 300 nm and (F) 900 nm pNIPAm films. (G) Statistical analysis of the number of paxillin-positive focal adhesions per cell in different groups. Initial cell-seeding density 40000 cells/cm$^2$, incubation time 48 h, scale bar 50 μm. Data is mean ± standard error of mean (SEM), * p<0.05 compared with control. The estimation of the number of focal adhesion per cell was carried out, first, for at least 10 cells from each group. The white arrows indicate focal adhesion sites.

Figure 3.9. Images of MS-5 cell detachment from (A) Thermox control, (B) 50 nm and (C) 80 nm pNIPAm films at 4 °C. Initial cell-seeding density 40000 cells/cm$^2$, incubation time 7 days, cell detachment time 25-30 min, scale bar 100 μm. The yellow arrow indicates a detaching MS-5 sheet.

Figure 4.1. Morphological characteristics of engineered MS-5 and HCEC cell sheets. (A) Phase contrast images of MS-5 and HCEC
growing on pNIPAm-coated surface prior to cell detachment. Scale bar: 100 μm. (B) The cell sheets began to detach from pNIPAm-coated culture dishes by reducing temperature to 4 °C. Scale bar: 100 μm. (C) Engineered MS-5 and HCEC cell sheets demonstrated significant contraction when lifted off the cell culture dish. Scale bar: 10mm. The yellow arrow indicates the detaching cell sheet. The white arrows indicate a ruptured cell sheet.

**Figure 4.2.** Live/Dead fluorescent staining of 2-week MS-5 and HCEC sheets before detachment, 1 h after detachment and 48 h after re-attachment.

**Figure 4.3.** Results of alamarBlue assay for 2-week MS-5 and HCEC sheets before and after detachment and 48 h after re-attachment to TCPS. Data is mean ± standard deviation (SD), no significant difference compared with control.

**Figure 4.4.** Immunofluorescent staining of 2-week MS-5 and 2-week HCEC before and after detachment. Phalloidin staining for F-actin (red), DAPI nuclear staining (blue). Scale bar: 20 μm.

**Figure 4.5.** Immunofluorescent staining of MS-5 and HCEC before and after detachment. Anti-paxillin antibody staining (green) and actin-phalloidin staining (red). Scale bar: 20 μm. White arrows indicate focal adhesion sites.

**Figure 4.6.** ECM deposition analysis of MS-5 and HCEC cell sheets. (A) Scanning electron microscopy images, showing 2-week MS-5 sheet secreted richer ECM than 2-week HCEC sheet. (B) H&E staining of MS-5 and HCEC cell sheets. MS-5 formed more cell layers and ECM than HCECs. The 2-week MS-5-sheet consisted of four-five cell layers with approximately 40-50 μm thickness, the 2-week HCEC-sheets were composed of approximately 2-3 cell layers with 10-20 μm in thickness.
Scale bar: 50 µm. (C) SDS-PAGE analysis of type I collagen in 2-week MS-5 and 2-week HCEC cell sheets. (D) Densitometric analysis of type I collagen in 2-week MS-5 cell sheets.

**Figure 5.1.** 3D hexagonal scaffolds fabricated by 2PP polymerization technique.

**Figure 5.2.** FTIR spectrum of poly (D, L-lactide) used for 2PP fabrication of 3D scaffolds.

**Figure 5.3.** SEM surface images of 3D scaffolds generated by 2PP polymerization technique. (A) Spatial arrangement of hollow cylinders within 2PP scaffolds. (B) The typical surface morphologies of the 2PP scaffolds. (C) 3D structural analysis of 2PP scaffolds.

**Figure 5.4.** In vitro cell growth of MS-5 stromal cells on 2PP scaffolds. (A and B) SEM images (top and side) of stromal cells cultured on 2PP scaffolds over 7 d. The yellow arrows indicate the cells covered the scaffold and the white arrows indicate cells migrated inside the scaffold. (C) AlamarBlue assay results indicate high metabolic activity of attached MS-5 cells after 7 d in culture.

**Figure 5.5.** In vitro cell growth of MS-5 sheet on 2PP scaffolds. (A) SEM of a MS-5 stromal cell sheet attached to 2PP-fabricated scaffolds after 7 d in culture. The yellow arrow indicates an attached MS-5 sheet. The white arrow indicates the cells migrated inside the scaffold. (B) AlamarBlue assay results: comparison between MS-5 single cell suspension and cell sheets seeded and grown on 2PP scaffolds for 7 d.

**Figure 5.6.** 3D polylactide scaffold fabricated by surface-selective laser sintering method.

**Figure 5.7.** FTIR spectrum of poly (D, L-lactide) used for SSLS fabrication of 3D scaffolds.
Figure 5.8. XRPD patterns of amorphous SSLS scaffolds.

Figure 5.9. SEM surface images of SSLS scaffolds. (A) The typical surface morphology of laser-sintered specimens. (B) The powder particles exhibit irregular morphology. The scaffold with pore sizes of about 700 μm in width and 560 μm in depth.

Figure 5.10. SEM analysis of MS-5 cell growth on SSLS scaffolds. (A) Most of the cells predominantly form dense cell clusters inside the pores of the scaffolds after 7 d in culture. The white arrows indicate cell clusters inside the pores. (B) The cells form well-defined processes that attach to the surface of granules. The yellow arrows indicate processes.

Figure 5.11. Cytotoxicity analysis of SSLS scaffolds. (A) Live/Dead assay of MS-5 cells grown on SSLS scaffolds for 7 d. (B) AlamarBlue assay results indicate high metabolic activity of attached cells after 7 d in culture.

Figure 5.12. In vitro cell growth of MS-5 sheets on SSLS scaffolds. (A) SEM image of a MS-5 sheet grown on the SSLS scaffold for 7 d in culture. The yellow arrow indicates an attached MS-5 sheet. The white arrow indicates a scaffold. (B) AlamarBlue assay results: comparison between single cell suspension and cell sheets seeded and grown on SSLS scaffolds for 7 d.

Figure 5.13. SEM of (A) native and (B) decellularized pericardial tissues.

Figure 5.14. Hematoxylin and eosin (H&E) staining of (A) native and (B) decellularized pericardial tissue samples. The white arrows indicate nuclei.

Figure 5.15. DAPI staining of native (A) and decellularized (B) pericardial samples. The white arrow indicates DNA.
**Figure 5.16.** Cytotoxicity analysis of pericardial samples. Live/dead assay of hMSCs on (A) fresh tissue (alive cells), (B) fresh tissue after treatment with DMSO (dead cells) and (C) decellularized tissue. (D) AlamarBlue assay results: the comparison of metabolic activity of hMSCs grown on native (fresh) and decellularized pericardial tissues after 7 d in culture.

**Figure 5.17.** Microscopy analysis of a multilayered hMSC sheet onto acellular bovine pericardial scaffolds. (A) SEM image of hMSC sheet cultured on bovine acellular pericardial tissue for 7 d. (B) H&E staining of cross-section of acellular BP scaffold with attached hMSC sheet after 7 d in culture. (C) AlamarBlue assay results: the comparison of metabolic activity between single hMSC suspension and hMSC sheets grown on decellularized pericardial tissues for 7 d. The white arrows indicate an acellular pericardial scaffold, the yellow arrows indicate an attached cell sheet.
LIST OF TABLES

Table 1.1. The spin-coating process troubleshouting. [53]

Table 1.2. Summary of main advantages and disadvantages of the methods commonly used for polymer film deposition.

Table 1.3. Overview of advantages and disadvantages of various natural and synthetic materials.

Table 2.1. PicoGreen® standard curve calculations.

Table 2.2. A detailed IHC tissue processing protocol.

Table 3.1. Average advancing water contact angle measurements taken at 40 °C on spin-coated pNIPAm films before and after thermal annealing.

Table 3.2. Thickness and RMS values of pNIPAm films.

Table 7.1. Summary of characteristics of MS-5 and HCEC sheets cultured for 1 week and 2 weeks.
LIST OF ABBREVIATIONS

2PP- Two-photon Polymerization;
3D - Three-dimensional;
AFM - Atomic Force Microscopy;
ANOVA- Analysis of Variance;
ATRP- Atom Transfer Radical Polymerization;
bFGF - basic Fibroblast Growth Factor;
BP- Bovine Pericardium;
CPC- Calcium Phosphate Cements;
DAPI - 4’,6-Diamidino-2-Phenylindole;
DCL- Decellularized;
DMEM- Dulbecco’s Modified Eagle Medium;
DMSO- Dimethyl sulfoxide;
DNA - Deoxyribonucleic Acid;
DNase- Deoxyribonuclease;
DPBS- Dulbecco’s Phosphate Buffered Saline;
EBP- Electron Beam Polymerization;
ECM - Extracellular Matrix;
EDX- Energy-Dispersive X-ray;
FAs- Focal Adhesions;
FBS - Fetal Bovine Serum;
FTIR- Fourier Transformed Infrared Spectroscopy;

GAGs- Glycosaminoglycans;

HBSS- Hank’s Balanced Salts Solution;

HCEC - Human Corneal Epithelial Cells;

HepG2- Liver Hepatocellular cells;

H&E- Haematoxylin and Eosin;

HGF - Hepatocyte Growth Factor;

hMSC- Human Mesenchymal Stem Cells;

HMDS-Hexamethyldisilazane;

HSPCs-Hematopoietic Stem/Progenitor Cells;

ICC- Immunocytochemistry;

LCST- Low Critical Solution Temperature;

MS-5-Mouse Bone Marrow-derived Stromal Cell Line;

PCL – Polycaprolactone;

PEG- Poly (Ethylene Glycol);

PHB – Poly (b-Hydroxybutyrate);

PLGA – Poly (Lactic-co-glycolic Acid);

PIGF - Placental Growth Factor;

PLLA- Poly-L-Lactide;

PMEDSAH- Poly [2-(Methacryloyloxy)-Ethyl Dimethyl-(3-Sulfopropyl) Ammonium Hydroxide];

PMT- Photomultiplier Tube;
pNIPAm -poly (N-isopropylacrylamide);

POEGMA – Poly [Oligo(Ethylene Glycol)] Methacrylate;

PU –Polyurethane;

PVA -Polyvinyl Alcohol;

RAFT-Reversible Addition-Fragmentation Chain Transfer Radical Polymerization;

RGD- Arginylglycylaspartic Acid;

RMS-Root-Mean-Square;

RNAse- Ribonuclease;

RT –Room Temperature;

SD- Standard Deviations;

SDF-1-Stromal cell Derived Factor-1;

SDS -Sodium Dodecyl Sulfate;

SDS-PAGE- Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis;

SEM- Scanning Electron Microscopy;

SMB- Skeletal Myoblasts;

SSLS –Surface-selective Laser Sintering Method;

TCPS- Tissue Culture Polystyrene;

TCP –Tricalcium Phosphate;

TE Buffer- Tris-EDTA Buffer;

TGF-β -Transforming Growth Factor-β;
VEGF - Vascular Endothelial Growth Factor;

XPS - X-ray Photoelectron Spectroscopy;

XRPD - X-ray Powder Diffraction;
DECLARATION

The work in this thesis is based on the research carried out at the School of Chemistry and National Centre for Biomedical Engineering Science (NCBES), College of Science, National University of Ireland Galway.

I, Nina Dzhoyashvili, hereby certify that this thesis has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a degree or qualification.
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DEDICATION

I dedicate this work to my parents, Marina and Alexander Dzhoyashvili, who have inspired me with their wisdom, kindness, generosity, and passion for hard work.
ABSTRACT

Several studies have shown very promising preclinical and clinical outcomes of cell therapy. However, direct cell injections offer little control over local retention and distribution of the injected cell suspensions leading to short-term therapeutic efficiency. The advantage of cell sheet-based approach for regenerative medicine is that tissue regeneration could be more effective if tissue-like constructs which are consisted of one or more types of cells and naturally derived, tissue-specific extracellular matrix (ECM) are used. Cell sheet tissue engineering technologies have been rapidly developed and applied in different areas of biomedical research, including regenerative medicine, cell-based drug screening assays and tissue and disease modelling. The preserved ECM and developed cell–cell and cell–matrix interactions in cell sheets may greatly support cell adherence to tissue after in vivo transplantation. Matrix materials as delivery vehicles might increase cell-to-cell and cell-to-tissue interactions and organization, cell sheet strength and survival. Many preclinical and clinical studies have reported the promising therapeutic potential of sheet-like cell transfer for the regeneration of skin, blood vessels, cornea, heart, lung, liver and bone. However, unlike the three-dimensional scaffold-based approach, engineered tissues created using cell sheets are not suitable for the production of organ-like structures that possess a complex 3D organizational architecture. This obstacle could be overcome by integrating multilayer cell sheets with 3D biomaterial scaffolds that could provide structural support to the construct.

Currently, poly (N-isopropylacrylamide) is widely used to fabricate thermoresponsive surfaces for cell sheet detachment. The substrates
when coated with pNIPAm or its copolymers enable the detachment of cells from culture dishes by temperature reduction below the lower critical solution temperature and without using digestive reagents that destroy extracellular matrix assembly, intercellular connections, and focal adhesion complexes. Many complex and expensive techniques have been employed to produce pNIPAm substrates for cell culture. The spin-coating technique allows rapid fabrication of pNIPAm substrates with high reproducibility and uniformity. The method of polymer deposition can significantly impact the biological properties of pNIPAm films. Several studies have investigated the dependency of cell behavior on the thickness of covalently grafted pNIPAm films. It was found that the thickness dependency of cell attachment and detachment is not observed for plasma induced polymerization method, but this dependency was shown for electron beam irradiation technique. There has been little investigation of cellular behavior on spin-coated pNIPAm films.

In this study, the dynamics of cell attachment, proliferation, and function on non-cross-linked spin-coated pNIPAm films of different thicknesses were examined. The thickness of pNIPAm films fabricated by spin-coating was shown to influence the cellular attachment and spreading. The cells grown on spin-coated pNIPAm surfaces showed significant changes in cell densities and perturbations in cellular cytoskeletal organization. It was found that 300 and 900 nm thick spin-coated pNIPAm films impeded cell attachment, while 50 and 80 nm pNIPAm substrates were the most preferable for cell attachment, functions, and detachment as a contiguous cell sheet. Next, biological properties of stromal and epithelial cell sheets after manipulation such as detachment from spin-coated pNIPAm films, transfer and re-attachment were assessed. The cell morphology, the pattern and speed of cell sheet...
recovery and total cell number in cell sheets were analyzed. In addition, the metabolic activity, proliferation and cell viability of cell sheets before and after detachment and re-attachment were also examined. To investigate cell sheet remodeling before and after detachment, the histological analysis of cell sheets and the immunofluorescent analysis of focal adhesion and actin stress fibers were performed. SEM images of ECM and the content of type I collagen were compared between lifted MS-5 and HCEC sheets in order to assess the role of extracellular matrix in cell sheet formation, maturation and detachment. These results can be valuable and used in tissue engineering purposes in providing a validation system for cell sheet systematic assessment, for drug testing and designing mechanically-balanced microtissues. Next, an integrated approach was applied to create tissue-like constructs from multilayered cell sheets and 3D natural (acellular pericardial matrix) or 3D synthetic (2PP-fabricated or SSLS-fabricated) scaffolds. Microscopy analysis showed that cell sheets together with ECM attached, homogeneously and continuously distributed and achieved confluence. These findings should promote further investigation of cell behavior and cell-cell and cell-ECM communication in 3D tissue-like microenvironments. The development of tissue constructs engineered from tissue-specific cell sheets and 3D scaffolds with precise microarchitecture can significantly promote the generation of implantable tissues and advance regenerative medicine.
1. GENERAL INTRODUCTION

1.1. Evolution of Tissue Engineering and Regenerative Medicine

The generation of complex bioartificial tissues, organs or life from non-living materials has been a matter of myths and dreams since the early history of men. During the last decades these myths and dreams became feasible through scientific discoveries and achievements and have been rapidly introduced into the therapeutic arena. The first collection of stories regarding the capacity of tissues and organs to regenerate may be related to the ideas of Ancients Greek mythology. In the famous myth, Prometheus had his liver regenerating every day after being pecked out by an eagle. The Biblical tale of Eve created from the bone tissue, the rib of Adam, is another famous metaphysical idea which is similar to the modern cloning techniques. The famous painting “Healing of Justinian” visualization of the legend of St. Cosmas and St. Damien is one of earliest instances depicting the limb transplantation. (Figure 1.1)

Figure 1.1. The healing of Justinian by Saint Cosmas and Saint Damian [by Fra Angelico, 1438].
During the period of the Middle Ages and the Renaissance, there was a revolutionary idea promoted by a number of scientists to generate living organisms using the methods of ancient chemistry and alchemy. Johann Wolfgang von Goethe introduces this vision in his fundamental work of literature Faust [1]:

*Look there’s a gleam! – Now hope may be fulfilled,*

*That hundreds of ingredients, mixed, distilled –*

*And mixing is the secret – give us power*

*The stuff of human nature to compound*

Goethe’s description of the creation of the artificial being, Homunculus, reflects the irrepressible dream of humankind to engineer life. (Figure 1.2)

![Figure 1.2. Dr. Faustus and his Homunculus.](image-url)
By mixing different substances and adding them to nonliving materials, Faust hopes to regenerate life. Interestingly, the description of the creation of Homunculus can be related to such modern technologies as cloning, genetic, stem cells and biomaterials-based techniques which are currently commonly used in tissue engineering and regenerative medicine.

The further evolution of biomedicine has been closely connected with the origin of cell biology, genetics, biochemistry and clinical medicine. The archeological findings of human skulls, containing a metallic implant in the jaw, are evidences that humans have attempted to replace missing teeth since the first or second century AD. [2] The history of modern tissue engineering has started from the use of skin grafts. Importantly, the results of skin transplantation were investigated not only at clinical level, but were confirmed by animal experimental work. [3] Breakthroughs in the development of skin sheets used in clinical practice were made by Heinrich Bunger who first successfully transplanted skin graft. [4] The improvements in tissue engineering have been further advanced by fundamental biological work. Rudolf Virchow described in his “Cellularpathologie” that tissue regeneration is dependent on cell proliferation. [5] This concept highlighted the essential role of cellular components in tissue regeneration and led to the development of in vitro cell cultivation. Ljunggren and Jolly were the first researchers to attempt to cultivate cells outside the body [6], and Harrison demonstrated active growth of cells in culture. [7]

The outstanding Russian–born scientists Maximov and Friedenstein devoted their lives to the study of stem cells. [8, 9] Their theories on stem cells were too far ahead of their time and were met with skepticism at first. In 1924, Maximov presented a theory of hematopoiesis and a concept of hematopoietic stem cells. Maximov used
extensive histological findings to identify a singular type of precursor cell within mesenchyme that develops into different types of blood cells.[10] In 1970, Friedenstein and colleagues reported the clonogenic potential of fibroblast-like cells with a high replicative capacity in vitro. [11] The researcher isolated for the first time adherent colony forming unit-fibroblasts (CFU-F), which were multipotential and were able to differentiate into osteoblasts, chondrocytes, adipocytes and hematopoietic supporting stroma. [12, 13] The scientific concept of stem cells developed by Maximov and Friedenstein inspired other scientists and medical doctors to develop hematopoietic stem cell transplantation and other methods of regenerative medicine.

The novel concept of assembly of cells in three-dimensional structures has been introduced in the early 1970s. Green undertook a number of experiments to generate cartilage tissue using a chondrocytes cultured in combination with a bone scaffold. Green performed successful experiments investigating the reparative potential of autologous and homologous chondrocyte transplantation. He used decalcified bone as a type of scaffold for cell transplantation. Green was also the first to use the rabbit as a model to study cartilage repair. Although his success was hampered by the limitations of technologies of the times, his work was a cornerstone for the future of cartilage repair as well as a pioneer in the field of tissue engineering. [14] First innovations in scaffold-free cell sheet engineering technologies have been made by Burke and Yannas who generated skin by a culture of dermal fibroblast or keratinocyte sheets and used them for the regeneration of burn wounds. [15] The modern concept of tissue engineering emerged in the late 1980s. Firstly, the term “tissue engineering” was randomly used in cases of surgical manipulation of tissues and organs or when prosthetic devices or biomaterials were
used. Currently, the term “tissue engineering” is defined: “Tissue Engineering is the application of the principles and methods of engineering and life sciences toward the fundamental understanding of structure-function relationships in normal and pathologic mammalian tissue and the development of biological substitutes to restore, maintain, or improve function.” [16]

As the field of tissue engineering and regenerative medicine is established as a central discipline in biomedicine nowadays, it seems interesting to speculate about the future of biomedical research. The aim is to tackle the biological complexity of organs and to engineer 3D system that functions as reliably as the real thing. The understanding and control of cell growth and differentiation in 3D microenvironment could give a crucial key to achieving this goal. In this case, research in a clinically optimal cell sourcing and cell-cell and cell-biomaterial interactions may improve the outcome of tissue healing. Moreover, the angiogenic and immunologic control during tissue regeneration are not yet at the forefront of the daily work, but considered to be highly strategic issues. With respect to the near future, the important concept supported by the largest number of specialists is safety and quality control of tissue engineering constructs oriented towards clinical application and manufacturing.

1.2. Smart Thermoresponsive Polymers for Cell Sheet Fabrication

1.2.1. Definition, Principle of Action and Quality Control of Thermoresponsive Cell Culture Dishes

Surface engineering using thermoresponsive polymers has been stimulated by the rising demand for active and switchable biointerfaces. [17] Among different types of thermoresponsive substrates, the
pNIPAm-based ones have a particularly high application potential due to exceptional dynamic response of such surfaces. (Figure 1.3) Upon application of a stimulus, thermoresponsive polymers undergo changes on a molecular scale, which are manifested also as macroscopic changes in their physicochemical properties. [18, 19] The development of such “smart” substrates that dynamically change their surface characteristics in response to applied stimuli, thereby mimicking the dynamic properties of biological systems, is an important research area in the field of tissue engineering. Such surfaces can modulate biomolecule activity [20], protein immobilization [21] and cell adhesion or migration and therefore, they can be useful in diverse applications in regenerative medicine, controlled drug delivery, and diagnostics, and as tools for the study of basic cell biology. Moreover, thermoresponsive substrates may be designed to accommodate active biomolecules (growth factors and drugs) and released on demand upon application of a certain stimulus. [22]

![Chemical structure of pNIPAm](image)

**Figure 1.3. Chemical structure of pNIPAm.**

In the 1990s, Okano’s research group in Japan has pioneered in this method, using electron beam polymerization uniformly spreading
isopropylacrylamide monomers in 2-propanol that were cross-linked and covalently immobilized onto the surfaces of tissue culture polystyrene (TCPS) dishes. The grafted pNIPAm made the cell culture surface sensitive to ambient temperature. At 37 °C, thermoresponsive surface becomes hydrophobic allowing various types of cells adhere and grow on the grafted pNIPAm-modified dishes. These cultured cells detach when the temperature is decreased below 32 °C because at this temperature, the surface becomes hydrophilic owing to changes in the hydration/dehydration of pNIPAm. [23]

Using different technologies for preparing thermoresponsive surfaces, the quality of thermoresponsive cell culture dishes has been investigated in many studies. The quality of thermoresponsive cell culture dishes significantly influences cell attachment, proliferation and detachment. To determine whether the deposition of pNIPAm was successful, it is very important to assess pNIPAm-modified surfaces using analytical techniques. Qualitative and quantitative analyses of pNIPAm films can be performed by scanning electron microscopy (SEM), atomic force microscopy (AFM), optical profilometry, X-ray photoelectron spectroscopy (XPS), Fourier transform infrared spectroscopy (FTIR) and contact angle measurements. Notably, pNIPAm deposited on polymeric substrates requires sensitive and selective detection because signals of pNIPAm polymer are generally similar to those of the polymeric substrates. SEM can be used for the characterization of a uniform coverage of pNIPAm on the surface. AFM is ideal to determine the morphology of pNIPAm films. XPS is usually used to determine the surface chemistry of a sample. FTIR is a powerful analytical tool characterizing pNIPAm deposition and identifying chemical bonds and molecular structure. Contact angles can be used to check the cleanliness and homogeneity of polymer films. There are different methods to
examine the thickness of polymer films. The thickness of pNIPAm films can be successfully measured optically by viewing optical profilometry and employing a laser ablation technique. \[24-26\]

1.2.2. Thermoresponsive Film Fabrication Methods

1.2.2.1. Electron Beam Polymerization

The choice of the fabrication method may significantly affect the physicochemical and biological characteristics of polymer film surface. The methods which are used to fabricate thin pNIPAm films can be divided, according to preparation procedure, into two major categories: covalently polymerization bonding \([27-29]\) and polymer coating. \([30-34]\) Polymerization and grafting by irradiation employing electron beam (EB) plasma as the irradiation source is widely used to deposit the polymer on the substrate. During electron beam polymerization (EBP), high energy electron beam irradiation leads to the cleavage of chemical bonds in a suitable chosen initiator which in turn initiate free radicals the polymerization of the deposited monomers. The polymerized monomers have been deposited on a substrate. By changing the conditions of the reaction, such as monomer concentration and the energy of the electron beam, the thickness of the polymer layer can be carefully controlled. \([35, 36]\) The EBP of NIPAm monomers has been widely used to fabricate pNIPAm-modifed thermoresponsive cell culture dishes capable of supporting the attachment and growth of a variety of adherent cell types. \([37-39]\) The cells grown on thermoresponsive substrates can be rapidly detached by lowering the temperature below the low critical solution temperature (LCST) of the deposited polymer. Several studies have shown the dependency of cell behavior on the thickness of covalently grafted pNIPAm films. In particular, the pNIPAm films with thickness higher than 20 nm fabricated by electron beam irradiation impede cell
attachment, and the optimal thickness was found to be between 15 and 20 nm. [28]

1.2.2.2. Plasma Polymerization

During plasma polymerization process, the polymer coatings are directly deposited on the substrate. It is possible to produce polymer films without use of a solvent. The high energy plasma initiates monomer fragmentation and recombination that cause to the rearrangement of the polymer structure. [40-42] Importantly, several studies have shown that plasma polymerized pNIPAm-modified TCPS can be successfully used for cell growth and expansion. [43] Interestingly, the thickness dependency of cell attachment and detachment has not been observed for plasma induced polymerization method in comparison to electron beam irradiation technique. [29] This is probably due to the differences in the process of the polymer film deposition. However, covalent polymerization bonding techniques are too expensive and too complex to be used in the general practice.

Polymer coating employs the deposition of pNIPAm copolymers with a layer-by-layer technique and physically polymer coating methods including physical adsorption, solvent casting and spin-coating.

1.2.2.3. Physical Adsorption

During physical adsorption, a polymer solution is deposited on the chosen substrate. The attractive forces between the depositing solution and the substrate occur what lead to the formation of a physically adsorbed film. Importantly, it has been confirmed that physically adsorbed films of pNIPAm retain their LCST property and can precipitate out of solution above the LCST. Above the LCST, polymer-polymer interactions dominate over polymer-solvent interactions.
resulting in the adsorption of the polymer on the substrate. This method is simple and can be employed in the preparation of cell culture substrates. This technology is mostly used to prepare nanometer thick thermoresponsive films. [44] Importantly, it is also possible to coat samples with non-planar geometries as well. However, the thickness can be increased by increasing the time of incubation, or the concentration of polymer solution.

1.2.2.4. Solvent Casting

Solvent casting is another effective method for fabricating pNIPAm films. The advantage of this method is that it allows fabricating of thin polymer films with thickness within micrometer scale. Due to its simplicity, the method does not require expensive and technologically complex equipment and therefore can be employed in many research laboratories. A solution of the polymer is prepared in absolute ethanol, and deposited and spread evenly over the substrate. Once the polymer solution solvent gradually evaporates, the film solidifies and tethers to the substrate. As the thickness of the polymer film is dependent on the concentration of the polymer solution, and may be changed by adjusting the volume of deposited polymer solution. [45] The solvent casting technique has been widely and successfully used for the production of cell culture substrates. [46] However, it is important to note that during the static drying, the internal film stress may cause the contraction, deformation and fracture of the polymer films. This undesired effect affects the uniformity and therefore the quality of the polymer films. [47]

1.2.2.5. Spin-Coating

Spin-coating has been emerged from semiconductor and nanotechnology R&D and industrial sectors. Spin-coating is an incredibly effective and
important technique for thin pNIPAm film preparation and can be utilized to produce high-quality uniform films. Spin-coating has been used for several decades for the application of thin films. It is widely used in the manufacture of integrated circuits, optical mirrors, magnetic disk for data storage, device of solar cells, detectors, sensors, insulating layers for microcircuit fabrication such as polymers (where it can be used to create thin films with thickness below 10 nm), flat screen display coatings, antireflection coatings and conductive oxide. In biomedical research, spin-coating technique is used for the preparation of cell culture supports. [48, 49] Spin-coating is able to produce a thin film (a few nm to a few μm) evenly deposited across the surface of a desired substrate. The rotation of the substrate at high speed (in the present study 4000 rpm) allows the centripetal force combined with the surface tension of the solution pulls the liquid coating into an even covering. During the rotating, the solvent then evaporates to leave the desired material on the substrate in an even covering. At low concentrations the thickness of a film is approximately linearly dependent upon the concentration of the polymer, however as concentrations increase this will affect the viscosity of the polymer solution and thus a non-linear relationship will develop. [24] The exact thickness of a film will depend upon the material concentration and solvent evaporation rate (which in turn depends upon the solvent viscosity, vapour pressure, temperature and local humidity) and so for this reason film thickness curves for a new polymer are most commonly determined empirically. The spin speed can then be adjusted to give the desired film thickness. [25]

In general, a dynamic spin-coating is a controlled process that gives low substrate-to-substrate variation. This is because the solvent has less time to evaporate during the spinning process. As shown in Figure 1.4, the
process of spin-coating includes deposition, spin-up, spin-off and evaporation. [50]

Figure 1.4. Schematic illustrating the basic stages of the dynamic spin-coating method.

During the deposition stage, the solution is dispensed on a desired substrate. During this stage, the substrate is accelerated to the desired speed. Spreading of the solution takes place due to centrifugal force.

Due to the centripetal force induced by the rotation, the polymer solution slowly rotates with the spinning substrate, evenly spread on the substrate without loss of solution, and the overall system remains balanced.

The second stage is when the substrate is accelerated up to its final, desired, rotation speed. This stage is usually characterized by aggressive fluid expulsion from the surface by the rotational motion. The much of the polymer solution is lost during this step, while the system becomes imbalanced. This stage is also characterized by gradual fluid thinning. Mathematical analysis of the flow behavior shows that if the liquid exhibits Newtonian viscosity (i.e. is linear) and if the fluid thickness is initially uniform across the film (albeit rather thick), then the fluid thickness profile at any following time
will also be uniform. However, depending on the surface properties and rotation speed, there may be small beads along the periphery of a polymer coating. *(Figure 1.5)*

![Figure 1.5. Schematic illustration of the edge bead effect of spin-coating.](image)

This effect depends on the shape of a substrate. The circular substrates are more favorable for spin-coating deposition. [48, 51] This side effect of spin-coating might be an undesirable in the electronics industry, but in the present study, the bead effect did not have a negative effect on cell attachment and growth.

Finally, the fluid eventually becomes thin enough to co-rotate and the system regains balance. This results in the formation of thin film on the substrate. The fourth stage is when the substrate is spinning at a constant rate and solvent evaporation dominates the coating thinning behavior. The removal of residual solvent is important to stabilize the film and prevent particulate contamination. [52]
1.2.2.6. Typical Problems that can be Encountered during the Spin-Coating of Substrates

There are several factors affecting the spin-coating process. Following issues should be considered as specific process problems. Table 1.1 summarizes the possible film defects that can occur during spin-coating. [53]

**Air bubbles**

Air bubbles may develop if polymer solution temperature was not adjusted to the temperature of the work place before the coating step. In addition, if the air humidity is too high, this fact may be responsible for the formation of bubbles. An inaccurate application of the polymer with pipettes may also lead to bubbles and thus cause inhomogeneity of the film.

**Pinholes**

Particles in fluid or particles on substrate surface prior to dispense result in the formation of pinholes. To avoid the appearance of pinholes, the surface of the substrate should be cleaned.

**Comets and streaks**

Comets or streaks are the most commonly appearing coating defect. These defects are known occur as lines extending from the center of the substrate. These defects appear if particles stick to the substrate and the fluid flow around each impediment causes a streak those points radially outward. To avoid the appearance of comets, the surface of the substrate should be cleaned. The defects occur from capillary forces that become unbalanced during spin-coating as a result of the solvent evaporation process. If spin speed and acceleration setting is too high or if fluid is
not being dispensed at the center of the substrate surface, it can also result in defects.

**Swirling pattern**

A Swirling pattern may be observed if the spin time is too short or if the fluid is striking substrate surface off center.

**Uncoated areas**

The uncoated areas on the polymer film usually indicate the insufficient volume of the polymer solution used for film preparation.

**Center circle (chuck mark)**

A center circle which is the same size as the spin chuck may appear on the film coating. It is recommended to switch to a Delrin spin chuck.

**Poor reproducibility and film quality**

The quality and reproducibility of polymer films appear to be poor if a poor substrate is used, or if the tip on the micropipette used to dispense the polymer solution is damaged. Therefore, it is important to use a high-quality substrate, tips, micropipettes, and other equipment. Furthermore, it is important to ensure that the substrate is centered properly.
Table 1.1. The spin-coating process troubleshooting. [53]

<table>
<thead>
<tr>
<th>Film defects</th>
<th>Specific process problems</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air bubbles</td>
<td>High air humidity; Inaccurate application of fluid; The temperature of dispensed solution is not adjusted to ambient temperature;</td>
</tr>
<tr>
<td>Pinholes</td>
<td>Air bubbles, particles in fluid or on substrate;</td>
</tr>
<tr>
<td>Comets and streaks</td>
<td>Spin speed and acceleration setting is too high; Particles exist on substrate surface prior to dispense; Fluid is not being dispensed at the center of the substrate surface;</td>
</tr>
<tr>
<td>Swirling pattern</td>
<td>The spin time is too short; The fluid is striking substrate surface off center;</td>
</tr>
<tr>
<td>------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Uncoated areas</td>
<td>Insufficient volume of the polymer solution used for film preparation;</td>
</tr>
<tr>
<td>Center circle</td>
<td>If the circle is the same size as the spin chuck, switch to a Delrin spin chuck;</td>
</tr>
</tbody>
</table>
1.2.2.7. Advantages and Disadvantages of Common Polymer Film Preparation Methods

Electron beam polymerization

Successful cell proliferation and detachment is observed on EBP-modified substrates. Cell proliferation is strongly favored on thin thermoresponsive films. However, the method requires complicated technical support, maintenance and highly qualified professionals, therefore the technique is expensive. Moreover, it is difficult to control the molecular weight, density and macromolecular morphologies of pNIPAm-grafted substrates. [54]

Plasma polymerization

This technique is attractive compared to traditional polymerization, because it leads to an increase in efficiency. Importantly, it only has one step, is solvent-free and it can be used to polymerize a large range of monomers. The coatings are thin, nanometer thick, smooth, generally uniform films. It also has an advantage in that these films can be deposited onto almost all solid materials and has been shown to have no effect on the original, mechanical properties of the substrate. Thus it is geometry-independent and requires little or no substrate pre-treatment. However, the main disadvantage of plasma polymerization is that, because of the fragmentation of the monomer, it can be hard to control chemical functionality. The monomer choice offers control over the functional groups present at the surface. However, by careful control of pressure, power and flow rate, the degree of fragmentation of the monomer molecules can be varied and so changes the density of the functional groups on the surface. Another disadvantage is that high-cost vacuum conditions are generally needed.
**Spin-coating**

As evidenced by its maturity, spin-coating has many advantages in coating operations with its biggest advantage being the absence of coupled process variables. Film thickness is easily changed by changing spin speed, or switching to a different viscosity of polymer solution. Another advantage of spin-coating is the ability of the film to get progressively more uniform as it thins, and if the film ever becomes completely uniform during the coating process, it will remain so for the duration of the process. [55] It is low-cost and fast operating system. The maturity of spin-coating implies many of the issues involved in spin-coating have been studied and a lot of information exists on the subject. [56] However, the disadvantages of spin-coating are few, but they are becoming more important as substrate size increases. Large substrates cannot be spun at a sufficiently high rate in order to allow the film to thin. The biggest disadvantage of spin-coating is its lack of material efficiency. Typical spin-coating processes utilize only 2–5% of the material dispensed onto the substrate, while the remaining 95–98% is flung off into the coating bowl and disposed. [57] If economically feasible manufacturing costs are to be maintained, a method for improving this material utilization is of primary importance.

**Solvent casting**

The method is simple, convenient and inexpensive. Defects depend on a variety of parameters, such as film thickness, film substrate interface adhesion, polymer properties and residual solvent. As mentioned above, as the polymer solvent evaporates polymer fills the voids left behind, but if the concentration of polymer in the solution is extremely low then
there may not be enough polymer units left to fill the voids created and hence divots may occur.

**Physical adsorption**

It is possible to form multilayer films, where the layers of adsorbed polymer weakly bound and can be detached easily. However, despite the simplicity of the method, the poor stability of the adsorbed films makes it hard to produce reproducible and uniform polymer films. **Table 1.2** summarizes the main advantages and disadvantages of the methods commonly used for polymer coating deposition.

**Table 1.2. Summary of main advantages and disadvantages of the methods commonly used for polymer film deposition.**

<table>
<thead>
<tr>
<th>Method of Film Preparation</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
</table>
| **Electron Beam Polymerization** | - high resolution (nm scale coating possible)  
- highly controllable;  
- high uniformity;  
- good reproducibility; | - expensive;  
- restrict access;  
- needs personal training; |
| **Plasma Polymerization** | - covalent bonding to the substrate;  
- one-step, solvent-free, highly controllable;  
- high uniformity and good reproducibility; | - the use of crosslinking agent impairs film biocompatibility;  
- expensive;  
- restrict access;  
- needs personal training; |
| **Physical Adsorption** | - convenient, simple, inexpensive;  
- possible to produce | - poor reproducibility; |
<table>
<thead>
<tr>
<th></th>
<th>ultra-thin film; available in any research laboratory;</th>
<th>-drying process depends on the thickness of the substrate; suitable only for planar substrate; low range of thickness variation; poor reproducibility;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Solvent Casting</strong></td>
<td>-convenient, inexpensive; available in any research laboratory;</td>
<td>substrate has to be planar; restrictions in terms of substrate geometry;</td>
</tr>
<tr>
<td><strong>Spin-Coating</strong></td>
<td>-convenient, fast, inexpensive; high uniformity and reproducibility; available in any research laboratory;</td>
<td></td>
</tr>
</tbody>
</table>

### 1.2.3. Cell Adhesion

Cell adhesion is involved in stimulating signals that regulate cell behavior such as cell differentiation, cell cycle, cell migration, and cell survival. [58] Biomaterials used in the preparation of cell culture substrates or in scaffolds for tissue generation are needed to promote the cells’ adhesion, subsequent proliferation, and expansion. Therefore, the affinity of cells to substrate is a crucial factor limiting the design of biomaterials.
Cells adhere to other cells or an extracellular matrix through localized sites. The adhesion sites are formed by transmembrane proteins called integrins to anchor the cell to an extracellular matrix. [59] The integrins are attached to the tensile members of the cytoskeleton, the actin filaments, through the focal adhesion complex. The focal adhesion complex is a highly organized cluster of molecules. [60] The function of the cytoskeletal structure, as a pathway for force transmission to the cytoskeleton, is to hold the nucleus and maintain the shape of the cell. [61-63] Therefore, integrins play an important role in mechanotransduction through focal adhesion proteins connecting the integrin domains to the actin filaments. [60] The focal adhesion formation is important in cell signaling to direct cell migration [64], proliferation, and differentiation for tissue organization, as well as cell-biomaterial interactions. [65, 66]

The process of static in vitro cell adhesion is characterized by three stages. The initial stage includes the attachment of the cell body to its substrate (stage 1), followed by the flattening and spreading of the cell body (stage 2), and then by the organization of the actin skeleton with the formation of focal adhesion between the cell and its substrate (stage 3). [67, 68] The initial adhesive interaction between the cells and the substrate are driven by the specific integrin-mediated adhesion and starts with the binding of single receptor-ligand pairs. [69] After initial attachment, cells continue flattening and spreading on the substrate, resulting in the decreasing of cell height and increasing of contact area. During cell spreading, actin microfilaments appear to be accompanied into bundles and the strength of adhesion becomes stronger. This will initiate the subsequent receptor-ligand bonds and quickly enhance in number, thus increasing the total adhesion strength. The spreading process is the combination of continuing adhesion with the
reorganization and distribution of the actin skeleton around the cell’s body edge. [70]

1.2.4. Biophysical Factors Influencing the Cell Adhesion and Detachment from Thin Thermoresponsive Films

There is a predictable relationship between surface physicochemical properties and cell behavior at the interface. There appears to be no answer to this question at the moment, considering the complexity of the process involved. It has to be kept in mind that in most cases surface chemistry is also related to surface topography.

1.2.4.1. Surface Chemistry and Electrical Charge

Knowledge of how functional groups affect cell adhesion properties can be used to modify biomaterial surfaces to enhance cell adhesion. The most common functionalities investigated with relation to biomaterial interactions are the carboxyl (-COOH), hydroxyl (-OH), amino (-NH2), and methyl (-CH3) groups. Biomaterial-bearing –COOH displays a negative charged functionality on material surfaces. Surfaces with –COOH also have shown an increase in cell growth. Studies on protein adsorption have shown that such proteins as fibronectin and albumin are more easily eluted from surfaces coated with –COOH. [71] Interestingly, it was revealed that this phenomenon is dependent upon the concentration of –COOH on the surface, as an increase in functional group density results in a higher negative charge on the surface, which was shown to inhibit cell growth. [72] Carboxyl functional surfaces showed high levels of two fibronectin domains (α5β1 and αvβ3) associated with structural and signaling components related to focal adhesions. [73] Adherence and growth rates of human aortic endothelial cells (HAEC) on plasma polymerized poly (vinylacetic acid) films were measured as functions of the surface density of -COOH groups and
plasma deposited film thickness. Endothelial cells exhibited increased cell adherence and proliferation with increasing -COOH surface densities. [74]

The hydroxyl group functionality (-OH) represents a neutral, hydrophilic surface. It is suggested that an increase in oxygen containing functionalities is proportional to cell growth. [75] Fibronectin adsorbed onto -OH functional groups (in comparison with –CH3 functional group) show high levels of α5β1 levels leading to increased cell adhesion strength as well as increased levels of structural signaling components related to focal adhesions. [73]

Amine group (-NH2) functionality displays a positive charge to the biomaterial surface. -NH2 surfaces promote the exposure of high density bound receptors as well as focal adhesion components by adsorbed fibronectin. [73] These favorable protein adsorption profiles lead to increased endothelial cell growth. [76] Preferable adhesion, growth, and matrix formation were also shown with fibroblasts on –NH2 surfaces. [77, 78] The cells cultured on -NH2 had already begun the formation of focal adhesion plaques, linked to increased cell spreading on the surfaces. [77]

The -CH3 group is the major component of commonly used polymeric materials and provides a hydrophobic surface on biomaterials when the charge is neutral. It is generally accepted that the hydrophobic –CH3 functionality promotes protein adsorption, usually in conformations unfavorable for desired cellular interactions. [73]

The results indicate that optimization of the functional group surface density could produce significant enhancements in initial adhesion and subsequent growth of the cells.
1.2.4.2. Surface Wettability and Protein Adsorption

It has been observed that cell adhesion and proliferation reveal dependence on surface wettability.\cite{79} Surface wettability (i.e. the surface hydrophilicity and hydrophobicity) is determined by water contact angles ($\Theta$) as Vogler suggested a definition of biologically hydrophilic surfaces having a $\Theta$ less than 65°\cite{80}. $\Theta$ is closely related to surface energy and a classic definition of $\Theta$ is given by the Young’s equation

$$\cos\Theta = (\gamma_{SV} - \gamma_{SL})/\gamma_{LV}$$

where $\gamma$ is surface energy and $\gamma_{SV}$ is the surface energy (J/m$^2$) between solid and vapor, $\gamma_{SL}$ is the surface energy between solid and liquid, and $\gamma_{LV}$ is the surface energy between liquid and vapor. Many studies so far have demonstrated a proportional relationship between surface energy and the adsorption of hydrophilic cell adhesive proteins (such as fibronectin and vitronectin). It is speculated that hydrophilic (i.e. high surface energy) surface have a higher affinity for cell adhesive proteins and, subsequently, promote cell functions than hydrophobic biomaterials.

The surface wettability (i.e. surface energy) conditions influence cell adhesion and growth through protein adsorption process. Numerous studies have reported different cell behaviors on cell culture surfaces with different wettability, which are likely related to a different pattern, i.e. the type, quantity and conformation, of adsorbed proteins from serum containing media. \cite{81-83} It is considered that due to the low water content on hydrophobic surfaces the latter rapidly interacts with the internal hydrophobic protein domains which attach, unfold from the core of the protein and spread over the surface. On the other hand, hydrophilic surfaces tend to interact with charged and polar groups of protein’s surface, which attach but did not change their conformation, i.e. do not
unfold and spread. [84, 85] It is well known fact that albumin, which is a primary component of fetal bovine serum, readily adsorbs to the surface and resists cell attachment, while vitronectin or fibronectin are responsible for cell adhesion. [86] It is necessary to note that protein molecules adsorb to the surface from multiprotein serum-containing media in a competitive mode according to their concentration, size and affinity to the surface. Hydrophobic surfaces tend to firmly adsorb larger and the most abundant proteins like albumin [87-89], which undergo structural rearrangements and modifications with a greater extent than on hydrophilic surfaces. [90, 91] Hydrophilic surfaces adsorb a lower quantity of proteins [92], but due to small conformational changes, albumin doesn’t occupy the whole surface and allows lower concentrations of cell adhesion proteins to attach to the surface. Moreover, due to a low affinity for hydrophilic surfaces, albumin can be replaced with time by proteins with a higher affinity like fibronectin. [89, 93] Therefore, hydrophobicity and hydrophilicity of a surface can significantly alter cell behavior including cell orientation, morphology and cytoskeleton arrangement. An interesting study performed by Kleinman et al. showed that non-adhesive protein albumin can maintain the exposure of active integrin-binding RGD sites in fibronectin. [94] Such relationships between albumin and fibronectin increases cell adhesive activity of adhesion proteins on hydrophilic surface. It was previously revealed that contact angle on pNIPAm films increases with increasing film thicknesses. [24] Figure 1.6 illustrates the relationship between multicomponent protein layer and pNIPAm surfaces of different hydrophobicity. Hence, when serum containing media is added to cell culture surface, albumin adsorbs and dominates rather on hydrophobic surfaces while cooperates with fibronectin and other adhesive proteins supporting their active conformation on hydrophilic surfaces. [89, 93]
Figure 1.6. Illustration of the multicomponent protein layer adsorbed from serum-containing cell culture media on hydrophilic and hydrophobic surfaces of pNIPAm polymer films with increased thickness and hydrophobicity. (A) Albumin adsorbs to the hydrophilic surface along with adhesive proteins allowing the latter to expose their cell-binding sites. The adherent cell has a flattened shape. (B) Albumin rapidly and firmly adsorbs to the hydrophobic surface, spreads and affects the adsorption of adhesive proteins in their active conformation. The cells adhere and spread over more hydrophilic surface while loosely attaching on more hydrophobic pNIPAm surface. The non-adherent cell has a spheroid shape. Albumin is represented in red and adhesive proteins are in green. [24]
1.2.4.3. Surface Roughness and Topography

Richards et al. compared the attachment of fibroblasts to the surface of plastic, titanium and steel substrates. [95] The attachment to samples was measured by measuring the total area of focal adhesion of cells. Interestingly, the results showed that material roughness had no effect on the area of adhesion. Later, Lampin et al. studied adhesion of chick embryo vascular and corneal explants on PMMA for a series of different roughnesses generated by sandblasting. [96] The potential for adhesion increases with increasing roughness. However, it was presumed that this may reflect a thicker extracellular matrix laid down on the rougher surface as revealed by electron microscopy. Conversely, Anselme et al. studied the adhesion of osteoblasts to a titanium alloy either polished or sandblasted. [97] Measuring a fractal dimension parameter, they found that cells on the rougher sandblasted surfaces never reached confluence. They concluded that lower adhesion was associated with the rougher (less organized) surface. Therefore, the organization of the surface was defined as the critical parameter in cell adhesion. In contrast to these results, Delligianni et al. found that the adhesion of human bone marrow cells to hydroxyapatite surfaces polished with various grades of grit showed a marked increase with increasing roughness. [98] Interestingly, Hallab et al. found that the increase in adhesion with surface roughness seemed to occur with the polymeric materials they tested, and was not found in metals. [99] Indeed they conclude that surface energy is a more important factor in determining cell adhesion than surface roughness, although it is, of course, difficult to separate the effects of these two variables. Lange et al. used a model of MG-63 cells adhering to titanium surfaces of varying roughnesses, and made an interesting observation that cells spread more easily on smooth surfaces, and that integrin expression increased on rough surfaces. [100] This observation was
also confirmed by Linez-Battaillon et al. [101] As above, a recent study by Ponsonnet et al. indicates that surface free energy derived parameters may be more important than simple roughness in determining certain stages, such as spreading, of cell adhesion to biomaterials. [102] Thapa et al. has suggested that nano- (rather than micro-) scale roughness is of biological importance. Using alkali or acid treatment of test surfaces of poly (lactic-co-glycolic acid) (PLGA), polycaprolactone (PCL) and polyurethane (PU), they produced nanoscale surface dimensions and showed that the adhesion of bladder smooth muscle cells was enhanced by nanoscale roughness. [103] These studies suggest a confusing picture of the relationship between material roughness and cell adhesion. This is likely to be a result of the varying experimental procedures and parameters used: metals versus polymers and ceramics, initial adhesion versus spreading, roughness produced by sandblasting or grit polishing, various ways of actually measuring roughness. In addition, the effect of surface topography may depend on the type of cells. It is also likely that the important interaction is between the proteins of the ECM and the surface, before cells actually adhere and spread, which suggests that the surface roughness of the cell itself is of importance.

1.2.4.4. Polymer Film Thickness

In the context of film thickness, the amount of pNIPAm deposited on tissue culture polystyrene by electron beam polymerization method greatly influences cell adhesion behavior. [104] Bovine endothelial cells (ECs) adhere to pNIPAm-grafted cell culture dishes at 37 °C when the grafted amount is less than 2.2 μg per cm². Decreasing the temperature enables the adhered cells to detach from the surface when the grafted amount of pNIPAm is between 1.4 and 2.2 μg per cm². [28] The effects of temperature-induced cell adhesion/detachment have been observed on an ultrathin pNIPAm layer with a thickness of 15–20 nm. The optimal
thickness of grafted pNIPAm on the cell culture dishes, fabricated by electron beam polymerization, is approximately 20 nm. [28, 105] Cell adhesion at 37°C dramatically decreases on surfaces grafted with a thicker pNIPAm layer (>30 nm), and pNIPAm-modified TCPS with a polymer thickness less than 15 nm fails to allow cell detachment below LCST. It is presumed that this thickness dependency is thought to be mainly due to the restricted hydration and mobility of grafted pNIPAm chains on the thermoresponsive surface. Within the ultrathin layer (<15 nm), the motion of pNIPAm chains is strongly restricted owing to the tethering of the chains on the TCPS surface. In addition, the hydrophobic surface of TCPS would decrease the hydration of grafted pNIPAm chains in the vicinity. These factors are thought to prevent the pNIPAm chains from being hydrated even when the temperature is lowered below the LCST. [106] It was found that atom transfer radical polymerization (ATRP)-fabricated pNIPAm brushes with thickness between 20 and 45 nm are the most suitable for HepG2 cell adhesion and detachment. [107] In contrast, temperature-controlled cell attachment/detachment properties of pNIPAm-modified surfaces, created by the plasma polymerization method, are independent of the polymer thickness. [29]

As it was shown for spin-coating technique, the thickness of polymer pNIPAm films also influences the cellular attachment and spreading. The spin-coating technique allows rapid fabrication of pNIPAm substrates with high reproducibility and uniformity. The results suggested that more hydrophilic 50 and 80 nm thin pNIPAm films are more preferable for cell sheet fabrication, whereas more hydrophobic 300 and 900 nm thick spin-coated pNIPAm films impede cell attachment. These changes in cell behavior were correlated with changes in thickness and hydration of pNIPAm films indicating that the control of pNIPAm film thickness
using the spin-coating technique offers an effective tool for the control over cell attachment and proliferation. [24]

1.2.5. Mechanisms of Cell Detachment from the Thermoresponsive Cell Culture Dishes

Cell detachment from the surface of a thermoresponsive cell culture dish undergoes a unique process when compared with the conventional cell culture method. When the temperature is lowered below the LCST, a pNIPAm film begins to hydrate. This hydration change initiates cell detachment by reducing the interaction of cells with the pNIPAm surface. Moreover, it was also suggested that cell detachment from thermoresponsive polymer surfaces is an active process and must require cell metabolic activity. Indeed, cell detachment from thermoresponsive cell culture dish is induced by varying intracellular events, such as signaling and cytoskeleton reorganization, because cell detachment requires dynamic changes in cellular morphologies. [108] While trypsinization causes damage to the cell membrane and ECM, the low temperature-induced detachment of cells from the thermoresponsive cell culture dish preserves their cellular structure and functions. Notably, when the cells recovered by this method were sub-cultured onto another TCPS dish, the cells maintained their adhesiveness, proliferation, growth, and secretion activities at a level nearly identical to the primary cultured cells. Thus, the cell culture method using the thermoresponsive cell culture dish is considered to be a powerful tool for investigating the molecular machinery involved in cell–surface interactions during the detachment process.
1.3. Cell Sheet Engineering

1.3.1. Advantages of Cell Sheet-based Therapy

The transplantation of cells as a single cell suspension is still widely used for cell therapy. However, many cells are known to be lost after transplantation, which leads to the short-term therapeutic effect. The cell sheet engineering can overcome these problems. As mentioned above, the cell sheets harvested from pNIPAm films maintain their ECM, which makes the cell sheets easily adhere to any biomaterials or host tissues without sutures. As the cell sheets can be transplanted without sutures, this procedure saves time, reduces risk from biological materials and can avoid suture-related problems, such as inflammation and scars. This approach offers several distinctive therapeutic advantages for different organs, including cornea [109], esophageal epithelium [110] or oral mucosa epithelium. [111] Transplanted cell sheets can replace injured tissue and compensate for impaired function. The transplantation of hepatic cell sheets improve albumin production [112], pancreatic cell sheets attenuate high glucose concentration in type 1 diabetes. [113] Skeletal myoblast sheets can be used for the treatment of heart diseases. [114-116] Skeletal myoblasts have been reported to secrete various growth factors, such a vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), stromal cell-derived factor-1 (SDF-1), placental growth factor (PlGF) and angiogenin. Moreover, skeletal myoblasts also produce some proteases, such as matrix metalloproteinases-2, -9 and -10. These growth factors control endothelial cell proliferation and migration, thus contributing to angiogenesis. HGF and SDF-1 induce the mobilization of endogenous stem cells from the heart and mesenchymal stem cells from bone marrow. [117] Therefore, in contrast to cell suspension delivery, the cell
Sheet transplantation enables the proper delivery of growth factors to achieve an appropriate level of growth factors that in turn induces and controls a complex of metabolic events. Importantly, using cell sheet technology, it is possible to combine different types of cells. The combination of skeletal myofibroblasts with mesenchymal stem cells significantly enhances the secretion of pro-angiogenic growth factors that improves angiogenesis and reduces fibrosis. [114, 118] Another important benefit of cell sheet transplantation is their immunomodulation potential. It is well known fact that MSCs can significantly attenuate immunoreactions. This aspect makes mesenchymal stem cells (MSCs) sheet transplantation especially favorable for the treatment of heart diseases such as myocarditis, myocardial infarction and heart failure. The MSC sheets are capable of long-term engraftment and may support heart healing by modulating chronic inflammation. Several studies reported that adiponectin produced by adipocytes has multiple protection functions, including anti-inflammatory, antifibrotic and anti-hypertrophic activity. [115] Due to these effects, the delivery of adipocyte cell sheets may decrease the infarcted and fibrotic areas and decrease the hypertrophy of myocardium. Such multilayered cell sheets not only contribute to the recovery of injured tissues, but also can provide structural support. Cell sheet technology is also enabling to create the three-dimensional hepatic tissues by stacking several layers of hepatocyte sheets accompanied by blood vessels. The results showed that liver-like tissue performed liver-specific function and showed appropriate level of metabolic activity including the secretion of albumin. [112, 119]
1.3.2. Biomedical Applications of Engineered Cell Sheets and Results of Preclinical Studies

The most attractive mechanism of tissue engineering and regenerative medicine is the replacement of injured tissues with appropriate tissues. Transplanted cell sheets not only replace injured tissues, but also compensate for impaired function. Recent progress in stem cell biology has enabled the creation of cell sheets which are involved in tissue regeneration through paracrine effects, including the secretion of pro-angiogenic growth factor, anti-inflammatory mechanism and cytoprotection.

The heart diseases are one of the leading causes of death in developed countries. The heart is an attractive target organ for cell sheet-based therapy because the human left ventricle has been reported to be mainly composed of cardiomyocytes and fibroblasts, and partly composed of neurons and vascular cells such as endothelial cells and smooth muscle cells. Therefore, the development of three-dimensional layered cardiac sheets with sufficient vascularization is currently considered a promising strategy. Currently, various types of cells derived from bone marrow, adipose tissue, heart tissue or skeletal muscle tissue have been applied for cardiac regeneration. The principle cause of heart failure is the loss of cardiomyocytes due to mainly such injuries as myocardial infarction, myocarditis and valvular diseases. Pathological processes associated with the apoptosis of functioning cardiomyocytes lead to persistent inflammation, compensatory hypertrophy and insufficient angiogenesis associated with fibrosis, left ventricular remodeling and chronic heart failure. [120]

Mesenchymal stem cells are the leading cell source for cell sheet transplantation for heart diseases. Many studies have shown that MSC sheet transplantation, regardless of autologous or allogeneic cells,
improve the cardiac function. However, it well known that the transplantation of dispersed suspension of MSC results in insufficient therapeutic effects due to poor retention of graft cells in severe ischemic diseases. Such short-term and low therapeutic efficacy could be due to the aggressive microenvironment which is present in infarcted myocardium. Cell sheet transplantation has been presumed to be an effective method to prolong graft cell retention in ischemic tissue.

Chang et al. generated multilayered cell sheets from bone marrow-derived MSCs using thermoresponsive cell culture dishes. As discussed above, these cell sheets preserved the basal ECM proteins. After the cell sheets were harvested, they were examined for their adhesion properties onto native porcine heart tissue. For investigating the adhesion properties of the basal and apical sides, the multilayered cell sheets were transplanted onto the surface of the heart's left ventricle. Multilayered cell sheets were histologically investigated at different time points after transplantation. The results showed that only the basal side of multilayered cell sheets containing secreted ECM significantly enhanced the sheets adhesion onto the surface of heart just 30 minutes after transplantation. [121]

Because cell sheet delivery is capable of long-term engraftment, implanted cells enable a complex spatiotemporal cascade of angiogenic growth factors. To increase the therapeutic efficacy of cell sheet transplantation, Tanaka and colleagues cultured bone marrow-derived MSC (BM-MSC) sheets under hypoxic conditions. [122] It was revealed that hypoxic preconditioning significantly increased the production of VEGF in BM-MSC sheets. In vivo experiments confirmed that the implantation of the preconditioned BM-MSC sheets accelerated angiogenesis in the peri-infarcted area and decreased the infarcted area.

Ishikane et al. have announced fetal membrane–derived MSC (FM-MSC) sheets as an alternative source to BM-MSCs. The FM-MSCs and
BM-MSCs sheets were transplanted into the scarred myocardium after myocardial infarction. Both FM-MSC and BM-MSC sheets had significantly improved cardiac function and reduced myocardial fibrosis compared with the untreated control group. [123]

Skeletal myoblast (SMB) sheets have also been used for cell therapy of heart diseases. The transplanted cell sheets induce myocardial regeneration without lethal arrhythmia. MSCs are known to have pro-survival and anti-apoptotic effects on co-cultured cells in vitro. The adding MSCs to the SMB cell sheets revealed to enhance smooth muscle cell sheet survival post-transplantation and improve their therapeutic effects. [124]

Transplantation of cardiomyocytes that are derived from human induced pluripotent stem cell-derived cardiomyocytes (hiPS-CMs) is another promising approach in cell sheet technology. Kawamura et al. suggested the advanced method of cell sheet transplantation with enhanced blood supply for the treatment of cardiac ischemia. The cell sheets were created from hiPS-CMs using thermoresponsive dishes and transplanted to porcine hearts with the omentum flap. The omentum flap, which is known to have rich vasculature, was used as a source of blood supply. The results showed that the survival rate of hiPS-CMs supported with the omentum was significantly higher than in those without it. In addition, vascular density in the transplanted area was significantly greater with the omentum that confirmed the therapeutic benefits of vascular-rich hiPS-CM sheet transplantation. [125]

While MSCs have shown success in cell sheets for myocardial repair, examination of cell sheets in the field of wound healing has been limited. Cellular therapies have shown a great promise in the treatment of wounds. Human MSC (hMSC) sheets are capable of accelerating the wound healing process. Chen et al. have developed hMSC sheets and
pre-vascularized hMSC transplanted to the wound bed and covered the cell sheet constructs with autologous skin graft. [126] The study in rats showed that hMSC sheets significantly reduced skin contraction and improved cosmetic appearance. Moreover, the treated group experienced the least hemorrhage and necrosis, lowest inflammatory cell infiltration, highest neovascularization and a robust blood microcirculation in early stages required for graft survival and tissue regeneration. In addition, the skin grafts preserved the largest amount of skin appendages such as hair follicles and sebaceous glands, and developed the smallest epidermal thickness. It was presumed that the superior therapeutic effects were attributed to the elevated presence of growth factors and cytokines in the pre-vascularized hMSC sheet, which provided a beneficial paracrine signaling during wound healing. Another source of mesenchymal stem cells, adipose tissue, has also shown a high potential in wound treatment. The human adipose-derived stem (ASCs) cell sheets displayed increased healing effect, and this effect was increased in the group where the triple layer cell sheets were used. [127]

The effectiveness of dispersed hepatocyte transplantation is not sufficient to restore metabolic functions of damaged tissues. It is difficult to control the engraftment efficiency and avoid unexpected engraftment in other organs because the transplanted cells are delivered into blood circulation before their liver engraftment. In addition, a large fraction of transplanted hepatocytes into the liver are rapidly rejected by the innate immune system. To achieve a more effective and long-term results of cell therapy, technical improvements in cell transplantation are required. The three-dimensional (3D) hepatic microtissues constructed from cell sheets could be valuable as a new experiment tool for an implantable tissue model for cell-based therapies and an efficient culture platform for bioartificial liver devices. Fujii et al. created multicellular cell sheets
consisted of hepatic non-parenchymal cells (NPCs) and adipose-derived stem cells, which produce various angiogenic factors. The cell sheets were harvested from thermoresponsive culture dishes. Further subcutaneous NPC-ASC sheet implantation revealed that NPCs formed functioning bile canaliculi, stored glycogen, produced albumin, showed signs of vascularization and survived subcutaneously without pre-vascularization. [128] It was mentioned above that iPS sheets might be a promising source of cardiomyocytes for heart regeneration. Due to their pluripotency, iPS-derived hepatocytes might be also effectively used for the treatment of liver failure. In addition, they can be produced on a large scale and generated from patients. Nagamoto et al. have used this attractive approach for the treatment of patients with liver failure. [129] The human iPS-derived hepatocyte sheets were attached onto the liver surfaces of mice with liver injury. This method reduced unexpected engraftment in organs other than the liver. Human albumin levels in the mice with human iPS-derived hepatocyte sheets were significantly higher than those in the intrasplenically-transplanted mice, suggesting the high potential for cell engraftment of the sheet transplantation procedure. Although subcutaneous liver tissue engineering is an attractive method to curative treat hepatic failure and inherited liver diseases, graft failure occurs frequently due to insufficient infiltration of blood vessels. Sakai et al. described a subcutaneous human liver construction consisting of human primary hepatocytes adhered onto a fibroblast layer. The engineered cell sheets showed superior expression levels of vascularization-associated growth factors (VEGF, TGF-β1 and HGF). The transplanted liver-like tissue contained glycogen stores, synthesized coagulation factor IX, and showed significantly higher synthesis rates of liver-specific proteins in vivo than tissues from hepatocyte-only sheets. These results confirmed the benefits from the combination of co-cultured cell sheets. [130] Itaba et al. investigated the
effect of orthotopic transplantation of human mesenchymal stem cell-engineered hepatic cell sheets against acute liver injury. Transplantation MSC-engineered hepatic cell sheets enhanced liver regeneration and suppressed liver injury. The survival rates of the mice were significantly improved. [131] Another research group presented the technique where triple-layered hepatic tissues with a hepatocyte-specific polarity were fabricated by sandwiching a hepatocyte sheet (Hep sheet) between two endothelial cell (EC) sheets. Cell sheet stratification technology has been used for reconstituting highly functional 3D hepatic tissues in vitro. The morphological and functional characteristics of the triple-layered hepatic construct (EC-Hep-EC) were evaluated and compared with those of a double-layered hepatic construct with a single EC sheet (Hep-EC) and a Hep sheet only. Microscopy analysis revealed that the ECM was deposited in the space between the ECs and hepatocyte cell sheets, with the signs of in hepatocyte polarization. In addition, hepatocyte-specific functions, including albumin secretion, ammonia removal and the induction of cytochrome P450, were also highly preserved. The presented technology was simple in operation and successfully reproduced both cell-cell and cell-matrix interactions, thus closely mimicking the in vivo environment. [132] Baimakhanov et al. confirmed the therapeutic efficacy of multilayered hepatocyte sheet transplantation. In their study, co-cultured multilayered hepatocyte sheets were generated by disseminating hepatocytes onto fibroblasts and cultured on thermoresponsive cell culture dishes. The transplanted multilayered hepatocyte sheets showed high proliferative activity, thereby maintaining the liver function in vivo and providing metabolic support for rats with radiation-induced liver damage. The hepatocyte sheet showed the storage of albumin and glycogen, and the vascularization was observed around the cell sheets significantly improved the survival rate. [133]
The approach considering fibroblast sheets as a source of extracellular matrix, growth factors and cytokines for functional parenchymal cell expansion might be applied for pancreatic tissue regeneration. Matsushima suggested that dermal fibroblasts could be an alternative to MSCs. The fibroblast sheets fabricated using thermoresponsive cell culture dishes were used as a cell culture support for human islets. Microscopy analysis revealed that human islets incorporated into the fibroblast sheets while maintaining a three-dimensional structure and well-preserved extracellular matrix. The viability, survival rates of islet cells and insulin secretions in response to high-glucose stimulation were significantly higher in case of islet culture on fibroblast sheets in comparison with the islets-alone group and the co-culture with fibroblasts group. [134] MSCs are also known to have a protective effect on islet cells. Cell sheets developed using tissue engineering help maintain the function of the cells themselves. Hirabaru et al. performed a series of experiments using islets with MSC sheets to improve the therapeutic effect of islet transplantation. The islets co-cultured with MSC sheets were harvested from thermoresponsive dishes. The improvement of islet function and viability was shown in situ after islet-MSC sheet subcutaneous transplantation in severe combined immunodeficiency (SCID) mice with streptozotocin-induced diabetes. In the recipient mice, normoglycemia was maintained for at least 84 days after transplantation, and neovascularization was observed. These studies demonstrated that islet transplantation would be possible by using the MSC or fibroblast sheets as a scaffold for islets. [135]

1.3.3. **Cell Sheet Manipulation Methods**

Although cell sheets can be harvested from thermoresponsive dishes by simply decreasing the temperature, the static detachment process of cell
sheet can be a slow process depending on time of cell sheet cultivation and the amount of deposited ECM. Moreover, harvested cell sheets usually shrink, wrinkle and fold during the detachment processes. For accelerating the detachment of cell sheets and for harvesting an extended cell sheets, Tang et al. developed a cell manipulator. This cell manipulator consists of supporting hydrogel, a silicone rubber mold, and a plunger-like manipulator. [136]

The benefit of this method is that the cell sheets can be recovered with a manipulator without shrinkage. For harvesting, gelatin hydrogel made by a silicone mold is used as an adhesive surface. This manipulator can be used in combination with low-temperature treatment. However, a unifying transfer method of cell sheets has not yet been established. Further development of a simple and convenient technique, in which technical expertise is not essential, is still required. Tadakuma et al. developed a device for cell sheet harvesting composed of a scooping part and a handling part. [137] The inner plate and outer movable belt are tilted toward the edge of a cell sheet. After touching the cell sheet, the outer belt is moved out with the movement of the inner plate and scoops the cell sheet. After moving the device with the harvested cell sheet, the tip of the device is placed onto another surface and pulled into the device to release the cell sheet.

Matsuda et al. presented a device for fabrication of cell-sheet based tubular vascular-like tissue. [138] The device consists of a moving tray and a roller unit driven by mechanics. When a confluent cell sheet forms, a cell sheet seeded on the pNIPAm-gelatin-coated surface slides gently and smoothly under control of a rod driven by a linear motor. The temperature of the surface beneath the roller is around 4-5 °C that causes pNIPAm to dissolve in the cell culture media. Detached cell sheets are simultaneously attached to a gelatin-coated surface of a roller. The
continuous sliding of a cell sheet and rotating of a roller enable the formation of a tubular multilayered tissue.

Although cell sheet transfer devices can be used to detach and move cell sheets, the mechanical force directly applying on a cell sheet may affect cell viability, metabolic activities and long-term functions. Konishi et al. have designed a device for cellular manipulation. [139] The manipulation system is composed of soft microfingers to pinch and release cellular aggregates without damage. The fingertips are closed and opened by pneumatic balloon actuator (PBA). They were designed by taking into account of the size of a cellular aggregate.

The gap between the open fingertips is around 450 μm. Although the device was originally designed for a cellular aggregate, these soft, small and safe microfingers can be implemented and evaluated for the cell sheet manipulation. Patel and Zhang have developed a simple and effective micropipette based method to transfer and stack cell sheets. [140] The detached and floating cell sheets are collected by a modified micropipette tip fabricated by cutting the 1ml micropipette tip. The cell sheets are then released easily and rapidly.

Importantly, the analysis of cell viability confirmed that the micropipette method did not cause cell death during the cell sheet transfer.

1.4. Scaffold-based Tissue Engineering Technologies

1.4.1. Classification of Biomaterials Used in Scaffold-based Biomedical Research

Traditional methods of regenerative medicine implying the transplantation of dispersed stem cell suspension have been shown to be ineffective for tissue regeneration. Advances in materials chemistry and fabrication as well as processing technologies have led to the design of
three-dimensional cell culture matrices or scaffolds that can support cell growth, organization and differentiation on and within their structure. These 3D biostructures better represent the geometry, chemistry, and signaling environment of natural tissues and can enhance and improve tissue repair processes after transplantation. A variety of fabrication processes and biomaterials have been developed to meet the requirements of tissue engineering. The fabrication process affects the resulting scaffold architecture, physicochemical and biomedical properties. For example, freeze-drying method enables to create scaffolds with sponge-like structures [141], and electrospinning technique can manufacture matrices with aligned and ordered architecture. [142] In addition, the selected fabrication method and potential application of 3D scaffolds determine the selection of biomaterials. Biomaterials should be biodegradable and metabolized in the body without causing toxic or immunogenic problems. In general, biomaterials used for making the scaffolds can be classified into natural and synthetic, according to their sources. Natural biomaterials usually have superb biocompatibility so that cells can attach and grow with excellent viability. Moreover, apart from the excellent biocompatibility of the natural materials, growth factors preserved in natural ECM may further facilitate cell growth and remodeling. For example, heparin sulfate proteoglycans facilitate basic fibroblast growth factor (bFGF) activities [143], the arginylglycylaspartic acid (RGD) sequence on fibronectin provides bioactive cues to the cells for regulation of their activities [144] while well-organized bundles of collagen or elastin fibers stimulates preferred alignment of cells. [145] Other studies document the presence of transforming growth factor-β (TGF-β), basic fibroblast growth factor (b-FGF) and vascular endothelial growth factor (VEGF) upon decellularization of different tissue types.[146-148] The presence of active molecules within scaffolds increases the possibility of their
clinical use for tissue and organ regeneration. ECM which is remained after decellularization includes specific structural components such as collagens, elastins, trace cell adhesive proteins (fibronectin, vitronectin, osteopontin, glycosaminoglycans (GAGs)). However, natural materials have limited physical and mechanical stability, and therefore, they may not be suitable for some applications. Another issue is the potential immunogenicity, because natural material from allogenic and xenogeneic sources may be antigenic to the hosts. It is widely accepted that synthetic materials have better controlled physical and mechanical properties and can be employed for engineering of both soft and hard tissues. However, the major issue for synthetic biomaterials is biocompatibility because cells may have difficulties in attachment and growth on these materials. To improve their biocompatibility, different processes are used to modify the material surfaces, for example, coating with natural biomaterials such as collagen [149], mechanical blending of synthetic powder [150] or surface laser engineering. [151] The property of synthetic polymers such as porosity, degradation time and mechanical characteristics can be tailored for specific applications. In addition, synthetic polymers are often cheaper than natural materials. Many commercially available synthetic polymers show physicochemical and mechanical properties comparable to those of natural tissues. Importantly, synthetic polymers can be produced under controlled conditions. Table 1.3 summarized the advantages and drawbacks of each type of materials.
Table 1.3 Overview of advantages and disadvantages of various natural and synthetic materials.

<table>
<thead>
<tr>
<th>Type of material</th>
<th>Materials</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
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<tbody>
<tr>
<td>Synthetic</td>
<td>CPC, PCL, TCP, PHB, PLLA, PVA, POEGMA, PMEDSAH, pNIPAm, PEG</td>
<td>Easily fabricated with desired porosity, mechanical properties and degradation time; High reproducibility; Low immunogenicity; Long shelf-life; Easy to sterilize;</td>
<td>Difficulty in homogenous cell seeding; Lack of cell control over scaffold thickness; Highly porous scaffolds can have weak mechanical properties; Small pore size scaffold has low cell infiltration and integration with host tissue after implantation;</td>
</tr>
<tr>
<td>Natural</td>
<td>Matrigel, alginate, chitin, chitosan, gelatin, silk fibroin, collagen, fibronectin, hyaluronan, laminin, E-cadherin, vitronectin</td>
<td>More relevant to cellular microenvironment; Better maintained cell adhesion, growth and differentiation; Biodegradable; Easily available;</td>
<td>May provoke inflammatory reactions; Low mechanical properties; Difficult to sterilize; Short shelf-life; High cost;</td>
</tr>
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</table>
1.4.2. 3D Scaffold Fabrication Methods

Over the last decade, the development of fabrication technologies for 3D scaffolds has been an intensive area of research. A number of fabrication techniques have been developed and reported in the literature and these techniques can generally be classified into conventional and advanced techniques. Traditional techniques include solvent-casting, particulate-leaching, and freeze-drying, which can build scaffolds with interconnected porous structures. However, despite their porous structure, these techniques offer little capacity to precisely control pore size, pore geometry, pore interconnectivity, and spatial distribution of pores within the scaffolds. Ideally, scaffolds should not only provide a supporting structure but also the chemical, mechanical, and biological signals which are required to respond to environmental stimuli. As an alternative to traditional scaffold fabrication methods, including electrospinning and rapid prototyping techniques, have recently been developed in tissue engineering. The present study mostly focuses on tissue decellularization technique and laser rapid prototyping techniques such as two-photon polymerization technique and surface-selective laser sintering method.

Decellularized extracellular matrix is one of the most attractive natural scaffolds proceeded from allogenic or xenogenic tissues. This interest stems mainly from the concept that tissue-derived ECM may better integrate with host tissue than purely synthetic biomaterials. Many tissue types have been decellularized including heart valve [152], vessels [153], nerves [154], tendon and ligament [155] for engineering of ECM scaffolds. Specialized decellularization techniques are applied to remove cellular components. Decellularization is achieved by a combination of physical, chemical and enzymatic methods such as freeze-thaw cycles, ionic solutions, hypo- or hypertonic solutions which lyse the cellular
membrane. Next, the cytoplasmic and nuclear cellular components are destroyed and removed by the treatment with reagents such as Triton X-100, sodium dodecyl sulfate (SDS) or trypsin. There are many protocols used for the decellularization of tissues and organs. The production of bioactive ECM scaffolds requires keeping a balance between maintaining native ECM structure and the removal of cellular materials such as DNA, mitochondria, membrane lipids, and cytosolic proteins. For example, Tsuchiya et al. have shown that decellularization solutions at an extreme pH increase the efficacy of cell and cytoskeletal removal but can significantly affect the ECM constituents. Ionic detergents can completely disrupt cell membranes and effectively solubilize cytoplasmic membranes, lipids and DNA. To achieve effective decellularization without severe detriment to the ECM constituents, protocols that use SDS would use multiple low-concentration washes with short exposure time, or apply SDS at a decreased temperature. [156]

Enzymatic agents including proteases (trypsin, dispases), esterases (phospholipase A2), and nucleases (DNase and RNase) are used for cell removal because of their specificity for biologic substrate. Indeed, it is known that trypsin selectively cleaves cell adherent proteins on the carboxyl side of the amino acids arginine or lysine to detach cells from the tissue surface. However, long exposure to trypsin can damage the collagen matrix. Meyer et al. showed 0.5 % trypsin causes extensive damage to aortic valve ECM following 48 h exposure. [157] Brown et al. used 0.02 % trypsin for 1 h with small change in porcine adipose tissue architecture after decellularization. [158] Prasertsung et al. have found that 1 % trypsin not be used longer than 24 h to prevent collagen damage in porcine dermis. [159] DNase and RNase are endonucleases that hydrolyze deoxyribonucleotide and ribonucleotide chains, respectively. Generally, these enzymatic agents are added to detergent treatments to increase the effectiveness of decellularization after treatment with
detergents, to help remove residual deoxyribonucleic acid (DNA). [160, 161] Grauss et al. showed that a 24 h SDS treatment of rat aortic valves still had remaining nuclei. However, the additional treatment of tissue for 1 h step with DNase (0.2 mg/ml) and RNase (20 μg/ml) helped produce a completely acellular material based on histological analysis. [162] The potential benefits provided by decellularized scaffolds for replacement and reconstruction of damaged tissues are noteworthy, and the biologic effects of such materials may be critically dependent upon the methods used to manufacture these materials.

As mentioned above, for optimal tissue regeneration, 3D scaffolds need to fit anatomically into the tissue defects and closely mimic the biochemical and biomechanical environment of the target tissue. In addition, the main requirements for the production of 3D cell-culture systems are reproducibility and flexibility to control experimental variables. Ideally, the technique should be suitable for every cell type. However, practically, it is difficult to produce a single artificial scaffold that is capable of regenerating any human tissue. Nevertheless, it is possible to develop an accurate and versatile technological platform for the fabrication of well-defined scaffolds that is able to accommodate different cells. In this case, rapid prototyping techniques, based on computer-assisted design (CAD) with computer-assisted manufacturing (CAM), are of particular interest to produce complex objects with desirable shape and internal structure. [163] 3D material micro-structuring by femtosecond laser-induced two-photon polymerization is announced as an important tool in regenerative medicine. During two-photon polymerization, a tightly focused femtosecond laser pulse induces a crosslinking photoreaction in the polymer confined within the focal volume. As a rapid-prototyping technique, two-photon polymerization enables the fabrication of three-dimensional micro- and
nanostructures using computer models, with a spatial resolution down to 100 nm. [164] 2PP technique is suitable with many biocompatible polymeric materials, such as polyethylene glycol [165], polylactic acid and polycaprolactone [166], gelatin [167], chitosan [168], zirconium-based hybrids [169], and others. Importantly, the process of fabrication does not require clean room conditions and does not use hazard chemicals or high temperatures. The most beneficial property of 2PP for biomedicine is that the technique is capable of producing anatomically complex 3D structures. Moreover, the flexibility in controlling geometries and feature sizes makes this technique particularly appealing for fabrication of 3D scaffolds for tissue engineering. The femtosecond laser pulses focused into the volume of a photosensitive material and transfer liquid polymer into the solid state. The non-illuminated material is removed by an appropriate developer to reveal the fabricated structure.

The advantage of 2PP is that 3D computer-designed structures can be fabricated with resolution beating the diffraction limit of light. Due to the threshold behavior and non-linear nature of the 2PP process, a resolution beyond the diffraction limit can be realized by controlling the laser pulse energy and number of applied pulses. The best lateral resolution achieved is 100 nm. [170] Although 2PP technique is very precise, the small size of 2PP-fabricated scaffolds may preclude their use as an effective route for tissue-engineering-scaffold fabrication. Most 2PP-fabricated scaffolds are miniature and hard to handle in cell culture.

Selective laser sintering (SLS) method can produce larger 3D scaffolds than 2PP. The SLS method is based on sintering material particles by laser heating and melting. The controlled motion of the laser beam allows layer-by-layer construction of a 3D scaffold with the architecture defined by a computer model using fine-dispersed powder materials. The method of surface-selective laser sintering
(SSLS), developed by Bagrashvili and co-workers [171], is a version of the SLS technique. It is based on sintering polymer particles with the size 50–200 μm by laser radiation in the presence of a sensitizer (carbon or gold), added to the polymer powder in a small amount (mass concentration not greater than 0.1 %) for the absorption of radiation by nanoparticles. In this case, the sensitized heating occurs only in the near-surface zone of the polymer particles, where the absorber or sensitizer particles are located, and the particles become integrated, i.e. sintered. If the choice of laser irradiation parameters is correct, then the most part of the polymer particle is not heated, thus allowing layer-by-layer synthesis of three-dimensional polymer matrices without essential thermal degradation of the polymer and bioactive compounds, included in it. Although the use of nanoparticles (sensitizer) significantly improves SSLS method, these particles may produce cytotoxic effect. In the present study, SSLS method was employed to produce 3D scaffolds, in which water was used as a sensitizer of laser heating and particle sintering.

1.4.3. Biomedical Applications of 3D Scaffolds

The clinical application of donor organs is limited by the shortage of allogeneic organs and the need for lifelong immunosuppression, highlighting the importance of developing more effective therapeutic strategies. In the field of regenerative medicine, various 3D scaffold-based tissue engineering technologies have been recently developed using various biomaterials to address these limitations.
1.4.3.1. Decellularized 3D Scaffolds in Regenerative Medicine

Decellularized scaffolds derived mainly from various non-autologous tissues have become an emerging treatment approach in regenerative medicine. The interest to decellularization techniques has increased considerably in the last decade after Badylak and colleagues first reported the technique for the decellularization of porcine small intestinal submucosa (SIS) by using well-known chemical detergents in 1995. [172] Ott and colleagues showed that this technique is feasible for the decellularization of whole organs. [173] The study showed that rat heart can be decellularized while still preserving the interconnected vascular network and functional heart valves. Although there are promising results emerging from whole organ decellularization studies, the anatomical complexity of the organs constitutes a great challenge for their clinical application. Decellularized ECM derived from tissues may have more important regenerative potential for use in clinical practice. Various animal studies evaluate the efficacy of a number of decellularized tissue scaffolds. There are many xeno- and allograft products, which have been successfully transferred into clinical applications, derived from porcine dermis, porcine small intestinal submucosa (SIS), bovine dermis and bovine pericardium. Recent studies evaluate the potential use of decellularized adipose tissue in soft tissue repair. Some studies are seeking to discover the optimal products for the clinical function of decellularized adipose tissue in injectable gel form. [174, 175] In addition to adipose tissue, pre-clinical studies on decellularized SIS layer are still in progress to test alternative fields of clinical application. Oasis® wound matrix (Smith &Nephew, Inc., Andover, MA, USA) is an acellular product fabricated from decellularized porcine SIS. The matrix was examined in a clinical trial involving 120 patients with chronic leg ulcers and critical-sized skin
defects. The study showed 60% of complete healing by the end of the 12-week period, compared to 35% healing in the control treatment group and no recurrent ulcer in the SIS-treated patients after six months’ post-transplantation period. [176] AlloDerm® regenerative tissue matrix (BioHorizons, Birmingham, AL, USA) is another acellular product that has been developed for skin regeneration. Fourteen patients with an average of 200 cm² full-thickness wound surface area and deep skin loss were enrolled in a clinical trial in 2010. The study showed a significant increase in scar quality and skin function after implantation. [177] Bovine tissues are selected for their size compatibility in cardiovascular treatments. Pericardium tissue is an important material with its high collagen fiber content, non-thrombogenic properties and elasticity. [178] It is well known that heart diseases are the leading cause of adult deaths according to the World Health Organization. [179] Myocardial infarction, hypertension, chronic illnesses and aging cause the loss of cardiomyocytes function and heart failure. The heart valve is one of the main targets for tissue engineering. Metal-based artificial heart valves made from titanium, or carbon valves, are used for the treatment of heart valve diseases. However, to avoid clot formation on metal surfaces, patients become dependent for life on anticoagulants. In contrast, implantation of decellularized heart valves does not need lifelong use of anticoagulants. [180] Moreover, these constructs facilitate mural cell differentiation and integration between the graft and tissue in the final phase. [181] Importantly, decellularized cardiac ECM may accelerate the endogenous cardiac repair via myocardium-mimetic physiological mechanisms and stimulation of endothelial cell migration. [182] CardioCel® (Admedus IHS Inc., Malaga, Australia) is an acellular pericardium-based product stabilized with glutaraldehyde. Clinical trials showed effective treatment and no signs of calcification upon implantation. [183] SynerGraft® from CryoLife (CryoLife Inc,
Kennesaw, GA, USA) is clinically approved product, developed by the
decellularization of pig aortic valve. SynerGraft has been evaluated in
many clinical trials. However, in 2003, researchers reported failures due
to valve-rupture or valve degeneration. [184] Decellularized human
pulmonary valves are another alternative available for routine clinical
applications instead of artificial heart valves. [185] CryoValve®, a newly
presented product of CryoLife, is produced from pulmonary valves
through a patented decellularization protocol. The CryoValve-Syner
Graft was compared with standard allografts, showing similar clinical
results as with the standard “Ross procedure” which is a surgical
operation used for the replacement of defected aortic valve with patients
own pulmonary valve. [181, 186] Despite the success of clinical studies,
the introduction of decellularized graft transplantation into routine
clinical practice is going to take considerable time. However, there are
newly founded companies and prominent academic research groups that
specifically work on the decellularization techniques and have
knowledge and understanding of their applications in clinical practice in
the shortest time.

1.4.3.2. 3D Polymer Scaffolds in Regenerative Medicine
As an alternative to the production of ECM originating from
decellularized native tissues and organs, there is another promising
approach, which involves the use of in vitro cell cultures in 3D polymer
scaffolds for tissue engineering. This approach is based on the
production of the natural ECM by cultured cells \textit{in vitro} upon cell
seeding and expansion into 3D scaffolds. In this case, synthetic
polymers, ceramics and metals incorporated into 3D systems together
with cells and secreted ECM can be implanted into diseased organ for
tissue regeneration. As mentioned above, rapid prototyping techniques
are of particular interest due to their possibility to produce complex
objects with desirable shape and internal structure which is anatomically
close to native tissues. The number of engineering and molecular biology studies on 3D scaffolds fabricated by 2PP technique or surface-selective laser sintering methods is limited. These techniques have been shown to be useful in combination with fibroblasts, stromal cells, neuronal cells and also bone marrow mesenchymal stem cells.

The advances of 3D scaffolds fabricated by 2PP technique for tissue engineering and regenerative medicine have been shown in the series of studies performed at the Nanotechnology Department of the Laser Zentrum Hannover (Germany) led by Chichkov. The team designed 3D scaffolds with open vertical pores that facilitated homogeneous high-density cell seeding throughout the scaffold. In particular, Koroleva et al. have used 2PP to fabricate scaffolds with tightly controllable pore sizes and interconnections. [187] Master structures were fabricated by 2PP and regenerated in natural protein, fibrin, by a two-step microreplication procedure. Seeding of endothelial cells in fibrin scaffolds resulted in their directed lining and spreading within network of microreplicated pores. In another study, Koroleva and co-workers explored 2PP-fabricated and micromolding – replicated 3D scaffolds for peripheral neural tissue engineering. SH-SY5Y human neuronal cell line and primary cultured rat Schwann cells revealed orthogonally aligned, proliferated and organized actin thin filaments and focal contacts. [188] Later, the same research group showed osteogenic differentiation and bone matrix formation after seeding of human bone marrow stem cells and human adipose tissue derived stem cells into 2PP-fabricated Zr-Si scaffolds. Moreover, they investigated the role of mechanical properties of 2PP-fabricated scaffolds on bone matrix formation and found that hASCs produced higher amount of bone matrix on the scaffolds with lower Young's moduli and hardness values compared to the cells cultured on scaffolds with higher Young's modulus and hardness values. Another study related to bone tissue engineering confirmed that 3D
biodegradable 2PP scaffolds fabricated from novel star-shaped poly (D, L-lactide) materials support differentiation of hASCs toward the osteogenic lineage and promote bone tissue regeneration after MSCs implantation into the cranial bone in mice. [189]

As another rapid prototyping technique, surface-selective sintering method leads to the fabrication of 3D scaffolds that have very precise microarchitecture. Kuznetsova and colleagues used SSLS scaffolds fabricated from polylactide powders and containing hydroxyapatite molecules for culturing of adipose tissue derived mesenchymal stem cells. The cells adhered and populated scaffolds nearly reached confluence. Moreover, due to appreciable cell migration, the cells filled the pores and grew from the surface into the scaffold. [190] Bukharova et al. reported no cytotoxic effect and no inflammation response after subcutaneous implantation of constructs engineered from SSLS scaffolds and MSCs in rats. [191] Kanczler and co-workers examined SSLS scaffolds for their biocompatibility in vitro and in vivo studies. Fetal femur derived cells attached and successfully proliferated on SSLS poly (D, L)-lactic acid scaffolds. Sirius red and Alcian blue positive staining confirmed matrix deposition in vitro and in vivo. Moreover, the SSLS scaffolds seeded with cells and implanted into a murine femur defect model promoted bone tissue regeneration. [192] These results suggested that computer-assisted SSLS technique can be successfully employed for the fabrication of biocompatible and biodegradable scaffolds to fit complex tissue defect and provide complete tissue regeneration. Later, the research group led by Bagratashvili advanced and improved SSLS technique. While all above-mentioned studies have used carbon nanoparticles as a sensitizer, the group reported a novel protocol for surface-selective laser sintering of polymer particles, in which water was used as a sensitizer of laser heating. Antonov and colleagues showed that the presence of water restricts the temperature of the heated polymer,
preventing its thermal destruction. The method was successfully applied to produce biodegradable polymer scaffolds for tissue engineering. Rapid prototyping techniques provide an instructive example for the possible future development of 3D scaffold-based tissue engineering techniques. These studies illustrate the need for interdisciplinary cooperation, shown that this work closely relates to such diverse discipline as medical, biological, machine technical and biomaterial sciences. The potential of such cooperation can certainly be considered to be huge. Although many research groups are working on this topic, there is still a long way to go before clinical application can be realized. However, the efforts to improve these techniques to use in the treatment of patients have reached the status of practically possible and applicable.

1.4.4. Integration of Scaffold-based and Scaffold-free Approaches

Conventional local injection or scaffold-based deliveries of cell suspension without their endogenous extracellular matrix might impair tissue regeneration after implantation. In these cell-based therapies, transferring cells to the target site is crucial, and various materials are developed as vehicles to mimic the natural environment of a tissue as closely as possible. As an alternative to conventional cell delivery methods, cell sheet technology has been recently developed as a promising tool in tissue-like engineering. Instead of using proteolytic enzymes to detach cells from the substratum as in most cell culture experiments, the cultured cell sheets are harvested non-invasively from thermoresponsive substrates with deposited intact extracellular matrix, key iron channels, growth factor receptors, and preserved cell-cell and cell-ECM interactions. While considering the fragile structure of cell sheets, scaffolds may be useful for supporting cell sheets before implantation and 3D tissue reconstruction. The integration between cell
sheet engineering and tissue graft fabrication technology can promote the
development of novel medical devices that provide structural support
and improve tissue regeneration. Successful cell sheet integration into
3D scaffolds has been observed in several recent studies. Wang et al.
showed that cell sheet seeding technique shorten the construction time
but also improve the efficiency of cell attachment and expansion on the
surface of biomaterials. [194] The endothelial cell sheets successfully
attached to acellular bovine paricardia, proliferated and remained
metabolically active. Ma et al. examined incorporation of bone-marrow
derived MSC sheet into hydroxyapatite (HA) scaffolds. The incorporated
MSC sheets exhibited higher alkaline phosphatase (ALP) activity than
osteogenic cell suspension. Moreover, HA scaffolds with MSC sheets
showed higher calcium and collagen I deposition and increased
angiogenesis compared to the scaffolds seeded with dispersed cell
suspension. [195] The MSC sheets were used to create a tissue construct
from multilayered MSC sheets and sliced porous acellular bovine
pericardia. The in vitro analysis revealed that cell sheets inside the tissue
scaffold remained viable, uniformly distributed and tightly adhered to the
scaffold. After tissue graft implantation in rats with chronic myocardial
ischemia, the results revealed significantly improved heart function than
in the control group before implantation. [196] Another study confirmed
that MSC sheets significantly enhanced the cell adhesion onto the
surface of porcine heart after implantation. [121] In this work, Chang and
colleagues showed that such relatively short period of time, as 30 min,
was sufficient for ensuring the adhesion of the sheets to the heart tissue.
Importantly, the study provided evidence for the possibility of successful
clinical application of cell sheet technology to the cell therapy of
myocardial diseases.
1.4.5. Comparative Analysis. Advantages and Challenges, Risks and Future Directions

The ability to engineer or regenerate damaged tissue due to injury, aging, disease, or genetic abnormality holds great promise. Tissue engineering research aims to develop organ substitutes for treating pathological disorders and organ failures. Although this field has made many breakthroughs in 3D fabrication technologies during the past decades, there are still many challenges and questions that need to be addressed before 3D scaffolds can be fully applied in humans for therapeutic uses. In the field of biocompatible 3D scaffolds, the development of complex natural and synthetic scaffolds that mimic the native tissue architectures will persist. The 3D scaffolds must be biodegradable, non-immunogenic, and able to provide structural, mechanical and biological supports for cell adhesion and growth.

The rapid prototyping technologies including 2PP and SSLS methods have a lot to offer to the fields of tissue engineering and regenerative medicine. 2PP and SSLS scaffolds with porous structure and specified architecture allow spatially oriented cell proliferation and provide desired 3D tissue-equivalent. They are also biodegradable and biocompatible. However, commercially available systems for 2PP and SSLS synthetic scaffold fabrication are quite expensive and are not very easy and fast to adapt to different customers’ needs. Moreover, the range of clinical grade biocompatible and biodegradable biomaterials is still very scarce. In addition, although current 2PP and SSLS systems are very precise, they are also relatively slow. 2PP and SSLS scaffolds are miniature and are hard to handle in cell culture. Considering this issue, processing speed has to be increased manifold.
Tissue decellularization offers a promising alternative for the fabrication of 3D bioconstructs with precise micro- and nanoarchitecture, being affordable system in terms of costs. Recent accomplishments in tissue decellularization provide acellular tissue-derived scaffolds. Their main advantage over synthetic scaffolds is that the acellular scaffolds retain the nature-designed structure from the microstructure scale down to the nanoscale. Moreover, they preserve the structurally organized entities such as collagen, elastin, glycosaminoglycans, and fibronectin that enable favorable cell-cell interactions, tissue organization and biological functionality.

In terms of cell compatibility, pericardial-derived acellular scaffolds are in some ways similar to naturally-derived proteins and provide the best cellular recognition. The removal of cellular content and antigens from pericardial tissue also reduces the risk of inflammation and potential immune rejection of acellular pericardial tissue-derived scaffolds.

In contrast, the possible degradation products in SSLS scaffolds and unreacted groups in polymerized volume of 2PP scaffolds have to be considered.

Still having some disadvantages and being in an early development stage, rapid prototyping technologies including 2PP and SSLS enable better control of mechanics of 3D structures, geometry, porosity and rate of degradation. Importantly, 2PP and SSLS scaffolds provide not only cell attachment, but also further in-depth penetration. Regarding the acellular tissue-derived scaffolds, still much effort should be directed on the development of standardized decellularization protocols with the goal to advance in cell seeding and culture methods to promote tissue formation and maturation.
For that, issues including the vascularization and innervations within engineered tissue-like constructs (based on either synthetic or natural scaffolds) should also be further studied. Consideration should be made for multiple design and delivery approaches for in vitro tissue preparations. Other important issue is to increase our understanding of the basic principles governing tissue formation, function, and failure, including the assembly of multiple cell types and biomaterials into single multi-dimensional structure that can mimic the microarchitecture and function of native tissue. In addition to laboratory work, more pre-clinical research is required in the area of regenerating functional tissues in situ. Future studies are needed to assess the functional significance of these processes in various types of tissues, and the potential of implanted 3D scaffolds to control tissue regeneration in long-term. If successful, these strategies will solve the problem of organ donor shortage and can be used for surgical repair of tissue defects. The ability to provide the tools for replacing damaged tissues will have a dramatic impact on future medicine.

1.5. Main Aims/Objectiveness

1. In the present study, the first aim was to investigate the dependency of cell behavior on physical properties (thickness, surface morphology and wettability) of non-crosslinked spin-coated pNIPAm films. Cell response to pNIPAm films were analyzed in terms of cell attachment, growth, and detachment, metabolic activity, viability and proliferation rate, morphology and cytoskeleton organization. Additionally the series of pNIPAm films with different thicknesses was examined to assess the possibility of contiguous cell sheet recovery.

2. Further, to define the quality of cell sheets lifted from thin spin-coated pNIPAm films, cell morphology, total cell number in cell sheets
as well as the pattern and speed of cell sheet recovery, cytoskeleton organization, metabolic activity and cell viability of cell sheets before and after detachment and after re-attachment were analyzed.

3. Finally, the possibility of integration of cell sheets harvested from thermoresponsive pNIPAm films with 2PP scaffolds, 3D SSLS scaffolds or acellular pericardial scaffolds was investigated. As scaffolds can provide initial structural support for cell sheet seeding and attachment, the structural and material characteristics of 3D scaffolds were analyzed. Histological analysis and DAPI staining of acellular pericardial scaffolds was performed to evaluate the efficiency of decellularization. To exclude cytotoxic effect of scaffolds, the morphology of cells, viability and metabolic activity of cells were examined after seeding single-cell suspension and after transferring cell sheets. The attachment and distribution of cell sheets into 3D structures were also assessed.
1.6. References


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2. MATERIALS AND METHODS

2.1. Materials

The polymer pNIPAm with Mn = 20000-40000, anhydrous ethanol (200 proof, > 99.5% assay), Triton-X-100, paraformaldehyde, Harris modified hematoxylin and Eosin Y, Sodium Dodecyl Sulfate (SDS), glutaraldehyde solution (25 wt. %), acetic acid glacial (≥ 99.85%), porcine gastric mucosa pepsin (≥ 2,500 U/mg), 0.5 M EDTA, 10 mM Tris-HCl and DMSO (anhydrous, ≥ 99.9 %) were purchased from Sigma-Aldrich (St. Louis, USA). PURASORB PDL 02A (poly (D, L-lactide) powder was purchased from Corbion (Amsterdam, Netherlands). Plastic consumables were purchased from Sarstedt (Nümbrecht, Germany). Dulbecco’s Phosphate Buffered Saline (DPBS), Hanks Balanced Salt Solution (HBSS) and Dulbecco’s Modified Eagles Medium (DMEM) were purchased from Lonza (Basel, Switzerland). Antibiotics (penicillin-streptomycin) and fetal bovine serum (FBS) were purchased from HyClone (Logan, USA). AlamarBlue® reagent, Live/Dead® viability/cytotoxicity kit, Quant-iT™ PicoGreen® dsDNA assay kit and the Silver Quest silver staining kit were purchased from Invitrogen-Thermo Fisher Scientific (Waltham, USA). Rabbit monoclonal anti-paxillin antibody (ab32084) and goat anti-rabbit secondary antibody (ab150077) were purchased from Abcam (Cambridge, UK). Phalloidin eFluor660 (50-6559-05) was purchased from Affymetrix (Santa Clara, USA). UltraCruzTM mounting medium was purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Heating dry bath from Torrey Pines Scientific (Carlsbad, USA) was used for careful temperature control.
2.2. Methods

2.2.1. Thermoresponsive Poly (N-isopropylacrylamide) Film Preparation Using Spin-Coating Technique

2.2.1.1. Solution Preparation

The appropriate amount of commercial pNIPAm depending on the desired final concentration of pNIPAm solution was put into a 15 ml tube and dissolved in absolute EtOH. The reason to use absolute ethanol is that pNIPAm is soluble in ethanol and water separately, it is not soluble in a mixture of both. This phenomenon is known as co-nonsolvency. In this study, 5, 10, 20, 40 and 80 mg/mL ethanol pNIPAm solutions were used for pNIPAm film fabrication, and 5 mg/ml ethanol pNIPAm solution was used for pNIPAm film fabrication for cell sheet harvest. [1]

2.2.1.2. Film Fabrication

Spin coaters deposit a very thin layer of onto the surface of the spinning substrate. All spin coaters consist of a metering device (such as a dispenser or nozzle), an apparatus to rotate the part to be coated, and a bowl/lid to contain the excess coating spun off the part. Spin coaters may be powered by an electric motor or other means and often feature variable rotational speeds to help control the coating thickness. In addition to paints and other coatings, spin coaters can also be used to dispense cleaning solutions or etchants in order to prepare a part surface for further coating. [1-3]
To produce thin pNIPAm films, the Laurell Technologies WS-400B-6NPP/LITE spin coater was used (Figure 2.1, A).

The key advantage of a spin-coating technique is the combination of reproducibility and versatility. The method allows very fast, gentle and nondeforming drying of thermoresponsive films with uniform surface which characteristics can be controlled very carefully. The technique
provides a more inexpensive and convenient way to prepare thermoresponsive cell culture surface and can be deployed in many university research laboratories. (Figure 2.1, B).

In this study, spin-coated pNIPAm films were fabricated by initially depositing a 150 μL aliquot of a 5, 10, 20, 40 and 80 mg/mL ethanol pNIPAm solution onto a slowly spinning, (150 rpm), substrate for 9 s followed by rapid acceleration to 4000 rpm for 30 s. [1]

2.2.1.3. Drying Process

The coated samples were slowly dried overnight in an ethanol saturated atmosphere and then left in a vacuum oven at 40 °C and 600 mbar for a minimum of 4 h to eliminate any residual solvent.

2.2.1.4. UV Sterilization

All films were sterilized under mild UV condition before cell seeding. The films were exposed to the UV in a laminar flow hood for 2 h.

2.2.2. Fabrication of Three-dimensional Star-shape Polylactide Scaffolds by Two-photon Fabrication Technique

2.2.2.1. Synthesis of Star-shaped Poly (D, L-lactide)

Synthesis of star-shaped poly (D,L-lactide)(PDLA) was performed by Dr. Peter Timashev, Institute of Photonic Technologies, Research Centre of Crystallography and Photonics RAS, Troitsk, Moscow, Russia.

Star-shaped poly (D, L-lactide) was synthesized by the ring-opening polymerization of D, L-lactide in bulk using Sn (Oct)$_2$ as a catalyst and pentaerythritol as an initiator in a Schlenk tube equipped with a magnetic stirrer bar. As an example of a typical procedure for the synthesis of star-
shaped poly (D, L-lactide), polymerization was carried out as follows ([D, L-lactide]/[initiator]=20). The melt of pentaerythritol (0.23 g, $1.7 \times 10^{-3}$ mol) in 5 g of D, L-lactide was prepared in a tube. Then the Schlenk reactor was charged by the catalyst solution (1 M) in toluene 0.56 mL ($5.6 \times 10^{-4}$ mol). After removing dichloromethane under the vacuum conditions, the pentaerythritol melt in D, L-lactide was added to the reactor. Then, the reaction vessel was immersed into an oil bath preheated to 130 ºC for 15 minutes for polymerization to give the desired poly (D, L-lactide). Yield: 99%; Mn (SEC) = 4530 g mol$^{-1}$; Mn(NMR)= 2600 g mol$^{-1}$; Mw/Mn=1.21. $^1$H NMR (CDCl3): $\delta$ (ppm) = 1.40-1.70 (m, H$^c$); 4.15 (m, H$^a$); 4.32(m, H$^d$); 5.05-5.20 (m, H$^e$). [4]

2.2.2.2. Fabrication of 2PP scaffolds

Fabrication of star-shaped poly (D, L-lactide) scaffolds was performed by Dr. Peter Timashev, Institute of Photonic Technologies, Research Centre of Crystallography and Photonics RAS, Troitsk, Moscow, Russia.

Two-photon absorption is a nonlinear absorption process whereby two photons are absorbed simultaneously by an atom or molecule, and an electron is promoted from a lower energy level (the ground state) to a higher energy level (an excited state). This concept was first proposed by Maria Goepert-Mayer in 1931. [5] Until the early 1980s, two-photon adsorption was used a spectroscopic tool. However, in late 1980s, Watt Webb suggested two-photon microscopy and imaging. [6] The two-photon excitation microscopy has several advantages over confocal microscopy due to its deeper tissue penetration, efficient light detection and reduced phototoxicity. The images of confocal microscopy are only based on one parameter, the local reflection. The two-photon imaging is
able to use three more parameters including emission spectrum, excitation spectrum and fluorescence lifetime. This enables a unique identification and characterization of 3D structures including different tissue microcomponents like collagen bundles, vascular structures, mitochondria and hemoglobin. [7]

Other applications, such as 3D scaffold fabrication, have been also suggested. Like two-photon microscopy, two-photon microfabrication technology involves the use of lasers to prepare the structure from bulk materials. The two-photon polymerization technique is an intelligent and promising technique for the fabrication of high quality three-dimensional tissue engineering scaffolds with precise microarchitecture.

The spectrum analyzer provides analysis and continuous monitoring of the laser emission spectrum. Together, the waveplate and a polarization-sensitive beam splitter adjust the transmitted laser power. The acousto-optical modulator (AOM) controls the radiation on/off regimes. Then, the telescope is designed to expand the beam to match the objective aperture. The quality inspection is performed online using CCD camera. [8]

A liquid solution was prepared by dissolving one unit of the star-shaped methacrylate-functionalized poly (D, L-lactide) in double the volume of dimethyl chloride. To obtain a photoactive material, 1 wt% of 4,4’-bis(dimethylamino)benzophenone photoinitiator was added to the solution and mixed for 2 h. The scaffolds were produced using a Ti: sapphire femtosecond laser system (Chameleon, Coherent, Germany), which delivers 150-fs pulses at 80 MHz repetition rate. For 2PP near-infrared femtosecond laser pulses was delivered by a Ti: sapphire oscillator (120 fs, 80 MHz, 780 nm). Briefly, 20×microscope objective (Zeiss, Epiplan) was used for focussing the laser beam into the volume of photoactive material. A drop of photosensitive PDLA was placed in polymeric 1 mm thick ring spacer enclosed in between two cover
glasses. The material is polymerized along the trace of the laser focus, which is moved in three dimensions. This allows the fabrication of any computer-generated 3D structure by direct laser “recording” into the photopolymer volume. In the case of a liquid monomer (such as 4-arm PDLA presented here), 2PP results in a localized conversion of liquid into the solid state. After 2PP processing, the scaffold structure was washed using 1:1 solution of 4-methyl-2-pentanone and 2-propanol to remove unpolymerized material. The 2PP-fabricated 3D scaffolds were composed of two layers of hollow cylinder arrays in a hexagonal arrangement within each layer. [4]

2.2.2.3. UV Sterilization

All scaffolds were sterilized under mild UV condition in a laminar flow hood for 3 h before cell seeding.

2.2.3. Fabrication of Three-Dimensional Star-shape Polylactide Scaffolds by Surface-selective Laser Sintering Method

Fabrication of SSLS scaffolds was performed by Dr. Peter Timashev, Institute of Photonic Technologies, Research Centre of Crystallography and Photonics RAS, Troitsk, Moscow, Russia.

2.2.3.1. Surface-Selective Laser Sintering of Poly (D, L-lactide) Polymer Particles

Selective Laser Sintering is a technique that uses laser as power source to form solid 3D scaffolds. This technique was developed by a student Carl Deckard and his supervisor Joe Beaman from Texas University in 1980s.
Unlike some other techniques, such as stereolithography (SLA) and fused deposition modeling, used for the fabrication of 3D scaffolds, SLS doesn’t need to use any support structures as the scaffold being printed is constantly surrounded by unsintered powder. The material to print with might be anything from nylon, ceramics and glass to some metals like aluminum, steel or silver. Due to wide variety of materials that can be used with this type of 3D printer the technology is very popular for 3D printing customized products. [9, 10]

In this study, fine dispersed PURASORB PDL 02A (poly (D, L-lactide) powder was used as a source material for sintering and creation of matrix structures. As the poly (D, L-lactide) is hydrophobic, the powder was hydrophilised by 1% solution of a hyaluronic acid (HA) to provide wetting. The samples were formed on SLS-100 machine developed at the Institute of Laser and Information Technologies, Russian Academy of Sciences (Moscow, Russia). Single-mode fiber laser (LS, 1.9, IRE-Polus) with a wavelength of 1.94 μm served as the source of continuous radiation, and water was used as a sensitizer of laser heating and sintering of particles. Diameter of laser beam of the surface of polymer particles was 200 μm. Polymer scaffolds with a developed structure were obtained. [11]

2.2.3.2. UV Sterilization

All scaffolds were sterilized under mild UV condition in a laminar flow hood for 3 h before cell seeding.
2.2.4. Fabrication of Three-dimensional Decellularized Pericardial Scaffolds

2.2.4.1. Decellularization of Bovine Pericardial Tissue

Bovine pericardia procured from a slaughterhouse were used as raw materials. The procedure used to remove the cellular components from bovine pericardial tissue was based on a method developed and characterized by Oswal et al. with some modifications. The sample was washed in PBS supplemented with 1 % penicillin-streptomycin antibiotics and left in sodium dodecyl sulfate (SDS, 0.1 % w/v) for 24 h, both in the presence of protease inhibitors. The tissue was finally treated with a nuclease solution (RNase/DNase), and then washed in sterile PBS supplemented with 1% penicillin-streptomycin antibiotics for 24 h with agitation, and then used for analysis or cell seeding. [12]

2.2.4.2. UV Sterilization

During the decellularization procedure, the samples were treated with sterile solutions supplemented with 1 % penicillin-streptomycin antibiotics and then sterilized under UV condition in a laminar flow hood for 4 h before cell seeding.

2.2.5. Physical Characterization of Biomaterials

2.2.5.1. Fourier Transform Infrared (FTIR) Spectroscopy

FTIR is used to measure roughness, height and depth of a sample surface. It yields topographic images and allows structure and detail to be seen with high resolution (1-10 nm) and without the need for rigorous sample preparation.
FTIR analysis was performed using a Shimadzu FTIR 830 Fourier transform infrared spectrometer with a golden gate diamond ATR accessory. (Figure 2.2) The light from an infrared light source is sent through an aperture hole to a mirror (the ‘beamsplitter’) that sends two equivalent beams (one reflected and one transmitted) to a fixed and to a scanning retroreflector mirror, respectively. After acquiring different optical path lengths, these two beams recombine on the beamsplitter and are sent to the sample and after this to a semiconductor detector. The detector signal as function of the optical path difference between the two mirrors is the interferogram. After fast Fourier transform calculation, the infrared spectrum is obtained. [13-15]

Spin-coated films were prepared on Thermanox plastic substrates or glass slides. A background scan was taken before every sample scan and this background was then subtracted. [4]
2.2.5.2. **pNIPAm Film Thickness Assessment**

*This work was done with the kind permission and assistance of Dr. Gerard O’Connor in the National Centre for Laser Applications, School of Physics, NUI Galway.*

2.2.5.2.1. **Laser Ablation**

An ArF excimer laser (ATL Atlex, Wermelskirchen, Germany) was used to ablate the selected areas on the thin pNIPAm film deposited on fused silica glass discs, 20 mm in diameter from UQG optics. The excimer laser operates at a wavelength of 193 nm with a pulse length of a few ns. Laser parameters included a pulse repetition frequency of 200 Hz at a fluence of 66 mJ/cm$^2$. A standard mask projection machining approach was used to shape the laser beam. An optical demagnification of 5× was employed to produce 9 periodically arranged ablated areas of approximately 400 μm×400 μm. [1-2]

2.2.5.2.2. **pNIPAm Film Thickness Measurements Using Optical Profilometry**

Inside an optical interference microscope, a light beam is split, reflecting half the beam from a test material, which is passed through the focal plane of a microscope objective. The other half of the split beam is reflected from the reference mirror.

When the distance from the beam splitter to the reference mirror is the same distance as the beam splitter is from the test surface and the split beams are recombined, constructive and destructive interference occurs in the combined beam wherever the length of the light beams vary. This creates the light and dark bands known as interference fringes. Since the reference mirror is of a known flatness – that is, it is as close to perfect flatness as possible – the optical path differences are due to height variances in the test surface. This interference beam is focused into a
digital camera, which sees the constructive interference areas as lighter and the destructive interference areas as darker. [16]

The top surface and film thickness data analysis capabilities enable precise measurement of various surfaces in multilayer processes. Special algorithms single out the film's top surface to measure its topography, while film thickness analyses identify the film's top surface and the substrate surface to calculate a film thickness map. These special analyses also permit topography measurement of the substrate surface. [17] Optical interference microscope was used to assess the thickness of pNIPAm films. (Figure 2.3)

![Optical interference microscope](image)

**Figure 2.3.** Light interference microscope ZygoNew View 100 (National Centre for Laser Applications, School of Physics, NUI Galway).

All measurements were made in air. The Zygo Newview 100 surface profiler’s field of view can vary from 6 mm × 4.5 mm down to 0.18 mm × 0.14 mm. It has a vertical range of 100 µm and it can measure roughness and step-heights in the range 0.1 nm up to 10’s of microns. Seans of three randomly selected areas along each scratch were recorded
on three different samples in order to insure statistical accuracy. The thickness of pNIPAm films was measured using white light interferometry (ZygoNew View 100) with an accuracy of 0.1 nm. The z-height distance between the remaining polymer and the underlying substrate was measured to accurately assess the thickness. Statistically relevant data was obtained by repeating all measurements three times, and scans of 5 randomly selected ablated windows were recorded on 3 different samples. The objective used for all measurements was a 20× Mirau, with zoom set at 0.5×. [1-2]

2.2.5.3. pNIPAm Film Roughness Assessment by Atomic Force Microscopy

The basic principle in AFM is that the microscope is able to scan the surface of a sample with a cantilever. When a laser is shone onto the top of the cantilever, the force interactions between the cantilever and the sample lead to minute deflections in the laser. These deflections are then detected by photodiodes that results in an accurate topographical map of the sample surface.

AFMs are able to resolve images up to the sub-nanometer scale, many times better than the optical diffraction limit. There are three main modes of operation in AFM. In contact mode, the force sensitive tip contacts with the sample moving across the surface and collecting topographical information. The contours of the surface are measured either using the deflection of the cantilever directly or, more commonly, using the feedback signal required to keep the cantilever at a constant position. Contact mode is suitable for hard substrates such as metals and crystals. In tapping mode, the cantilever is driven to oscillate up and down at or near its resonance frequency. When the cantilever comes close to the surface, Van der Waals forces, dipole-dipole interactions, and
electrostatic forces cause the amplitude of the cantilever's oscillation to change. The amplitude of this oscillation varies between several nm and 200 nm. A tapping AFM image is therefore produced by imaging the force of the intermittent contacts between the tip and the sample surface. The specimens do not require a partial vacuum but can be observed in air at standard temperature and pressure. Non-contact atomic force microscopy is a type of scanning probe microscopy. Non-contact AFM is mostly used for measuring soft biological samples. However, the main disadvantage of non-contact AFM is that the scanning techniques are generally slower in acquiring images, due to the scanning process. As a result, efforts are being made to greatly improve the scanning rate. [18, 19] In case of rigid and dry samples, non-contact, contact and tapping images may look the same. Tapping mode AFM has been established and successfully used for topographic studies of pNIPAm films. [1, 2, 20-22]

Figure 2.4. The Dimension 3100 Atomic Force Microscope (NCBES, NUIG).
In the current study, AFM images were obtained in tapping mode in air using a Dimension 3, 100 AFM (Digital Instruments, Santa Barbara, CA, USA), and Veeco 1–10 Ohm-cm phosphorus (n) doped Si tips and a matrix of 512×512 data points along the x–y plane were analyzed in a single scan. (Figure 2.4) AFM was used to assess the roughness of the deposited pNIPAm coatings using 10 μm×10 μm scans. Four 10 μm×10 μm scans were recorded at a scan rate of 0.5 Hz on each pNIPAm film to ensure statistical accuracy. The roughness of the films was reported as root-mean-square (RMS) roughness values. [1, 2]

2.2.5.4. Hitachi Scanning Electron Microscope with Energy-dispersive X-ray Analysis System

The first true scanning electron microscopy was built in the 1930s, and they have become commercially available 30 years after inventing. SEM has the ability to magnify objects from about 10 times up to 300,000 times with high resolution. The high resolution in SEM is about 10 nm. Moreover, SEM has depth of field, which is the height of a sample that appears in focus in an image. It is approximately 300 times more than the light microscopy. Objects of interest may be observed at close to 0 °K, or at higher temperatures, also wet or dry, and pressurized or in a vacuum. This feature can be used to obtain great topographical information. [23]

The basic principle of the SEM is that a beam of high-energy electrons, produced by an electron gun and controlled by a series of lenses and apertures, arrives to the surface of the specimen. This electron-sample interaction produces several types of signals that are captured by detectors.
The filament (emitter) which is made from Lanthanum Hexaboride (LaB6) crystal is used as an electron source. Heating is applied to the filament to excite electrons off the filament. The filament is placed in Wenhelt Cylinder that controls the electron beam. SEM uses two types of lenses to correct the path of the electron beam and direct it toward the specimen. The “condenser lens” control the intensity of the electron beam, and the “objective lens” focus the electron beam on the specimen. The final diameter of the electron beam is very important parameter, because by determining the diameter of the electron beam, the produced image can be different. The metal aperture between the two sets of lenses stops any electron that is off-axis or off-energy from going down to the objective lens that helps to narrow the beam. Due to the very narrow electron beam, SEM scans have a large depth of field. [24]

![Diagram](image)

**Figure 2.5. Schematic illustration of interaction volume of the electron beam with a sample.**

After the interaction between the electron beam and the specimen, four major components secondary electrons, backscattered electrons, X-rays,
and visible light are generated. (Figure 2.5) X-rays can be used to get elemental information about the specimen. The backscattered electrons provide great information about the chemical composition and topographical properties of the samples. However, the key player is the secondary electrons, and the real topographical information which is the goal of SEM can be obtained from imaging and analysis of secondary electrons. When secondary electrons hit the scintillator, the light is generated which is then directed to a photo-multiplier tube (PMT). Finally, the light is converted to electrons to produce electric signals which are converted to an image by an amplifier. The image is projected on the monitor providing valuable topographical information about the specimen. [25, 26] The Hitachi S-4700 Scanning Electron Microscope was used to analyze the samples. (Figure 2.6)

![Hitachi S-4700 Scanning Electron Microscope](image)

**Figure 2.6. Hitachi S-4700 Scanning Electron Microscope.**

1) Cells grown on a substrate (thermoreponsive films, 3D scaffolds, pericardial tissue samples and control culture dishes) were rinsed twice with pre-warmed HBSS.

2) The samples were fixed by adding enough 2.5 % glutaraldehyde in 0.1 M sodium cacodylate to each sample and left for 5 min at RT.
3) Samples were then rinsed three times with 0.1 M cacodylate buffer (5 minutes per rinse).

4) Samples were then dehydrated through a graded alcohol series: 50 % ethanol for 10 minutes, then 70 % ethanol for 10 minutes, 95 % ethanol for 10 minutes, 100 % ethanol for 10 minutes and, finally, 100 % ethanol for 10 minutes.

5) After dehydration, the samples were placed in HMDS for 5 minutes and air dried overnight.

6) The next day, the samples were placed on stubs with double-sided adhesive carbon tap sheets, and then sputter coated with gold using an Emitech™ K550X gold sputter coater. The specimens were examined at 15 kV in high vacuum mode.

2.2.5.5. Contact Angle Measurements

This work was done with the kind permission and assistance of Dr. Alexander Gorelov from UCD School of Chemistry & Chemical Biology, Dublin.

The “contact angle” is a characteristic of wetting properties of polymer film surfaces which is extremely important for the analysis of cell-surface interactions.

As it was described by Thomas Young in 1805, the contact angle of a liquid drop on an ideal solid surface is defined by the mechanical equilibrium of the drop under the action of three interfacial tensions (Figure 2.7):
\[ \gamma_{lv} \cos(\theta_Y) = \gamma_{sv} - \gamma_{sl} \]

where \( \gamma_{lv} \), \( \gamma_{sv} \), and \( \gamma_{sl} \) represent the liquid-vapor, solid-vapor, and solid-liquid interfacial tensions, respectively, and \( \theta_Y \) is the contact angle. The equation is usually referred to as Young’s equation, and \( \theta \) is Young’s contact angle.

Figure 2.7. Schematic illustration of a liquid drop on a solid surface with energy vectors and contact angle (\( \Theta \)) as described by the equation.

However, in practice, there exist many metastable states of a droplet on a solid, and the observed contact angles are usually not equal to \( \theta_Y \). There are a number of factors (such as contamination of the liquid, surface roughness, contamination of the surface, mechanical deformation of the surface by liquid, adsorption of the liquid by the surface leading to altered surface) which can influence the contact angle. Therefore, the phenomenon of wetting is more than just a static state.

There are many methods have been described to measure contact angles. However, only a few of them have been found to be widely applicable. One of the most frequently used is the goniometer-telescope measurement of sessile-drop contact angles. [27, 28] Commercial contact
angle goniometers employ a microscope objective to view the angle directly. In the static method a drop is deposited on a surface and the contact angle can be measured by looking at the drop through a goniometer (an instrument that measures contact angles). The dynamic method is similar to the static one but the drop of liquid which is deposited on a surface is modified. The droplet is being deposited via a syringe and the droplet’s volume is changed dynamically without increasing its solid-liquid interface area and this maximum angle is the advancing angle. Volume is then removed to produce the smallest possible angle, which is called the receding angle. The difference between those two measured angles is called contact angle hysteresis. [29, 30]

In this study, advancing contact angle measurements were performed on a home-built goniometer assembled on an optical rail from Newport Optics with opto-mechanical components from Newport Optics and Edmund Optics. DROPimage software marketed by Rame Hart and developed by F.K. Hansen was applied to determine contact angles. [30] pNIPAm - coated samples were placed in a temperature-controlled environmental chamber mounted on an adjustable tilt stage. Contact angles were taken at 40 °C, i.e. above the LCST of the pNIPAm polymer, and controlled using a thermocouple attached to the stage surface. A drop of ultrapure water was deposited on the surface with an initial radius of about 3 mm. For the advancing contact angle experiments, a thin stainless steel needle (gauge 22) was inserted in the center of the drop from above. The volume of a drop was increased by pumping liquid into the drop using a syringe pump. The pumping speed was maintained at the rate of advancing to below 0.5 mm/min. The values of advancing contact angles were taken and averaged between approximately 450 and 500 s. The values of contact angles were
measured in two different positions for each coverslip. Contact angle was also measured also after thermal annealing at 120 °C for 2 h in a vacuum oven. [1, 31]

2.2.5.6. X-ray Powder Diffractometry

This work was done with the kind permission and assistance of Ms. Katarzyna Gniado and Dr. Andrea Erxleben from School of Chemistry, NUIG.

X-ray powder diffraction data was collected on a Siemens D500 powder diffractometer (Munich, Germany) which was fitted with a diffracted beam monochromator. Diffraction patterns were recorded between 5° and 40° (2θ) using Cu Kα radiation (λ = 1.54 Å) with steps of 0.05° and a 2 s counting time per step. Simulated powder patterns were obtained from CDCC data, using the XPert Pro software. These were used to identify and differentiate between different polymorphs. [32]

2.2.6. Cell Culture Techniques

2.2.6.1. Thawing Cryopreserved Cells

Normally, cells are stored in liquid nitrogen for long-term cryopreservation. To thaw, the vials were removed from the liquid nitrogen and heated in a 37 °C water bath until almost thawed, after which they were transferred to a T75 tissue culture flask.

Immediately, a total of 10 ml of media pre-heated to 37 °C was added drop by drop, and the flask was placed into an incubator (37 °C, 5 % CO2). The following day, the media was changed in order to remove remnants of toxic DMSO.
2.2.6.2. Cryopreservation of Cells

The cryopreservation of cells is used in general cell culture practice to preserve them for future uses. First, the cell suspension was harvested by trypsinization or scraping and then spun down to a pellet by centrifugation. Then the pellet was re-suspended in a freezing medium containing 10% DMSO in FBS, aliquoted into sterile cryogenic vials and placed in the -80 °C freezer overnight. Finally, the cells transferred to liquid nitrogen storage for long-term maintenance.

2.2.6.3. Subculture of Adherent Cells

The mouse bone marrow (MS-5) stromal cell line, human bone marrow stromal/stem cells (MSCs) and human corneal epithelial cells (HCEC) were cultivated in DMEM, supplemented with 10% FBS, 1% penicillin-streptomycin antibiotics and maintained in a humidified incubator at 37 °C and 5% CO2.

Cells were checked microscopically daily to ensure they are healthy and growing as expected. Attached cells were grown in T75 flasks. When the cells were approximately 80% confluent, they reached the log phase of growth and required sub-culturing. To sub-culture, using aseptic technique, the media was carefully poured off from a flask with cells. The flask was gently rinsed with PBS twice and 0.5% trypsin solution (approximately 1 ml) was then added. Next, the flask was placed in the incubator at 37 °C and 5% CO2. The flask with detaching cells was observed periodically. As soon as cells detached, pre-warmed media (37 °C) was added to the flask (approximately 2-3 ml) in order to neutralize the trypsin. The cell suspension was then removed and centrifuged in a 15 ml tube at 1500 rpm. Finally, the cell pellet was re-suspended in fresh cell culture media. A haemocytometer was routinely used to calculate the number of cells in the suspension and the appropriate
amount of cells were re-seeded into new flasks at required split ratio. The new flask then was topped up with fresh culture media to required volume 10 ml for T75 flask. Human mesenchymal stem cells (hMSCs), murine stromal cell line (MS-5) and human corneal epithelial cells (HCEC) were received from the Regenerative Medicine Institute (REMEDI), NUIG, Galway. MS-5 and hMSCs are adherent cell exhibiting fibroblast-like morphology. HCEC are adherent cells exhibiting a cobblestone-like morphology. All hMSCs were used in passage numbers between 2 and 5.

2.2.6.4. Phase Contrast Microscopy

Microscopic images of all cell types grown on the various polymer films and control culture dishes were captured using an Olympus IX81 microscope and CellSens Dimension software (Japan).

2.2.6.5. Cell Seeding on pNIPAm Films

For cell culture experiments, pNIPAm films were sterilized under mild UV light for 2 h. The MS-5, MSCs and HCEC were cultivated in DMEM, supplemented with 10 % FBS, 1 % penicillin-streptomycin antibiotics. First, thermoresponsive films were placed on a dry chilling heating thermoplate set to 37 °C for complete temperature equilibration. Second, 2 ml of pre-warmed media (at 37 °C) were quickly added to the pNIPAm-coated dishes. Then, cell suspension was prepared as described above and left in a 15 ml falcon in a water bath at 37 °C for temperature equilibration. Finally, the required number of cells was seeded on each thermoresponsive polymer film. The cells were maintained in a humidified incubator at 37 °C and 5 % CO2.
2.2.6.6. Temperature-induced Cell Detachment from pNIPAm Films

Cell growth and detachment was microscopically observed using a Leica inverted-microscope (Leica, Solms, Germany) on either pNIPAm-coated or control cell culture dishes. The cells were rinsed with pre-warmed HBSS to remove any traces of serum. Cold serum-free DMEM was added to cells and the samples were left on a digitally controlled thermal/cooling plate set to 4 °C. Micrographs of cells were taken every 10 min on a phase contrast microscope to monitor cell detachment.

2.2.6.7. Static Cell Seeding on Acellular Pericardial Scaffolds

To evaluate the suitability of decellularized bovine pericardial scaffolds for cell attachment, 10mm x 10 mm x 5mm tissue samples were placed into a 12-well plate. Single cell suspension (5 x 10^6 cells/cm^2) or cell sheets were first seeded onto fibrous side of BP scaffolds in a culture medium volume of 200 μL. After 4 h of incubation in a humidified incubator at 37 °C and 5 % CO2, a final culture medium volume of 1.5 ml was added to each well of the plate. Seeded scaffolds were maintained in DMEM, supplemented with 10 % FBS, 1 % penicillin-streptomycin antibiotics at 37 °C, 5 % CO2, and culture medium was changed every day. Tissue pericardial scaffolds were removed from the experiment after 7 days and processed for analysis. To determine cell attachment, the scaffolds were analyzed by SEM analysis. To determine cell infiltration, repopulated BP tissue samples were processed for H&E staining.

2.2.6.8. Static Cell Seeding on 3D Polymer Scaffolds

The scaffolds were sterilized and left in 24-well plates. The single cell suspension (5 x 10^6 cells /ml) or cell sheets were added to scaffolds in the culture medium volume of 200 μL per scaffold and left in a humidified
incubator at 37 °C and 5 % CO2 for 4 h. The culture medium was then topped up to its full working volume. After 1 day, the scaffolds with seeded cells were put in other plates and cultured under standard conditions.

2.2.7. Assessment of Cell Proliferation, Viability and Metabolic Activity

2.2.7.1. Quant-iT™ PicoGreen® Assay

The total amount of DNA in cells was determined by the Quant-iT™ PicoGreen® Assay kit according to the manufacture protocol. In brief, PicoGreen® is a fluorescent dye which selectively binds to solubilized double strand DNA. The procedure is described for 6 well plates or 35 mm cell culture Petri dishes. The quantities of solution described below are sufficient for 26 samples and the calibration curve. (Table 2.1)

1) The cells were grown on the polymer pNIPAm films or control cell culture dishes. The cells grown on thermoresponse pNIPAm films were placed on the chilling-heating dry bath set to 37 °C to prevent unwanted cell detachment.
2) The culture media was removed and the samples were rinsed three times with pre-warmed HBSS.
3) 1.5 mls of double distilled H2O was then added to each sample.
4) The cells were then placed in a -80 °C freezer for 30 minutes. After 30 minutes, the cells were removed and allowed to defrost at room temperature on the bench for 30 minutes. This cycle of freeze-thawing was repeated three times in order to ensure complete cell lysis.
5) During the 2nd free-thaw step the following solutions were prepared.
5.1. The 20x TE buffer was diluted in 20 times to prepare 1x TE buffer (for example 8.075 ml of double distilled water was added to 425 µl of 20x TE buffer).
5.2. The 100 µg/ml DNA stock was diluted in 50 times to prepare 2 µg/ml DNA (for example, 588 µl of 1xTE was added to 12 µl of the 100 µg/ml DNA).
5.3. The 2 µg/ml DNA was diluted in 40 times to prepare 50 ng/ml DNA (for example, 585 µl of tube 1xTE was added to 15 µl of 2 µg/ml DNA).
5.4. The PicoGreen® dye was diluted in 200 times, and the tube was covered in aluminum foil (for example, 5174 µl of 1x TE was added to 26 µl of the PicoGreen® dye). As the PicoGreen is photosensitive, the solution was prepared just before it was required.
5.5. The calibration curve was prepared in a 96 well plate during the final freeze-thaw step (Table 2.1).
5.6. 100 µl of cell lysate from each sample was transferred to a 96 well plate after the final freeze-thaw step, and 100 µl of the diluted PicoGreen® was then added to each well containing either cell lysate or the standard curve.
5.7. The samples were prepared in triplicate on independent days.
5.8. Fluorescence was measured using a Thermo Scientific Varioscan FlashMultimode plate reader at an excitation wavelength of 480 nm and an emission wavelength of 530 nm. Fluorescence emission intensity was then plotted versus DNA concentration.
5.9. In a set of culture tubes, the cell suspension (the same type of cells as in experimental groups) with a known number of cells was serially diluted to obtain a range of samples containing known numbers of cells from $10^5$ to $5 \times 10^6$. The total number of DNA was measured in each tube as described above. The calibration curve of total cell DNA versus known numbers of cells was plotted. The number of cells in samples of
interest (in experimental groups) was obtained using this calibration curve.

Table 2.1. PicoGreen® standard curve calculations.

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<tr>
<th>DNA Stock 50 ng/ml</th>
<th>DNA Stock 2 μg/ml</th>
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<td>DNA (ng/ml)</td>
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2.2.7.2. AlamarBlue™ Assay

The metabolic activity of cells grown on pNIPAm films, 3D scaffolds and control cell culture Petri dishes was assessed using an alamarBlue assay according to the manufacturer’s instructions. The alamarBlue reagent or Resazurin is an oxidized form of redox indicator that is blue in color and non-fluorescent. After incubation with viable cells, the reagent becomes fluorescent and changes color converting to its highly fluorescent pink form, resorufin. (Figure 2.8) This change can be detected using fluorescence or absorbance measurement.
It is important that alamarBlue™ is a non-destructive assay and the cells can be cultured and used for further experimentation. The alamarBlue™ dye must be protected from light during the experiment. [33]

1) The cells grown on pNIPAm films were kept on a chilling heating dry bath set to 37 °C in order to prevent premature cell detachment.
2) A 10 % alamarBlue™ solution in HBSS was prepared in a laminar flow hood with the lights off. The tube containing the 10 % alamarBlue™ was wrapped in aluminum foil in order to protect it from light. 2 ml of the 10 % dilution was suitable for one well of a six well plate or for a single 35 mm Petri dish.
3) The culture media was removed from the cells, and the samples were then rinsed three times with pre-warmed HBSS (2 ml) in order to remove any residual media.
4) The 10 % alamarBlue™ solution (2 ml) was then added to each sample.
5) The samples were then covered with aluminum foil and transferred to the incubator for 1 hour for pNIPAm films and 3 h for 3D scaffolds.

6) After the incubation time, the samples were removed from the incubator and 100 µl was taken from each sample in triplicate and transferred to a well in a 96 well plate.

7) The 10 % alamarBlue™ was used as a negative control.

8) The samples were prepared in triplicate on independent days.

9) Fluorescence was measured using a Thermo Scientific Varioscan FlashMultimode plate reader at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. The number of viable cells in a sample was calculated using trypan blue assay. Cell metabolism was expressed as the fluorescence per cell after the fluorescence was divided by the number of vital cells in a sample.

**2.2.7.3. Trypan Blue Viability Assay**

Trypan Blue staining was used during routine sub-culturing to discriminate between viable and non-viable cells. Trypan blue is a dye which selectively stains dead cells.

The mechanism of trypan blue staining is based on it being negatively charged and therefore not interacting with cells unless the membrane is damaged. Non-viable cells will be stained blue, viable cells will be unstained. Trypan Blue should be sterile filtered before using it in order to get rid of particles in the solution that would disturb the counting process.

The viable cells grown on pNIPAm films, 3D scaffolds and in cell sheets were routinely counted using trypan blue exclusion analysis. The cell suspension was diluted 1:4 with 0.4 % Trypan Blue solution. The mixture was allowed to equilibrate at room temperature for five minutes. Next, the hemocytometer chamber was used to count the number of healthy and dead cells. The percentage viability was calculated. Using a
pipette, 100 µL of Trypan Blue-treated cell suspension was applied to the hemocytometer.

![Hemocytometer gridlines](image)

**Figure 2.9. Hemocytometer gridlines.**

The 16 corner squares indicated in **Figure 2.9** were used as a unit for counting. Using a counter, the live (unstained) cells were counted in one set of 16 squares. It is important that the cells were only counted within a big square, on the right-hand and the bottom boundary line. Following the same guidelines, dead cells stained with Trypan Blue were also counted for a viability analysis. Totally, the cells were counted in 5 sets of 16 corners. To calculate the number of viable cells per 1 ml, the average cell number from each of the sets was multiply by $10^4$, then multiply by 5 (to correct for the 1:5 dilution from the Trypan Blue addition), and the final value was the number of viable cells/ml in the original cell suspension. [34]

### Live/Dead Cell Viability Assay

A Live/Dead cell viability assay was used for cell viability analysis. Titration was performed for both calcein-AM and ethidium homodimer-1
to define optimal dye concentration according to the manufacture protocol. A solution of 1 μM calcein-AM and 2 μM ethidium homodimer-1 in HBSS was mixed thoroughly, and then added to the cells for 20 min at 37 °C. The dead cells were prepared by the treatment of cultured cells with cell death inducing agent, dimethyl sulfoxide (DMSO), for 20 min. The staining with calcein-AM resulted in bright green fluorescence in the cytoplasm of undamaged viable cells. Ethidium homodimer stained dead cells red. The samples were prepared in triplicate on independent days. The fluorescent staining was observed using an Olympus IX81 fluorescence microscope (Olympus) and images were captured using a DP72 CCD camera (Olympus) linked to CellSens Dimension software (Olympus). [1]

2.2.8. Fluorescence Microscopy and Imaging Analysis

2.2.8.1. Confocal Microscopy

The invention of the confocal microscope is usually attributed to Marvin Minsky, who produced a working microscope in 1955. In a confocal fluorescence microscope, the specimen is illuminated by a laser. The light coming from the laser passes through an (excitation) pinhole, is reflected by a dichroic mirror, and focused by a microscope objective to a small spot in the specimen. Coherent light emitted by the laser system (excitation source) passes through a pinhole aperture that is situated in a conjugate plane (confocal) with a scanning point on the specimen and a second pinhole aperture positioned in front of the detector (a photomultiplier tube). As the laser is reflected by a dichromatic mirror and scanned across the specimen in a defined focal plane, secondary fluorescence emitted from points on the specimen (in the same focal plane) pass back through the dichromatic mirror and are focused as a confocal point at the detector pinhole aperture. [35]
The significant amount of fluorescence emission that occurs at points above and below the objective focal plane is not confocal with the pinhole and forms extended Airy disks in the aperture plane. Because only a small fraction of the out-of-focus fluorescence emission is delivered through the pinhole aperture, most of this extraneous light is not detected by the photomultiplier and does not contribute to the resulting image. The dichromatic mirror, barrier filter, and excitation filter perform similar functions to identical components in a wide field epi-fluorescence microscope. Refocusing the objective in a confocal microscope shifts the excitation and emission points on a specimen to a new plane that becomes confocal with the pinhole apertures of the light source and detector. [36, 37]

Immunocytochemistry (ICC) was used to detect specific peptides or protein antigens in the cell using antibodies that binds with cellular proteins via specific epitopes and can be detected using fluorescence microscopy. [1]

1) Cells grown on thermoresponsive pNIPAm films were stained at 37 °C in order to prevent premature cell detachment. Cells were rinsed briefly in HBSS and then fixed in 4 % paraformaldehyde. The cells were allowed to fix for 15 min at room temperature.
2) The cells were then rinsed 3 times in HBSS for 5 min each.
3) A 0.2 % of Triton-X was added to each sample and allowed to incubate for 10 min and then it was removed. The cells were then rinsed in HBSS for 5 min.
4) 3 % goat serum was used to inhibit the non-specific protein interactions for 1 h at room temperature. Cells were then incubated with rabbit monoclonal anti-paxillin antibody at 1:250 dilution at 37°C for 1 h.
5) The next day the cells were washed three times in HBSS for 5 min at a time to decrease background staining.

6) The fluorochrome-conjugated secondary antibody was added to the cells for 1 h at room temperature in the dark. Rhodamine phalloidin was used to visualize F-actin in cell cultures. As rhodamine phalloidin staining (at 1:500 dilution) is fully compatible with other fluorescent stains, it was added to secondary antibody and incubated at RT for the last 20 min of staining.

7) The cells were then rinsed in HBSS and covered samples were mounted with UltraCruz mounting medium containing 1.5 μg/mL DAPI for nuclear counter staining.

8) Microscopic images of all cell sheets before and after detachment were captured using an Olympus IX81 inverted microscope and Andor IQ™ software.

*This procedure was done with the kind permission and assistance of Prof. Peter Dockery and Dr. Kerry Thompson of the School of Anatomy, NUI Galway.*

### 2.2.8.2. Imaging Analysis

Focal adhesions (FAs) are specialized adhesive structures that contribute to the organization of actin cytoskeleton and thus play a crucial role in signaling network regulating cell adhesion and spreading. Since FAs play crucial role in multiple cellular processes, their quantitative analysis can provides valuable information for cell behavior analysis. Here, the quantitative analysis of FAs was performed using step-by-step approach described earlier by Horzum. [38] The estimation of the number of focal adhesion per cell was carried out, first, for at least 10 cells from each group. To improve the reliability of our data, we then selected those groups for which a non-significant difference, compared to controls, or between pNIPAm substrates, could potentially become significant
(p<0.05) if the number of measured cells was increased. In this case, the p values were recalculated for a higher number of cells. Raw image data which were greyscale single channel images without any special preprocessing were used for analysis. All image processing steps are carried out using the ImageJ software which provides a wide range of processing and analysis approaches possible via not only built-in functions but also numerous plugins. This approach can be applied to quantify a variety of fluorescent images comprising focal signals from FAs, one of the many complex signaling structures in a cell.

2.2.9. ECM Deposition Analysis of Cell Sheets

2.2.9.1. Short-term Decellularization of Cell Sheets

To prepare and analyze ECM from cells, MS-5 and HCEC cell sheets cultured for 2 weeks were decellularized using the treatment with low concentration of SDS as previously described. [39] The cell sheets were shaken for 30 min in decellularization solution containing 0.05 wt% SDS, 10 mM Tris, and 25 mM EDTA and then washed with HBSS. For SEM imaging, the decellularized cell sheets were fixed in 2.5 % glutaraldehyde for 10 min, dehydrated in a graded ethanol series, air-dried and finally sputter-coated with gold and examined using a Hitachi S-4700 SEM. The specimens were examined at 15 kV in high vacuum mode.

2.2.9.2. Extraction, Isolation and Densitometric Analysis of Type I Collagen

The cell sheets were digested with 150 µl pepsin (porcine gastric mucosa) solution containing phenol red (100 µg/ml pepsin in 0.05 N acetic acid) per cell sheet for 2 hours at 37 °C and continuous shaking (200 RPM) and neutralised with 1 N NaOH. Samples for SDS-PAGE were prepared using appropriate dilution of water and 5X sample buffer.
Finally, 15 µl per sample solution per well was loaded on the gel (5 % running gel / 3 % stacking gel) after 5 minutes heating at 95 °C. Mini-PROTEAN Tetra Electrophoresis System (Bio-Rad, Ireland) was used for the electrophoresis run. The low-voltages (about 50 V) were applied for the initial 30 minutes, followed by 120 V for the remaining time. The gels were washed gently in ultrapure water and stained using Silver Quest™ (Invitrogen, Ireland) silver stain kit, according to the manufacturer instructions. Images of the gels were taken after brief washing with water. In order to quantify type I collagen, the gel densities (GeneTools software, Syngene, Ireland) of collagen α1(I) and α2(I) chains were evaluated and compared with the band densities of standard type I collagen (Symatese Biomateriaux, France).

[40]

2.2.10. **Histological Analysis**

2.2.10.1. **Hematoxylin and Eosin Staining**

The specimens (pericardial tissue scaffolds or cell sheets) were fixed in 10 % formalin for 24 h, embedded in paraffin using an automated tissue processing machine, and sectioned using a microtome. 5µm sections were mounted on slide, dried and deparaffinized and rehydrated according to the protocol. (Table 2.2)
Table 2.2. A detailed IHC tissue processing protocol.

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<td>Xylene</td>
<td>with agitation</td>
<td>5 min</td>
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<td>(3 or 4 times)</td>
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<td>Xylene</td>
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<td>5 min</td>
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<tr>
<td>(3 or 4 times)</td>
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<tr>
<td>100% ethanol</td>
<td>with agitation</td>
<td>1.5 min</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>with agitation</td>
<td>1.5 min</td>
</tr>
<tr>
<td>70% ethanol</td>
<td>with agitation</td>
<td>1.5 min</td>
</tr>
<tr>
<td>Distilled water</td>
<td>with agitation</td>
<td>1.5 min</td>
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<tr>
<td>Distilled water</td>
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Next, the samples were stained with hematoxylin and eosin (H&E) according to manufacturer’s instruction. Dehydration was done through a series of ethanol changes (70 %, 80 %, 90 % and 100 %). Finally, the slides were cleared in xylene for 5 minutes and mounted using DPX. Images were captured with upright microscope Olympus BX51 with DP70 Color Camera. Thicknesses of cross-sectioned cell sheets were measured from digitalized images using ImageJ software (NIH, Bethesda, MD, USA). [41]

2.3. Statistical Analysis

Statistical analysis was performed using Statistica 8.0 software. Values are expressed as mean ± standard deviation (SD). The error bars refer to SD where 6 separate samples from each control or experimental group were used. The analysis was performed in triplicate on independent days. If normal distribution was confirmed using Kolmogorov-Smirnov test and Shapiro-Wilk’s W test, student t-test were conducted to compare independent groups and multiple comparisons were made using one-way ANOVA test. Otherwise, non-parametric Kruskal-Wallis test for
multiple comparisons and Mann-Whitney test for two independent
groups were carried out. Statistical significance was defined as p-value
<0.05. [42, 43]
2.4. References

1. Dzhoyashvili, NA.; Thompson, K.; Gorelov, AV.; Rochev, YA. Film Thickness Determines Cell Growth and Cell Sheet Detachment from Spin-Coated Poly (N-isopropylacrylamide) Substrates. ACS Applied Materials & Interfaces. 2016, 8 (41), 27564–27572.


11. Antonov, EN.; Krotova, LI.; Minaev, NV.; Minaeva, SA.; Mironov, AV.; Popov, VK.; Bagratashvili, VN. Surface-selective Laser Sintering of Thermolabile Polymer Particles Using Water as Heating Sensitizer. Quantum Electronics. 2015, 45, 1023-1028.


27. What is Contact Angle? 


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https://www.thermofisher.com/order/catalog/product/DAL1025

34. Counting Cells Using a Hemocytometer. 


40. Huang, CY.; Kuo, JM.; Wu, SJ.; Tsai, HT. Isolation and Characterization of Fish Scale Collagen from Tilapia by a Novel Extrusion-hydro-extraction Process. Food Chemistry. 2016, 190, 997-1006.


3. FILM THICKNESS DETERMINES CELL GROWTH and CELL SHEET DETACHMENT from SPIN-COATED POLY (N-ISOPROPYLACRYLAMIDE) SUBSTRATES

Some parts of this study were adapted with the permission from a published work:

Dzhoyashvili NA, Thompson K, Gorelov AV, Rochev YA. Film Thickness Determines Cell Growth and Cell Sheet Detachment from Spin-Coated Poly(N-Isopropylacrylamide) Substrates. ACS Applied Materials & Interfaces. 2016, 8 (41), 27564–27572. DOI: 10.1021/acsami.6b09711 Copyright 2016, American Chemical Society.
3.1. Introduction

A short-term goal of regenerative medicine is the development of functional tissue substitutes created from a patient’s own autologous cells that can be applied in clinical practice. [1] Cell sheet transplantation provides a promising therapeutic approach for tissue regeneration because it does not cause inflammation and enables improved integration and attachment to a host tissue. [2, 3] Biomaterials play a significant role in the creation of synthetic cell culture substrates. pNIPAm is one of the most studied intelligent biomaterials widely used for cell sheet harvesting. [4, 5] The substrates when coated with pNIPAm or its copolymers enable the detachment of cells from culture dishes by temperature reduction below the lower critical solution temperature and without using digestive reagents which destroy extracellular matrix assembly, intercellular connections and focal adhesion complexes. The preserved ECM and developed cell-cell and cell-matrix interactions in cell sheets greatly supports cell adherence to natural or polymer scaffolds, or directly to tissue after in vivo transplantation. Despite pNIPAm’s biocompatibility, bulk pNIPAm coatings display resistivity to cell adhesion. [6] This fact can present a significant obstacle for cell sheet fabrication. The choice of deposition method can significantly affect the surface bioactivity. The methods which are used to fabricate thin pNIPAm films can be divided, according to preparation procedure, into two major categories: covalently polymerization bonding and polymer coating. Polymerization and grafting by irradiation employing electron beam plasma as the irradiation source is widely used to deposit thin pNIPAm on the substrate. [7, 8] In addition to the irradiation procedure, pNIPAm can be covalently grafted by two other techniques: atom transfer radical polymerization (ATRP) and reversible addition-fragmentation chain transfer radical polymerization (RAFT). However,
covalent polymerization bonding techniques are too expensive and too complex to be used in the general practice. Polymer coating employs the deposition of pNIPAm copolymers with a layer-by-layer technique [9] and physically polymer coating methods including physical adsorption [10], solvent casting [11] and spin-coating. [12, 13] Several studies have shown the dependency of cell behavior on the thickness of covalently grafted pNIPAm films. In particular, the pNIPAm brushes with thickness higher than 20 nm fabricated by electron beam irradiation impede cell attachment, and the optimal thickness was found to be between 15 and 20 nm. [14] It was found that atom transfer radical polymerization (ATRP)-fabricated pNIPAm brushes with thickness between 20 and 45 nm are the most suitable for HepG2 cell adhesion and detachment. [15] While the cell behavior on covalently grafted pNIPAm brushes is well understood, there are a limited number of studies which systematically investigated cellular adhesiveness of spin-coated pNIPAm films.

The key advantage of a spin-coating technique is the combination of reproducibility and versatility. The method allows very fast, gentle and non-deforming drying of polymer films with uniform surface which characteristics can be controlled very carefully. [16] The technique provides a more inexpensive and convenient way to prepare thermoresponsive cell culture surface and can be deployed in many research labs.

pNIPAm coating thicknesses critically influence the material’s thermoresponsive properties. In regard to a pNIPAm covalently grafted substrate, it was shown that thin pNIPAm films impede water penetration and increase hydrophobicity. In contrast, thick pNIPAm films are more hydrophilic and that could be due to larger freedom and mobility of pNIPAm brushes. [17] In case of the spin-coated pNIPAm films, the thickness is also closely related to surface wettability. Muller-
Buschbaum’s group recently investigated the behavior and interfacial interaction with water of non-crosslinked spin-coated pNIPAm films. [18] In a humid atmosphere and at room temperature, pNIPAm films start to adsorb water in a one-dimensional manner in height. Water molecules permeate through the surface, diffuse into accessible free space between linear pNIPAm chains and accumulate in the polymer film. As a result, pNIPAm films might swell and increase water adsorption with time. It was also found that pNIPAm films become more hydrophilic with decrease in thickness. [18, 19]

Surface wettability (i.e. hydrophobicity and hydrophilicity) is closely linked with surface energy which describes the interaction of the surface with a range of materials outside the surface. It is a fact that materials with a higher surface energy have a higher affinity to water and consequently are more hydrophilic. Otherwise, hydrophobic surfaces have low surface energy and therefore expel water. The cell adhesion properties of pNIPAm films are closely related to protein adsorption. Considering surface hydration, protein adsorption and material’s cell adhesion properties, high surface energy substrates tend to resist protein adsorption, while low surface energy substrates better adsorb proteins from the surrounding medium. [20, 21] The influence of surface wettability on protein adsorption is not completely understood, but it is considered that due to the low water content on hydrophobic surface the latter rapidly interacts with internal hydrophobic protein domains which attach, unfold from the core of the protein and spread over the surface. On the other hand, hydrophilic surfaces tend to interact with charged and polar groups of protein’s surface, which attach but did not change their conformation, i.e. do not unfold and spread. Hence, wettability of a material’s surface is important for the adsorption of proteins and can be
considered as a crucial factor in the evaluation of material’s biocompatibility.

Nash et al. previously showed that 3T3 cells attached and grew on spin-coated films regardless film thickness. [16] In contrast, in the present study, different cell lines including epithelial, endothelial and stromal cells grew inconsistently on spin-coated pNIPAm films. To systematically investigate the dependency of cell behavior on thickness, surface morphology and wettability of spin-coated pNIPAm films, mouse stromal (MS-5) cells were seeded and examined in terms of cell attachment, growth, and detachment, metabolic activity, viability and proliferation rate, morphology and cytoskeleton organization. The stromal cells including MS-5 cells have been commonly used for biocompatibility evaluation of biomaterials. [22-24] Moreover, they provide a supportive environment for stem cells [25, 26], including human hematopoietic stem/progenitor cells (HSPCs). [27, 28] The thickness of pNIPAm films was measured optically by viewing optical profilometry and employing a laser ablation technique. To analyze pNIPAm film morphology and wettability, the films of different thicknesses were characterized using atomic force microscopy and contact angle measurements, respectively. Immunofluorescence staining of F-actin and paxillin was used to examine the cells grown on pNIPAm films. The actin cytoskeleton is connected to the extracellular matrix via macromolecular complexes, such as focal adhesions. Paxillin, the major focal adhesion adaptor protein, binds several proteins that contribute to the organization of actin cytoskeleton and thus plays a crucial role in signaling network that can regulate cell adhesion and spreading. [29] The possibility to detach the cells in the form of a contiguous sheet from pNIPAm films of different thicknesses was examined by lowering the temperature.
The results of present study may be important for chemists improving polymer properties, engineers optimizing the polymer production processes and biomedical scientists specializing in cell sheet based tissue engineering.

3.2. Results and Discussion

3.2.1. Physical Characterization of pNIPAm Films

3.2.1.1. pNIPAm Film Thickness Measurements

The thickness range measured from dry pNIPAm films is presented in Figure 3.1. The results are in agreement with our previously obtained thickness measurements. [16]

![Figure 3.1. Polymer film thickness as a function of polymer concentration.](image)
3.2.1.2. Contact Angle Measurements of pNIPAm Films

Advancing contact angles of the coated films give the information regarding surface energy properties used for more detailed qualitative assessment of the wettability or hydrophobicity/hydrophilicity of film surface. The advancing contact angle for water on spin-coated pNIPAm films with different thicknesses are summarized in Table 3.1. It was shown earlier for thick pNIPAm films that while pNIPAm becomes hydrophobic over its LCST, the polymer matrix still contains a certain degree of water. [30, 31] The same results were obtained by Ryder and co-authors. [32] Using fluorescence emission of 3-hydroxyflavone probes, they revealed that dry non-crosslinked pNIPAm films have significant water uptake capacity even above LCST when pNIPAm exists in a more hydrophobic and collapsed state. According to our results, 50 and 80 nm dry pNIPAm thin films were quite hydrophilic at 40 °C, while the contact angle of thicker pNIPAm films gradually increased at the same temperature (Figure 3.2). This implies that thinner pNIPAm films adsorbed water and become more hydrophilic with a decrease in thickness. Our data are corroborated by the results obtained in Muller-Buschbaum’s group. [18, 19] They recently showed that water can be accumulated by non-crosslinked spin-coated pNIPAm films with time and that pNIPAm films become more hydrophilic with decrease in thickness. This indicates that spin-coated non-crosslinked pNIPAm films showed a positive relationship between hydrophobicity and film thickness. Films with thickness below 300 nm show advancing contact angle which stays constant across the sample (Figure 3.3, A-D). However, a “sawtooth” pattern was observed for 300 and 900 nm-thick films in the advancing contact angle (Figure 3.3, E-F). It is known as “stick-slip” behavior of a moving drop. Such behavior is the result of the advancing leading edge sticking to the ridge formed on the surface,
which causes an increase in contact angle. While more liquid is pumped into the drop, the drop front jumps to a new location resulting in reduction in contact angle again. We name the angle just prior to the drop front slipping the “slip angle”. The angle after the drop has jumped to a new position is named the “stick angle”. Stick-slip behavior was previously examined and explained on pNIPAm thick solvent cast films where it was more pronounced. [30] According to our results, such slip-stick behavior was more obvious on 900 nm-thick coated films, than on 300 nm-thick films and was not observed on 50, 80 and 120 nm pNIPAm films indicating thickness dependence. The reported contact angle for thick 300 and 900 nm pNIPAm films corresponds to stick contact angle which is taken after the liquid drop reaches its equilibrium state.

As heat-treatment is widely employed in industrial processes for stabilization of material microstructure, the pNIPAm surface was analyzed after thermal annealing. The applied heat provides the energy which is needed to relieve internal stress which can occur during spin-coating and to order pNIPAm linear chains after spin-coating and thus to enhance the stability of pNIPAm polymer thin films. Contact angle measurement was repeated on pNIPAm films after the coated films were dried and then annealed for 2 h at 120 °C under vacuum. It was found that after annealing the trend of surface wettability did not change and the stick-slip effect was still observed on 300 and 900 nm pNIPAm films (Figure 3.2 and Table 3.1). These results indicate that the spin-coating process allows the preparation of thin films with stable and uniform surface structure and that spin-coating technique itself does not influence pNIPAm film physicochemical properties such as wettability.
Table 3.1. Average advancing water contact angle measurements taken at 40 °C on spin-coated pNIPAm films before and after thermal annealing. The a) indicates a stick angle.

<table>
<thead>
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<th>pNIPAm film thickness (nm)</th>
<th>Water contact angle (Degrees)</th>
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<tr>
<td></td>
<td>as coated</td>
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<tr>
<td>50</td>
<td>49 ± 1</td>
</tr>
<tr>
<td>80</td>
<td>49 ± 1</td>
</tr>
<tr>
<td>120</td>
<td>52 ± 1</td>
</tr>
<tr>
<td>300</td>
<td>57 ± 2(^a))</td>
</tr>
<tr>
<td>900</td>
<td>60 ± 3(^a))</td>
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</table>

Figure 3.2. The dependence of contact angle on pNIPAm film thickness before and after thermal annealing at 120 °C under vacuum.
Figure 3.3. Contact angles measured on (A) Thermanox control, (B) 50 nm, (C) 80 nm, (D) 120 nm, (E) 300 nm and (F) 900 nm pNIPAm films at 40 °C.

3.2.1.3. Surface Roughness Evaluation of pNIPAm Films

The values of root mean square (RMS) roughness analysis showed no significant differences between the groups and were comparable with flat non-coated glass slides (RMS 4.5 ±2.0 nm). (Table 3.2) This indicates
that all dry spin-coated pNIPAm films were assessed to be smooth and flat regardless of film thickness.

Table 3.2. Thickness and RMS values of pNIPAm films.

<table>
<thead>
<tr>
<th>Polymer concentration</th>
<th>Thickness of polymer films (nm)</th>
<th>RMS value (nm)</th>
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<tbody>
<tr>
<td>0.5%</td>
<td>50</td>
<td>6.8 ±2.2</td>
</tr>
<tr>
<td>1%</td>
<td>80</td>
<td>4.4 ±3.2</td>
</tr>
<tr>
<td>2%</td>
<td>120</td>
<td>3.9 ±2.6</td>
</tr>
<tr>
<td>4%</td>
<td>300</td>
<td>7.3 ±4.1</td>
</tr>
<tr>
<td>8%</td>
<td>900</td>
<td>5.3 ± 3.2</td>
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3.2.2. Cell Cultures on pNIPAm Films of Different Thicknesses

MS-5 cell attachment and proliferation were monitored for a period of 7 days and analyzed by microscopic examination, metabolic activity, viability assessment and DNA content analysis.
3.2.2.1. Cell Adhesion, Growth and Morphology Analysis

A

B

C
Figure 3.4. Bright field microscopy images of MS-5 cells cultured on (A) Thermanox control, (B) 50 nm, (C) 80 nm, (D) 120 nm, (E) 300 nm and (F) 900 nm pNIPAm films. Initial cell-seeding density 40000 cells/cm$^2$, incubation time 48 h, scale bar 100 μm.

According to microscopic observation, after 24 h MS-5 cells cultured on Thermanox control were already confluent and after 48 h they grew in layers on top of each other. MS-5 cells grown on 50 and 80 nm pNIPAm films presented similar morphology. The cells were fully spread and had a large cell area with numerous well-defined and fully stretched pseudopodia, indicating a good cell attachment. In contrast, MS-5 cells on 120 nm did attach and proliferate but formed short rudimentary processes and did not display the fibroblast-like shape. MS-5 cells seeded on 300 and 900 nm pNIPAm films tended to grow in loosely attached clumps, did not flatten out and generally had a rounded morphology. (Figure 3.4)
Figure 3.5. Adhesion and proliferation of MS-5 grown on pNIPAm films of different thicknesses (50-900 nm). Initial cell-seeding density 40000 cells/cm$^2$, incubation time 12 h, 24 h and 48 h. Data is mean ± standard error of mean (SEM), * p<0.05 compared with control. Cell numbers were calculated based on a calibration curve of DNA amount vs. known cell numbers.

The DNA content analysis confirmed significant variability in cell densities on spin-coated pNIPAm films of different thicknesses. (Figure 3.5) During the first 12 hours after cell seeding, the number of attached cells on 50 and 80 nm pNIPAm films was comparable to that on Thermanox controls with no significant difference. The cells attached to 120 nm pNIPAm films but cell density was 38.3 %, i.e. significantly less than on Thermanox control. The cells seeded on 300 and 900 nm pNIPAm films loosely attached, and therefore the cell density was less than 10 %. The DNA content after 12 h and 48 h of incubation on 120 nm and particularly on 300 and 900 nm pNIPAm films were still significantly lower when compared with Thermanox controls.
3.2.2.2. Cell Viability and Metabolic Activity Analysis

Figure 3.6. Cell metabolic activity of MS-5 grown on control slides and pNIPAm films with different thickness. AlamarBlue assay test results. Initial cell-seeding density 40000 cells/cm$^2$, incubation time 48 h. Data is mean ± standard error of mean (SEM), no significant difference compared with control.

The decrease in proliferation rate of MS-5 cells which preferably formed clumps on 300 nm and 900 nm pNIPAm films was accompanied by decrease in metabolic activity that may be a consequence of poor cell attachment. (Figure 3.6) It is interesting to note that while the MS-5 cells on thick pNIPAm films displayed a slow proliferation rate, their metabolic activity still remained at a high level. This discrepancy could imply that while the cells remained in a semi-quiescent state they were attached, spread out and able to uptake sufficient nutrients to meet the metabolic demands. This suggestion is supported by the study where Coller and co-authors demonstrated high metabolic rates in quiescent fibroblasts. [33] The authors concluded that while quiescence signals
often inactivate TOR kinase, resulting in reduced cell growth, it is not always associated with reduced metabolism. In contrast, MS-5 cells on thick 300 and 900 nm pNIPAm films were loosely attached and therefore, easily detached from the surface. The analysis of cell viability from the Live/Dead assay results did not reveal any significant differences in cell survival among groups. The percentage of viable cells grown on pNIPAm films reached almost 100% for all tested samples, as indicated in Figure 3.7. However, there was a limitation, with performing a live/dead assay on 300 and 900 nm thick pNIPAm films due to low number of adhered cells on the surface.
Figure 3.7. Live/dead assay images representing cell viability of MS-5 cells on (A) uncoated control glass slides (alive cells), (B) uncoated control glass slides after treatment with DMSO (dead cells), (C) 50 nm, (D) 80 nm, (E) 120 nm, (F) 300 nm and (G) 900 nm pNIPAm films. Initial cell-seeding density 40000 cells/cm$^2$, incubation time 48 h, scale bar 100 μm.

3.2.2.3. Actin Cytoskeleton Organization and Focal Adhesion Assessment

The changes in cell morphology were accompanied by changes in actin cytoskeleton organization. (Figure 3.8, A-F) The qualitative immunofluorescence analysis indicated that the cells grown on 50 and 80 nm pNIPAm surfaces exhibited a well-spread shape with long, thin filopodia and a well-organized pattern of numerous and straight actin stress fibers that is similar to the control group. The cells grown on the 120 nm pNIPAm surface had shorter filopodia with a smaller number of
short actin filaments, and the intact actin stress fibers of cells seeded on 300 and 900 nm pNIPAm surfaces were mostly lost. Quantification of cells expressing paxillin shows that 50 and 80 nm pNIPAm films had similar number of paxillin-positive focal adhesions as the cells grown on bare Thermanox controls. In contrast, paxillin was poorly expressed in cells grown on 120 nm pNIPAm films, and cells on thick 300 and 900 nm pNIPAm films formed significantly fewer paxillin-positive focal adhesions as cell grew in loosely attached clumps. (Figure 3.8, G)
Focal adhesions per cell vs. Thickness of pNIPAm films (nm)

Diagram showing the relationship between focal adhesions per cell and thickness of pNIPAm films. The graph indicates a decrease in focal adhesions as the thickness of the pNIPAm films increases, with significant differences marked by asterisks for thicknesses of 120 nm, 300 nm, and 900 nm compared to the control.
Figure 3.8. Immunofluorescence microscopy images of paxillin (green) and actin (red) staining of MS-5 cells cultured on (A) control slides, (B) 50 nm, (C) 80 nm, (D) 120 nm, (E) 300 nm and (F) 900 nm pNIPAm films. (G) Statistical analysis of the number of paxillin-positive focal adhesions per cell in different groups. Initial cell-seeding density 40000 cells/cm², incubation time 48 h, scale bar 50 μm. Data is mean ± standard error of mean (SEM), * p<0.05 compared with control. The estimation of the number of focal adhesion per cell was carried out for at least 10 cells from each group. The white arrows indicate focal adhesion sites.

Therefore, the analysis of adhesion, proliferation, viability and metabolic activity, morphology and cytoskeleton organization revealed that 50 and 80 nm pNIPAm films are the most compatible coatings to support cell growth and maintain cell function. The 120 nm pNIPAm films did not affect MS-5 metabolic activity and viability, but significantly reduced the rate of cell growth. 300 nm and 900 nm thick pNIPAm films were not suitable for long-term cell culture due to poor cell attachment.

Many surface characteristics, conditions and components of surrounding environment influence cell adhesion and growth through protein adsorption process. Numerous studies have reported different cell behaviors on cell culture surfaces with different wettability, which are likely related to a different pattern, i.e. the type, quantity and conformation, of adsorbed proteins from serum containing media. [20, 21, 34] It is considered that due to the low water content on hydrophobic surfaces the latter rapidly interacts with the internal hydrophobic protein domains which attach, unfold from the core of the protein and spread over the surface. On the other hand, hydrophilic surfaces tend to interact with charged and polar groups of protein’s surface, which attach but did not change their conformation, i.e. do not unfold and spread. [35, 36] It
is well known fact that albumin, which is a primary component of fetal bovine serum, readily adsorbs to the surface and resists cell attachment, while vitronectin or fibronectin are responsible for cell adhesion. [37] It is necessary to note that protein molecules adsorb to the surface from multiprotein serum-containing media in a competitive mode according to their concentration, size and affinity to the surface. Hydrophobic surfaces tend to firmly adsorb larger and the most abundant proteins like albumin [38-40], which undergo structural rearrangements and modifications with a greater extent than on hydrophilic surfaces. [41, 42] Hydrophilic surfaces adsorb a lower quantity of proteins [43], but due to small conformational changes, albumin doesn’t occupy the whole surface and allows lower concentrations of cell adhesion proteins to attach to the surface. Moreover, due to a low affinity for hydrophilic surfaces, albumin can be replaced with time by proteins with a higher affinity like fibronectin. [40, 44] Therefore hydrophobicity and hydrophilicity of a surface can significantly alter cell behavior including cell orientation, morphology and cytoskeleton arrangement. An interesting study performed by Kleinman et al. showed that non-adhesive protein albumin can maintain the exposure of active integrin-binding RGD sites in fibronectin. [45] Such relationships between albumin and fibronectin increases cell adhesive activity of adhesion proteins on hydrophilic surface (Figure 9). Hence, when serum containing media is added to cell culture surface, albumin adsorbs and dominate rather on hydrophobic surfaces while cooperates with fibronectin and other adhesive proteins supporting their active conformation on hydrophilic surfaces. [21, 40, 44]

In case of the spin-coated pNIPAm films, the surface wettability is closely related to thickness. Muller-Buschbaum’s group recently described the mechanism of interfacial interaction of non-crosslinked
spin-coated pNIPAm films with water. [18, 19] pNIPAm films start to adsorb water in a one-dimensional manner in height and accumulate water molecules into accessible free space between linear pNIPAm chains. These studies that corroborate our findings also showed that pNIPAm films become more hydrophilic with decrease in thickness. We showed that the cells attached, rapidly proliferated and reached confluency on the more hydrophilic 50 and 80 nm pNIPAm films with similar surface wettability, attached but did not spread on moderately hydrophilic 120 nm pNIPAm films and loosely attached and formed spheroid clusters on more hydrophobic 300 and 900 nm pNIPAm films. Our results are in accordance with the results of numerous studies which have shown that hydrophilic surfaces, in comparison with hydrophobic substrates, have higher levels of cell attachment, spreading, proliferation and differentiation.[46-49] Therefore, it is unlikely that cell attachment and growth is a single-valued function, but other factors including chemical and structural nature of material, the method of preparation and the influence of wettability on conformational changes in adsorbed proteins should be considered.

3.2.3. Cell Detachment Analysis

To complete the analysis of cell behavior on spin-coated pNIPAm films with different thicknesses, we analyzed the detachment of MS-5 cells grown on pNIPAm films for 7 days.
Figure 3.9. Images of MS-5 cell detachment from (A) Thermanox control, (B) 50 nm and (C) 80 nm pNIPAm films at 4 °C. Initial cell-seeding density 40000 cells/cm², incubation time 7 days, cell detachment time 25-30 min, scale bar 100 μm. The yellow arrow indicates a detaching MS-5 sheet.

Qualitative image analysis demonstrated the typical pattern of cell detachment from pNIPAm films after the temperature decreased down to 4 °C. We observed that the cells detached from pNIPAm polymer films with different thicknesses in different ways. After the temperature was dropped, cells grown on Thermanox control surfaces did not detach, as expected, but the cells grown on pNIPAm 50 and 80 nm films detached as a contiguous cell sheet for 25-30 min. (Figure 3.9) The results of the study suggest that more hydrophilic 50 and 80 nm thin pNIPAm films are more preferable for cell sheet fabrication while more hydrophobic 300 and 900 nm thick spin-coated pNIPAm films impede cell attachment. The control of the thickness of pNIPAm films fabricated by the spin-coating technique with high reproducibility offers an effective tool for cell sheet-based tissue engineering.
3.3. Conclusions

The thickness of polymer pNIPAm films fabricated by spin-coating is shown to influence the cellular attachment and spreading. The contact angle of pNIPAm films is gradually increased with increasing of film thickness. It is unlikely that this change in surface wettability is related to the process of film fabrication, because the annealing treatment of spin-coated pNIPAm films resulted in a similar trend in the contact angle values. The roughness analysis showed that all dry spin-coated pNIPAm films were assessed to be smooth and flat regardless of film thickness.

Although the cells grow on spin-coated pNIPAm surfaces, there are significant changes in cell densities and perturbations in cellular cytoskeletal organization. In agreement with previous studies, 300 and 900 nm thick spin-coated pNIPAm films impede cell attachment. Although other spin-coated pNIPAm surfaces attach and release cells, 50 and 80 nm pNIPAm substrates would be more preferable for applications such as cell sheet engineering, because cell attachment, functions and detachment as a contiguous cell sheet are more successful on thinner pNIPAm films.

The reason might be due to changes in surface hydration with film thickness changes and relationships between pNIPAm film surface and multicomponent cell culture media containing proteins of different size, concentration and affinity towards the surface.

This study is important for the assessment and design of thermoresponsive surfaces for cell sheet-based tissue engineering using commercially available pNIPAm. The results suggest that it is possible to control the cell behavior on thermoresponsive surface by changing the thickness of non-crosslinked pNIPAm films and employing fairly fast and reproducible spin-coating technique. This should also be taken into
account during the design of new pNIPAm-based thermoresponsive surfaces for biological applications, especially for cell sheet fabrication.
3.4. References


4. FABRICATION of FUNCTIONAL HIGH-DENSITY CELL SHEETS for TISSUE ENGINEERING USING SPIN-COATED POLY (N-ISOPROPYLACRYLAMIDE) THIN FILMS

4.1. Introduction

The current goal in developing cell sheet engineering technology is to provide an improved approach for generation of viable tissue-like constructs. Success requires the characterization of cell sheets before and after detachment from a substrate. Cell sheet tissue engineering technologies have been rapidly developed and applied in different areas of biomedical research, including regenerative medicine [1-3], cell-based drug screening assays [4, 5], and tissue and disease modelling.[6-9]

Several studies have shown that cell sheet transplantation has been effective in the treatment of severe diseases. Most experimental studies have used cell sheets for scaffold-free tissue engineering. [10-12] As the cell sheets can be transplanted without sutures, this procedure saves time, reduces risk from biological materials and can avoid suture-related problems. This approach offers several distinctive therapeutic advantages, especially for cornea, esophageal epithelium or oral mucosa regeneration. [13-15]

Currently, the most effective way of cell sheet generation involves the use of pNIPAm-based thermoresponsive substrates. [16-19] pNIPAm dissolves in cell culture media when the temperature drops below its low critical solution temperature of about 32 °C. Among many methods, spin-coating technique has been shown to provide an inexpensive and convenient way to prepare thermoresponsive polymer films which thickness and surface characteristics can be carefully controlled. [20,21] Proteolytic enzymes generally used to detach adherent cells can
potentially damage cell surface proteins, including ion channels, growth factor receptors, ECM and affect their downstream signal transduction pathways. [22] In contrast, thermoresponsive polymer-based technology enables fabrication of contiguous and viable cell sheets composed of cells and functional extracellular matrix avoiding the use of proteolytic reagents. In cell sheets, the cells are embedded in ECM and delivered together with secreted growth factors that might increase survival rate and the ability of cells to engraft into damaged tissue after transplantation. [23, 24] Different components of the cytoskeleton network are important in regulation of cell sheet integrity in the absence of a substrate. [25, 26]

It was previously found that 50 nm spin-coated pNIPAm films enable harvesting of contiguous cell sheets. [20] The main goal of this study was to examine the quality of lifted cell sheets harvested from 50nm spin-coated pNIPAm films. Two different types of cells, mouse stromal cell line (MS-5) and human corneal epithelial cells (HCEC), were examined and compared for their biological properties. In particular, the cell morphology, time of cell sheet recovery as well as the metabolic activity and cell viability of cell sheets before and after detachment and after re-attachment were examined. The histological analysis of cell sheets and the immunofluorescence analysis of paxillin and actin fibers were performed. Paxillin was examined because it is an important structural focal adhesion component which plays a central role in the coordination of numerous regulatory proteins involved in cytoskeletal reorganization during cell adhesion. [27-29] The knowledge obtained from this systematic characterization of cell sheets may be required for better control of cell sheet functions. SEM images of ECM and SDS-PAGE analysis of type I collagen were performed in MS-5 and HCEC lifted cell sheets. These results can be valuable and used in tissue
4.2. Results and Discussion

4.2.1. Generation and Morphology of MS-5 and HCEC Lifted Cell Sheets

Despite the wide use of pNIPAm-modified thermoresponsive surfaces, there is a need to characterize cell sheet harvesting from pNIPAm films as the harvesting process may influence cell sheet properties. Microscopy analysis showed that MS-5 with spindle-shaped morphology and HCEC with normal cobblestone-like cell morphology attached, expanded and reached confluence on pNIPAm-coated surface (Figure 4.1, A). Immediately after reducing the temperature, the cells became less spread and flattened and started to detach. The cells actively detached from the dish edges only, followed by the complete detachment of the rest of the cell sheet from the substrate. The perimeter of detaching cell sheets was torn, resulting in significant contraction of the lifted sheet. (Figure 4.1, B) MS-5 were successfully harvested as a contiguous cell sheet at 1 week and 2 weeks, and HCEC cell sheets were detached only at 2 weeks. (Figure 4.1, C)
Figure 4.1. Morphological characteristics of engineered MS-5 and HCEC cell sheets. (A) Phase contrast images of MS-5 and HCEC growing on pNIPAm-coated surface prior to cell detachment. Scale bar: 100 μm. (B) The cell sheets began to detach from pNIPAm-coated culture dishes by reducing temperature to 4 °C. Scale bar: 100 μm. (C) Engineered MS-5 and HCEC cell sheets demonstrated significant contraction when lifted off the cell culture dish. Scale bar: 10mm. The yellow arrow indicates the detaching cell sheet. The white arrows indicate a ruptured cell sheet.

To assess cell sheet detachment from pNIPAm substrate, we compared the time of detachment of MS-5 and HCECs cell sheets. MS-5 and HCEC successfully grew on pNIPAm surface for 7 days and 14 days and formed confluent monolayers at 37 °C. Visual tracking revealed that MS-5 grown 7 days and 14 days successfully detached in 7-15 min. In contrast, HCEC grown on pNIPAm surface for 7 days showed poor
detachment, and those cultured on pNIPAm surface for 14 days can detach as an intact contiguous sheet within 30-40 min.

4.2.2. Viability and Metabolic Activity Evaluation of MS-5 and HCEC Sheets

To assess cell sheet behavior, viability and metabolic activity assays were applied to cells under two conditions: prior to cell sheet detachment and after cell sheet lifting. Also, the harvested 2-week cell sheets were re-attached to tissue culture polystyrene dishes and cell behavior was analyzed 48 h after cell sheet re-attachment. The cells remained 100% viable at all processing steps, with no significant differences among tested groups and controls. In particular, a viability test was done on detached and re-attached MS-5 and HCEC cell sheet with similar results, indicating that the manipulation with cell sheets such as harvesting from pNIPAm-coated substrate and further re-attachment to TCPS dishes did not significantly damage the cells (Figure 4.2).
Figure 4.2. Live/Dead fluorescent staining of 2-week MS-5 and HCEC sheets before detachment, 1h after detachment and 48 h after re-attachment.

As a quality control for the generated cell sheets, the metabolic activity was assessed after cell sheet detachment and re-attachment. (Figure 4.3) The results of alamarBlue assay showed that 2-week MS-5 and 2-week
HCEC cell sheets have lower metabolic activity than initially seeded control cells, but the difference was not significant.

Figure 4.3. Results of alamarBlue assay for 2-week MS-5 and HCEC sheets before and after detachment and 48 h after re-attachment to TCPS. Data is mean ± standard deviation (SD), no significant difference compared with control.

The data indicate that the high cell-density cells remained viable and highly metabolically active. Our results corroborated the findings of the study performed by Coller’s research team [30] They showed that contact inhibited primary fibroblasts remain highly metabolically active. Another study published by the Blagosklonny group showed that contact inhibited cells did not undergo senescence and retained proliferative potential after splitting.[31] These studies together with our results may imply that the hyper-confluent cell sheets could retain viability and high metabolic activity after detachment and re-attachment indicating important implications for cell sheet-based technology.
4.2.3. Cytoskeleton Reorganization Analysis during Cell Sheet Detachment

At a high density after cell sheet detachment, the MS-5 and HCEC cell sheets shrunk significantly and changed in shape and size. The cell sheet detachment resulted in cell sheet contraction. DAPI nuclear staining showed that immediately after lifting, shrunk MS-5 and HCEC cell sheets exhibited significant increase in nuclear density. Cell sheet properties rely on the structure of actin cytoskeleton. [32] Actin cytoskeleton is an essential contributor to cell sheet formation, its mechanical strength and cohesion. The comparison of actin distribution before and after detachment indicated a structural difference between attached and detached cell sheets. The attached MS-5 spun abundant thin and parallel actin bundles. After detachment, actin formed cortical actin fibers along the cell membrane. The detached HCEC sheets also displayed cortical fluorescent ring of filamentous actin. (Figure 4.4) Paxillin is a key component of focal adhesion network, found in regions closely adherent to the substratum. The analysis revealed that paxillin as well as actin filaments in lifted cell sheets were organized into cortical networks. (Figure 4.5)
Figure 4.4. Immunofluorescent staining of 2-week MS-5 and 2-week HCEC before and after detachment. Phalloidin staining for F-actin (red), DAPI nuclear staining (blue). Scale bar: 20 μm.
Figure 4.5. Immunofluorescent staining of MS-5 and HCEC before and after detachment. Anti-paxillin antibody staining (green) and actin-phalloidin staining (red). Scale bar: 20 μm. White arrows indicate focal adhesion sites.
4.2.4. ECM Deposition Assessment of MS-5 and HCEC Sheets

SEM images of decellularized MS-5 sheets confirmed ECM protein depositions. (Figure 4.6, A) H&E staining also showed that MS-5 formed more cell layers and extracellular matrix than HCEC. When cross-sections of cell sheets were examined, 2-week MS-5 cell sheets were significantly thicker than 2-week HCEC cell sheets. The 2-week MS-5 cell sheets composed of 4-5 cell layers with an approximately 40-50 µm thickness, and extracellular matrix was abundant. In contrast, the 2-week HCEC-sheets were composed of approximately 2-3 layers with 10-20 µm in thickness, and the extracellular matrix was undeveloped or absent. (Figure 4.6, B) SDS-PAGE and complementary densitometric analysis revealed deposition of type I collagen in MS-5 sheets. (Figure 4.6, C)
Figure 4.6. ECM deposition analysis of MS-5 and HCEC cell sheets. (A) Scanning electron microscopy images, showing 2-week MS-5 sheet secreted richer ECM than 2-week HCEC sheet. (B) H&E staining of MS-5 and HCEC cell sheets. MS-5 formed more cell layers and ECM than HCECs. The 2-week MS-5-sheet consisted of four-five cell layers with approximately 40-50 µm thickness, the 2-week HCEC-sheets were composed of approximately 2-3 cell layers with 10-20 µm in thickness. Scale bar: 50 µm. (C) SDS-PAGE analysis of type I collagen in 2-week MS-5 and 2-week HCEC cell sheets. (D) Densitometric analysis of type I collagen in 2-week MS-5 cell sheets.

The difference in the ECM content and deposition between stromal and epithelial cell lines might result in the differences in cell sheet integrity,
strength and contraction during and after detachment. The smart pNIPAm-coated thermoresponsive substrates offer significant advantage for cell sheet technology allowing for the preservation of cell-ECM contacts after detachment, and therefore, proper functioning of cell sheets. [33] Previously, Canavan et al. have shown that ECM remained preserved and attached to the cell sheets after rapid recovery from pNIPAm-coated surfaces. [34, 35] The strong interactions between detaching cells and ECM might additionally facilitate rapid cell sheet recovery. Histological analysis confirmed that MS-5 stromal sheets composed higher number of cell layers with a dense network of ECM fibers whereas HCEC epithelial sheets revealed poor and undeveloped ECM formation. These data corroborated SEM and SDS-PAGE analyses which confirmed the deposition of ECM in MS-5 sheets. As fibroblastic stromal MS-5 cells produce higher amounts of ECM than HCEC, MS-5 cell sheets were stronger and detached significantly faster. In contrast, HCEC sheets were more fragile and partly broken after 1 week in culture that might also be due to a low content of extracellular matrix components. This might also result in a higher speed of MS-5 cell sheet recovery in comparison with HCEC cell sheets. Moreover, positive paxillin staining was observed in detached cell sheets that can indicate the possible preservation of cell-ECM interaction in recovered cell sheets. The preserved ECM and focal adhesions within cell sheets could greatly facilitate cell sheet re-attachment. Summary of morphological characteristics and histological evaluation of MS-5 and HCEC sheets as well as the results of cell activity assays cultured for 7 days and 2 weeks are presented in Appendix I.
4.3. Conclusions

Thus, the pNIPAm-coated cell culture dishes have advantage of producing viable and metabolically active cell sheets.

The cell sheets detached from pNIPAm-coated substrate are able to preserve structural integrity. Both stromal and epithelial cells harvested from thin spin-coated pNIPAm films as a single contiguous cell sheets are shown to exhibit a well-organized cell-dense structure with intact cell-cell junctions and deposited ECM.

The study highlights that the preserved focal adhesions in both stromal and epithelial cell sheets may promote successful re-attachment of lifted cell sheets. The cell sheet harvesting using thin spin-coated pNIPAm films represents a convenient and cost-effective technique for 3D tissue engineering.
4.4. References


5. TISSUE ENGINEERING: DELIVERY OF LAYERED CELL SHEETS USING THREE-DIMENSIONAL SCAFFOLDS

5.1. Introduction

Tissue engineering is aiming to develop biological substitutes to restore, replace or regenerate defective tissues. Cells, ECM and 3D scaffolds are the key components of engineered tissues. The scaffolds provide the structural support for cell attachment, subsequent ECM secretion and tissue development. Currently, there are an enormous variety of methods and biomaterials used for scaffold fabrication. [1-3]

The best 3D structured construct for an engineered tissue should mimic the target tissue in its native state. The biomaterials should be degradable and give a porous structure for efficient cell attachment, grow and penetration as well as efficient nutrient and metabolite transport. A tissue design should consider multiple parameters including the evaluation of scaffold material used as delivery vehicles, cell survival and behavior after attachment, cell-to-cell and cell-to-material interactions. In order to optimize the results of cell-to-material interactions, a standardized technique for the fabrication of 3D structures is needed. Importantly, this technique has to include the control over the key properties of 3D scaffolds such as geometry, surface topography, spatial resolution, mechanical properties, reproducibility and flexibility. All of these parameters are important to develop 3D structures that mimic the environment of in vivo tissues. Although there is no a single method for producing 3D structures that is suitable for every cell and tissue types and biomaterials, it is possible to establish a technological approach, which is accurate enough, reproducible and well-controlled for a wide range of biomedical applications. [4-6]
The scaffolds for tissue engineering can be created using polymers or native biomaterials. Traditional scaffold fabrication techniques often failed to produce 3D structures with high resolution and involved multiple steps. In the field of scaffolding, laser-based fabrication methods can provide a powerful tool to produce structures with complex architecture with appropriate pore sizes and distribution. [7, 8] Laser-based rapid prototyping technologies such as two-photon polymerization technique [9-12] and surface-selective laser sintering method [13-16] are very favorable methods for fabricating precisely defined tissue engineering scaffolds from biodegradable materials. Scaffold systems derived from decellularization of tissues have been popular due to their assured biocompatibility and bioactivity. [17, 18] The decellularized pericardial ECM has shown a particular promise as a delivery system for the cell therapy of cardiovascular diseases, skeletal muscle tissue regeneration, articular cartilage or meniscal tissue repair. [19-22] The ECM is the architectural foundation of organ development and homeostasis that serves as a scaffold to support proliferating cells and also provides a wide variety of biochemical and physical signaling during tissue morphogenesis or regeneration. [23, 24]

As with any functional tissue, successful tissue regeneration involves the proper delivery of cells with environmental cues favoring integration and survival of the implanted tissue-like graft. The big challenge of scaffold-based tissue engineering is related to a complete cell seeding of scaffolds avoiding uneven or insufficient cell attachment and migration into scaffolds. One of the first tissue-engineering techniques included the static seeding of concentrated cell suspension on scaffolds, which have been examined for bone [25], cartilage [26] and blood vessels [27] regeneration. This traditional method involves only one seeding step, and it has a risk of cell loss and low cell attachment. To prevent uneven
distribution and increase the number of attached cells, the high-density cell sheets may be applied to the scaffold surfaces. Recently, an approach of cell sheet engineering, in which cultured cells are harvested as a contiguous sheet, was developed and applied in tissue engineering. Several studies have demonstrated the regenerative potential of bioscaffold-assisted mesenchymal stromal cell sheets for heart regeneration. [28, 29] This integration between cell sheet engineering and tissue graft fabrication technology can promote the development of novel medical devices that provide structural support and improve tissue regeneration. Such constructs may increase treatment efficacy in patients with heart failure or heart valve disease.

Currently, the most effective way of cell sheet generation involves the use of pNIPAm-based thermoresponsive substrates. [30-33] The cultured cells grown on pNIPAm-coated dishes can be detached, in the form of an intact cell sheet. [34-36] Importantly, the thermoresponsive polymer-based technology enables fabrication of contiguous and viable cell sheets composed of cells and functional ECM. In cell sheets, the cells are embedded in ECM and delivered together with secreted growth factors that might increase survival rate and the ability of cells to engraft into damaged tissue after transplantation. [37]

The main goal of the present study was to assess the possibility of functional and structural integration of cell sheets harvested from thermoresponsive pNIPAm films with 3D 2PP scaffolds, 3D SSLS scaffolds and acellular bovine pericardial scaffolds. The cell sheets were harvested from thermoreponsive pNIPAm coatings. [35] The mouse bone marrow derived stromal cells or human mesenchymal stromal/stem cells were cultured on the surface of the pNIPAm-coated surfaces to form cell sheets. First, the material and topographic characteristics of 3D scaffolds were investigated. Next, the cytotoxic effect of scaffolds was
assessed by investigating the morphology of cells, their viability and metabolic activity as well as the efficiency of decellularization process. In addition, the attachment of cell sheets and the distribution of cells from cell sheets into 3D structures were examined. These results can be valuable and used in tissue engineering purposes in providing a validation for the development of construction through the integration of cell sheets with 3D natural or 3D polymer structures. Although this study focused on the use of stromal cell sheets, this tissue design can be applied to many other types of cells with minimal design and material changes.

5.2. Results and Discussion

5.2.1. Cell Sheet-seeding and Biological Analysis of Scaffolds Fabricated by Two-photon Polymerization Technique

5.2.1.1. Structural Analysis of 2PP scaffolds

Two-photon polymerization technique was used to create 3D porous scaffolds with hexagonal shape. (Figure 5.1)
Figure 5.1. 3D hexagonal scaffolds fabricated by 2PP polymerization technique.

FTIR spectra were collected to analyze the material characteristics of scaffolds. Star-shaped poly (D, L-lactide) was used for 2PP scaffold fabrication. (Figure 5.2)
In the FTIR spectrum of poly (D,L-lactide) bands were present at 2,994.036 and 2,943.099 cm\(^{-1}\) from asymmetric and symmetric valence vibrations of C-H, respectively. Asymmetrical valence vibrations of C-O-C of the aliphatic chain were present at 1,180.941 cm\(^{-1}\), and symmetrical valence vibrations of C-O-C of the aliphatic chain 1,081.709 cm\(^{-1}\). Accompanying bands at 1,747.522 cm\(^{-1}\) (valence vibration of C═O of aliphatic ester), 1,450.913 and 1,379.017 cm\(^{-1}\) (asymmetric and symmetric bending vibration of C-H from CH\(_3\), respectively), 1,265.621 cm\(^{-1}\) (the overlap C-H bending vibration and C-O-C stretching vibration) were also detected. A peak at 1632 cm\(^{-1}\) indicates a carbonyl group (-C=O) stretch.

The 2PP-fabricated 3D scaffolds were composed of two layers of hollow cylinder arrays in a hexagonal arrangement within each layer. SEM analyses revealed that the second layer of cylinders was fabricated with X and Y offsets to the previous layer of cylinders. (Figure 5.3, A)
Figure 5.3. SEM surface images of 3D scaffolds generated by 2PP technique. (A) Spatial arrangement of hollow cylinders within 2PP scaffolds. (B) The typical surface morphologies of the 2PP scaffolds. (C) 3D structural analysis of 2PP scaffolds.

Each single cylinder of this scaffold has a wall thickness of 70 μm and a diameter of 145 μm. The total height of the scaffold was 165 μm.
Neighboring cylinders bore against each other ensuring the mechanical stability of the structure. Between the layers, adjacent in the vertical direction, a lateral shift by the value of the half of a single cylinder radius was introduced. (Figure 5.3, B and C)

5.2.1.2. Cytotoxicity Evaluation of Microstructured 2PP Scaffolds

The cytotoxic character of 2PP scaffolds was analyzed. After an extended culture period of 7 days, MS-5 stromal cells adhered to the PDLA 2PP scaffold, covering the scaffold completely. (Figure 5.4, A) Scanning electron microscopy confirmed that stromal cells showed an extensive proliferation of cells over the scaffolds, with complete coverage. Moreover, the cells actively migrated inside the scaffolds. (Figure 5.4, B) Minimal degradation or loss of structural design of scaffolds was observed after culture for 7 days in vitro. As 2PP scaffolds had a strong fluorescence in all channels, fluorescent live/dead staining results were not representative for this type of scaffolds. AlamarBlue assay showed high metabolic activity of cells that was confirmed by the absence of statistically significant differences between the treatment group and control cells. (Figure 5.4, C) This may indicate that the scaffolds produced no toxic effects on grown cells.
Figure 5.4. In vitro cell growth of MS-5 stromal cells on 2PP scaffolds. (A and B) SEM images (top and side) of stromal cells cultured on 2PP scaffolds over 7 d. The yellow arrows indicate the cells covered the scaffold and the white arrows indicate cells migrated inside the scaffold. (C) AlamarBlue assay results indicate high metabolic activity of attached MS-5 cells after 7 d in culture.

5.2.1.3. Structural and Functional Analysis of Integrated System for 3D Assembly of Cell Sheets and 2PP scaffolds

Next, the possibility of cell sheet integration with 3D 2PP scaffolds was analyzed. The MS-5 stromal sheets lifted from 50 nm-thick spin-coated pNIPAm films were transferred on the 2PP – fabricated scaffolds. As 2PP-fabricated scaffolds have been shown not to impair cell behavior, their use as a vehicle for the delivery of layered cell sheets may be considered as a promising tissue-engineering approach. After a 7 day in culture, the seeded cell sheets were stuck to the scaffolds. SEM analyses indicated that the cell sheets were tightly adhered to the surface of the scaffolds. Moreover, the cells were found deep within the scaffolds. This implies that cells proliferated and migrated filling the pores of the scaffolds. (Figure 5.5, A)
Figure 5.5. In vitro cell growth of MS-5 sheet on 2PP scaffolds. (A) SEM of a MS-5 stromal cell sheet attached to 2PP-fabricated scaffolds after 7 d in culture. The yellow arrow indicates an attached MS-5 sheet. The white arrow indicates the cells migrated inside the scaffold. (B) AlamarBlue assay results: comparison between MS-5
single cell suspension and cell sheets seeded and grown on 2PP scaffolds for 7 d.

The comparison of metabolic activity between MS-5 single-cell suspension and MS-5 sheets grown on 2PP scaffolds for 7 d did not reveal any statistically significant differences. (Figure 5.5, B)

This work reported for the first time the biofabrication of 3D functional constructs via integration of cell sheets with 2PP scaffolds. Although these results focus on the generation of stromal cell sheets, such design of 3D cell sheet–seeded scaffolds can be applied to many types of cells with minimal changes. Compared to other technologies, 2PP not only allows great variability in size, shape and structural parameters, but also to produce 3D structures directly adaptable to any biological applications that require high-resolution patterning and precise microarchitecture such as tissue engineering. Considering scaffold-based cell sheet delivery approach, scaffolds can provide initial structural support for cell sheet seeding and attachment. The mechanical support of cell sheet adhesion and growth is critical for cell communications and proper tissue functions. The results demonstrated the great potential of 2PP technology for the fabrication of biocompatible scaffolds with controlled topology and high-resolution structural properties, enabling the design of 3D scaffolds for cell sheet delivery. Most natural tissues contain more than one cell type. For the future studies, arrangement of different cells within the cell sheets may further improve the functionality of scaffold-based cell-sheet constructs. In order to perform systematic studies on 3D cell sheet-seeded constructions, the scaffolds have to be created in a reproducible manner and follow a defined design. In this context, 2PP scaffolds should gain an increasing attention.
5.2.2. Cell Sheet-seeding and Biological Analysis of Scaffolds Fabricated by Surface-selective Laser Sintering Method

5.2.2.1. Structural Analysis of SSLS Scaffolds

Figure 5.6 presents a photograph of a poly (D,L-lactide) matrix fabricated via sintering of water-wetted powder.

Figure 5.6. 3D polylactide scaffold fabricated by surface-selective laser sintering method.

The SSLS method is used to produce the scaffolds for tissue engineering with given shape and porosity. In addition, microarchitecture of scaffolds can be varied within wide limits both by controlling the energy parameters of the sintering process and by modelling the trajectory of the laser radiation beam. The physicochemical properties of SSLS scaffolds were characterized by infrared spectroscopy and X-ray powder diffraction.
Figure 5.7. FTIR spectrum of poly (D, L-lactide) used for SSLS fabrication of 3D scaffolds.

In the FTIR spectrum of poly (D,L-lactide) bands were present at 2,995.082 and 2,945.082 cm\(^{-1}\) from asymmetric and symmetric valence vibrations of C-H, respectively. Asymmetrical valence vibrations of C-O-C of the aliphatic chain were present at 1,187.045 cm\(^{-1}\), and symmetrical valence vibrations of C-O-C of the aliphatic chain 1,090.081 cm\(^{-1}\). Accompanying bands at 1,757.032 cm\(^{-1}\) (valence vibration of C=O of aliphatic ester), 1,455.615 and 1,383.217 cm\(^{-1}\) (asymmetric and symmetric bending vibration of C-H from CH\(_3\), respectively), 1,271.511 cm\(^{-1}\) (the overlap C-H bending vibration and C-O-C stretching vibration) were also detected. (Figure 5.7)
Figure 5.8. XRPD patterns of amorphous SSLS scaffolds.

XRPD pattern of SSLS scaffolds did not show any well-defined peaks. These strong broad peaks suggest the amorphous characteristic nature of the scaffolds. (Figure 5.8) SEM analysis has confirmed XRPD results.

SEM analysis revealed that the surface morphology of the laser-sintered specimens was rough and contained a lot of pores. During the process of scaffold fabrication, heating and melting processes occurred in wetted powder, and the numerous pores appeared in the sintered structure due to both water evaporation and water "embedded" inside of the sintered volume. (Figure 5.9, A)
Figure 5.9. SEM surface images of SSLS scaffolds. (A) The typical surface morphology of laser-sintered specimens. (B) The powder particles exhibit irregular morphology. The scaffold with pore sizes of about 700 μm in width and 560 μm in depth.

The powder particles exhibit irregular morphology: certain portions of them looked relatively spherical and varied in size around 100 μm. The area occupied by a pore, as calculated using ImageJ software, ranged
from 0.42 to 0.52 mm$^2$, and the depth of a pore was around 560 μm. (Figure 5.9, B)

5.2.2.2. Cytotoxicity Evaluation of Microstructured SSLS scaffolds

On day 7 of culturing, although some of the cells were scattered over the surface of the scaffolds, most of the cells predominantly formed dense cell clusters inside the pores of the scaffolds. (Figure 5.10, A) The cells had well-defined processes that were attached to the surface of granules. (Figure 5.10, B)
Figure 5.10. SEM analysis of MS-5 cell growth on SSLS scaffolds. (A) Most of the cells predominantly form dense cell clusters inside the pores of the scaffolds after 7 d in culture. The white arrows indicate cell clusters inside the pores. (B) The cells form well-defined processes that attach to the surface of granules. The yellow arrows indicate cell processes.

For the assessment of scaffold cytotoxicity, the live/dead assay and alamarBlue assay were performed 7 d after the cell seeding. The results indicated the noncytotoxic character of the SSLS scaffolds. In particular, the live/dead staining confirmed high viability of cells grown on SSLS scaffolds. (Figure 5.11, A) These results corroborated with the results of alamarBlue assay. The cells grown on SSLS scaffolds showed high metabolic activity and no difference compared to TCPS control dishes. (Figure 5.11, B)
Figure 5.11. Cytotoxicity analysis of SSLS scaffolds. (A) Live/Dead assay of MS-5 cells grown on SSLS scaffolds for 7 d. (B) AlamarBlue assay results indicate high metabolic activity of attached cells after 7 d in culture.
5.2.2.3. Structural and Functional Analysis of Integrated System for 3D Assembly of Cell Sheets and SSLS scaffolds

SEM analysis revealed that cells were tightly adhered to the scaffold. This may be attributed to the fact that the endogenous ECM transferred with cell sheets may act as an adhesive agent for cell sheet attachment. After 7 days, a relatively uniform cell distribution over the scaffold was observed. (Figure 5.12, A)
Figure 5.12. In vitro cell growth of MS-5 sheets on SSLS scaffolds. (A) SEM image of a MS-5 sheet grown on the SSLS scaffold for 7 d in culture. The yellow arrow indicates an attached MS-5 sheet. The white arrow indicates a scaffold. (B) AlamarBlue assay results: comparison between single cell suspension and cell sheets seeded and grown on SSLS scaffolds for 7 d.

The comparison of metabolic activity between MS-5 single-cell suspension and cell sheets grown on SSLS scaffolds for 7 d did not reveal any statistically significant differences. (Figure 5.12, B)

In addition to two-photon fabrication technique, the surface-selective laser sintering is another method of tissue engineering which can be used to fabricate 3D structures with given shape, porosity and precise microarchitecture. Traditional scaffold fabrication techniques are limited in practical use, typically, because of lack of reproducibility. The ability of SSLS method to accurately fabricate scaffolds using computational designs has allowed the production of more precise structures. In the present study, SSLS scaffolds encouraged the adhesion of single-cell suspension as well as cell sheets. Moreover, the specific design of the SSLS scaffolds facilitated cell migration inside the
scaffolds due to pores within these structures. It was found that MS-5 sheets seeded on the scaffolds remained viable, implying that cell damage after cell sheet detachment, manipulation and attachment to the scaffold were minimal. To promote tissue regeneration, sufficient numbers of cells are needed to be delivered and maintained, and the cell sheet-seeded scaffolds can serve as an effective engineered constructs to deliver appropriate number of cells together with secreted ECM. ECM also provided a physical support to cells within lifted cell sheets. Importantly, cell sheets harvested from pNIPAm films preserved the endogenous ECM with integrated adhesive agents and secreted growth factors which regulate many cellular functions such as adhesion, migration and proliferation, and must be maintained for optimal cellular functions. Moreover, growth factors and enzymes which are incorporated into preserved ECM can additionally stimulate and enhance tissue regeneration. Therefore, the SSLS technology opens up new possibilities in bioengineering including the fabrication of bioactive structures with controlled biological properties and physicochemical characteristics. The integration of smart technologies such as cell sheet engineering and 3D rapid prototyping techniques such as two-photon polymerization technique or surface-selective sintering method offers the opportunity to produce anatomically fitting structures with the potential to improve implant integrity, reduce healing times and avoid implant rejection.

5.2.3. Cell Sheet-seeding and Biological Analysis of Acellular Pericardial Scaffolds

As it was already shown in the present study, the cell sheet-seeded synthetic scaffolds represent an attractive approach for cell delivery to damaged tissues. However, synthetic materials failed to recapitulate complex native tissue microenvironment. This drawback can be overcome by using decellularized native tissues, which act as ideal
supports for cell attachment and differentiation due to the presence of 3D porous topographies, as well as the macrostructure of native tissue. Bovine pericardial tissue has been adopted to simulate the architecture of human heart valves. Moreover, this tissue can adjust other conditions of heart tissue, such as nutrient transport, the native stress and stretch, blood flow during loading resembling the native functions of the heart.

5.2.3.1. Microscopy Analysis of Acellular Pericardial Scaffolds

Decellularized tissue scaffolds are widely used for many tissues such as tendons, whole hearts, and aortic heart valves. The primary goal of decellularization is to remove cellular components from the tissue while preserving the ECM. The decellularization is desired as the presence of antigens on the endogenous cells may activate the host’s immune response after scaffold transplantation. The protocol used in the present study was developed from the widely adopted methods. Following this protocol, the decellularized pericardial tissue was prepared by chemical washing (in SDS), which removed endogenous cells as well as cell debris by breaking the molecular interactions, and remained ECM intact. For a bulky tissue, such as pericardial tissue, the diffusion of decellularization solution through the tissue is a great challenge. Mechanical agitation, therefore, was additionally applied throughout the process to enhance the effect of chemical exposure. The continual irrigation with SDS and then with HBSS were performed to remove cell debris. A longer washing duration may effectively enhance the decellularization that depends on the size and thickness of bulky tissue samples.

The normal fibrous pericardium consists of a 1mm-thick layer of dense collagen with sparse interspersed elastic fibers. [38, 39] SEM analysis of bovine pericardial samples revealed connective tissue fibers in native as well as decellularized pericardial tissue on the fibrosa surface.
Figure 5.13. SEM of (A) native and (B) decellularized pericardial tissues.

In control samples, collagen and elastic fibers were arranged orderly. (Figure 5.13, A) Images of decellularized samples revealed a more porous microstructure and delamination of the multiple layers of
collagen bundles and elastic fibers in pericardial tissue. The arrangement of connective tissue fibers was somewhat disorganized as compared with control samples. (Figure 5.13, B)

Figure 5.14. Hematoxylin and eosin (H&E) staining of (A) native and (B) decellularized pericardial tissue samples. The white arrows indicate nuclei.
On H&E staining, control samples showed many cells scattered among connective tissue fibers, which were compact with an ordered arrangement. (Figure 5.14, A) Decellularized pericardial samples showed no cells, and the mesh of fibers was looser than in control samples. Moreover, lamellar arrangements of fibers were disturbed, with gaps between them. (Figure 5.14, B) Results of H&E stain were similar with DAPI staining. Although the presence of residual cell remnants, including DNA fragments and antigens may induce negative effects, it is believed that the existence of cell remnants should not cause severe immunoreactions to transplanted grafts derived from decellularization. Many blue fluorescent dots representing DNA were evenly distributed in control pericardial samples (Figure 5.15, A), with none in decellularized samples. (Figure 5.15, B)
5.2.3.2. Cytotoxicity Evaluation of Acellular Pericardial Scaffolds

Examination of the contact cytotoxicity showed that hMSC, which were grown adjacent to the fresh untreated bovine pericardial tissue, displayed negligible red fluorescence and strong green fluorescence indicating good viability (Figure 5.16, A). When the cells were treated with cell death inducing agent DMSO, the green fluorescent intensity was degraded to a low level, and the cells exhibited strong red fluorescence indicating worse viability of the cells (Figure 5.16, B). The decellularized pericardial samples displayed strong green fluorescence which indicated good viability of the cells. (Figure 5.16, C)

Figure 5.15. DAPI staining of (A) native and (B) decellularized pericardial samples. The white arrow indicates DNA.
Figure 5.16. Cytotoxicity analysis of pericardial samples. Live/dead assay of hMSCs on (A) fresh tissue (alive cells), (B) fresh tissue after treatment with DMSO (dead cells) and (C) decellularized tissue. (D) AlamarBlue assay results: the comparison of metabolic activity of hMSCs grown on native (fresh) and decellularized pericardial tissues after 7 d in culture.

Moreover, the results of alamarBlue assay confirmed high metabolic activity of cells grown on decellularized tissue with no significant difference compared to untreated controls (fresh tissue samples). (Figure 5.16, D)

5.2.3.3. Structural and Functional Analysis of Integrated System for 3D Assembly of Cell Sheets and Acellular Pericardial Scaffolds

To fully assess the benefits of cell sheet integration within 3D scaffolds, the cell sheets were applied not only to synthetic scaffolds but also to natural porous acellular tissue scaffolds. The natural scaffolds used in the present study were made from decellularized bovine pericardia. The cell sheets were engineered from human bone-marrow derived mesenchymal stem cells. As shown and discussed above, the decellularized bovine pericardial samples were composed of native ECM. These native ECM-
rich acellular scaffolds can serve as scaffolds for cell attachment and growth. The cell sheets from human bone-marrow derived mesenchymal stem cells were transplanted to acellular pericardial scaffolds.
Figure 5.17. Microscopy analysis of a multilayered hMSC sheet onto acellular bovine pericardial scaffolds. (A) SEM image of hMSC sheet cultured on bovine acellular pericardial tissue for 7 d. (B) H&E staining of cross-section of acellular BP scaffold with attached hMSC sheet after 7 d in culture. (C) AlamarBlue assay results: the comparison of metabolic activity between single hMSC suspension and hMSC sheets grown on decellularized pericardial tissues for 7 d. The white arrows indicate an acellular pericardial scaffold, and the yellow arrows indicate an attached cell sheet.

After 7 days, the cell sheets were found to adhere physically to the surface (Figure 5.17, A), and H&E staining of cross-sections revealed no obvious gaps between the transplanted cell sheets and the scaffolds. (Figure 5.17, B) This implies that multilayered sheet together with preserved ECM may significantly enhance the adhesion of the cell sheet to the tissue. The comparison of viability between hMSC single-cell suspension and cell sheets grown on acellular pericardial scaffolds for 7 d did not reveal any statistically significant differences. (Figure 17, C)
The current study presents an example of integrated design for tissue engineering where multilayered cell sheets were seeded and cultured on 3D polymer or natural scaffolds to create tissue-like structures. Our findings demonstrated the effective decellularization of bovine pericardium, with preserved biological properties. The majority of cells were removed during decellularization procedure, and those that remained behind were metabolically inactive. This implies that the presence of endogenous cell remnants allowed for the growth and survival of newly introduced cells. The preservation of pericardial tissue microarchitecture indicated that the presented decellularization procedure can be used to treat pericardial tissue.

Cell penetration through the scaffold is a great challenge for tissue engineering. Many studies have reported that cultured cells may not penetrate, migrate and become well distributed in scaffolds from native tissues. In the present study, the tissue recellularization using cell sheet seeding technique showed that cells migrated within the decellularized scaffold with limited penetration. The reason of limited penetration may be due to the loss of growth factors during the decellularization process, which promote cell migration. From the SEM images, the distortion of collagen fiber meshwork in the decellularized scaffold was observed. This may be caused by the decellularization chemicals such as SDS that affected a meshwork of collagenous and elastic fibers and native tissue microstructure. These factors may thus be contributory factors that additionally may prevent deep penetration of seeded cells. In addition, the present study illustrated only the behavior of implanted cell sheets into the acellular pericardial matrix within a relatively short period and in vitro conditions (7days). Further in vivo studies are required to investigate the long-term effects after the implantation of hMSC sheets on decellularized pericardial tissue. Therefore, the findings suggest that
decellularized pericardial tissue support the functional behavior of hMSC sheets and may serve as a functional framework for tissue engineering, because the biological properties and the characteristics of the native architecture were preserved. It might further provide a microenvironment for the attachment and proliferation of stem cells as well as heart-specific cells, with their further implantation and tissue regeneration.

5.3. Conclusions

The present study has shown that 3D scaffolds, produced by 2PP technique, SSLS method and by decellularizing pericardial tissue, are able to provide a functional niche for cell sheet attachment and function.

Indeed, the findings here indicated that MS-5 and hMSC cell sheets attached and remained viable and metabolically active after seeding on 3D structures. Due to appropriate biological properties, 3D 2PP scaffolds, SSLS scaffolds and acellular pericardial scaffolds encouraged the adhesion of cell sheets. Moreover, considering the fact that cell sheets remained viable and metabolically active, the cell damage after cell sheet detachment and transfer to the scaffolds was minimal.

Next, the 2PP and SSLS scaffolds produced with high accuracy facilitated cell migration inside the scaffolds due to specific design and pores within these structures.

Studying different parameters of decellularization protocols and culturing conditions are suggested to further promote cell migration inside the acellular pericardial scaffolds and therefore, improve the outcome of tissue recellularization and the engineering of structurally complex tissues.

To promote tissue regeneration, cell sheets provide sufficient numbers of cells needed to be delivered and maintained. Therefore, the cell sheet-
seeded scaffolds can serve as an effective engineered constructs for cell colonization of 3D scaffolds.
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6. GENERAL CONCLUSIONS

The present study aimed to explore the possibility to design functional 3D assembly from tissue-like cell sheets and 3D scaffolds. The most effective way of cell sheet generation involves the use of pNIPAm-based thermoresponsive substrates. Compared to complex and expensive covalent polymerization bonding techniques, conventional spin-coating method has been successfully employed to produce pNIPAm coatings with high reproducibility and uniformity. Considering the lack of detailed information about cell adhesive and cell sheet detachment properties of spin-coated pNIPAm films, the cell behavior on pNIPAm films of different thicknesses were firstly examined.

The results suggest that it is possible to regulate the cell behavior on thermoresponsive coatings by changing the thickness of non-crosslinked spin-coated pNIPAm films. The thickness of pNIPAm films determines cellular attachment and spreading: 300 and 900 nm thick spin-coated pNIPAm films impede cell attachment, while thinner pNIPAm films (in this study, 50, 80 and 120 nm pNIPAm films) are more preferable for cell attachment, growth and proliferation. The contact angle measurements on pNIPAm films revealed the increase of surface wettability with increasing of film thickness. The changes in surface hydration with changes of film thickness might affect protein adsorption from multicomponent cell culture media containing proteins of different size, concentration and affinity towards the surface. Therefore, it is unlikely that cell attachment and growth is a single-valued function. Several factors including chemical and structural nature of material, the method of polymer surface fabrication, physicochemical characteristics...
of polymer films and the influence of wettability on conformational changes of adsorbed proteins should be considered.

Although thin spin-coated pNIPAm surfaces attach and release cells, there are significant differences in cell densities and the pattern of cell detachment. As pNIPAm films are considered to be used for cell sheet engineering, cell detachment as a contiguous cell sheet are more successful on 50 and 80 nm pNIPAm films. Therefore, the results of the present study may be important for the assessment and design of pNIPAm-based thermo-responsive surfaces used for cell sheet-based tissue engineering.

Cell sheet technology has been emerging as a promising and clinically applicable tool for tissue regeneration. In order to engineer more functionally improved cell sheet constructs, it is necessary to define the quality of lifted cell sheets. In the present study, a series of quantitative assessments of stromal and epithelial cell sheets harvested from spin-coated pNIPAm films revealed that lifted cell sheets remain viable and metabolically active and exhibit a well-organized cell-dense structure. In particular, the cell sheets detached from pNIPAm-coated substrates are able to preserve ECM, focal adhesions and structural. Importantly, lifted stromal and epithelial cell sheets can successfully re-attach to a new substrate.

It has been announced that cell sheet technology requires no scaffolds. However, in order to fabricate more complex and functional 3D tissues, 3D scaffold-based techniques can be combined with cell sheet engineering methodology. Considering scaffold-based cell sheet delivery approach, scaffolds can provide initial structural support for cell sheet seeding and attachment. The mechanical support of cell sheet adhesion and growth is critical for cell communications and proper tissue
functions. This interdisciplinary approach could produce interesting and promising results. In the present study, mesenchymal stromal cell sheets harvested from thermoresponsive pNIPAm films were successfully integrated with 3D synthetic (from biodegradable poly (D,L –lactide) biomaterials) or natural (from acellular pericardial tissue) scaffolds. Regarding 3D synthetic scaffolds, cell sheets are able to attach and the cells remain viable, metabolically active and can penetrate deep inside the scaffolds. In case of acellular pericardial scaffolds, the cell sheets can readily attach appearing alive and metabolically active but the cells migrated within the decellularized scaffold with limited penetration. Such difference in cell accommodation might be due to the structural difference between synthetic and natural scaffolds. 3D synthetic scaffolds were fabricated by two-photon polymerization technique or surface-selective laser sintering method based on computer-assisted design (CAD) in order to obtain desirable shape and precise internal structure of 2PP and SSLS scaffolds. The main benefit of a decellularized tissue is the presence of 3D native ECM and porous topography of native tissue. However, the distortion of a meshwork of collagen and elastic fibers in the decellularized scaffold might occur during the procedure that might prevent deep penetration of seeded cells. In other words, the acellular native scaffolds created from bovine pericardial tissue provides 3D environment with less controllable structural pattern and microarchitecture. Therefore, compared to decellularization procedure, 3D printed technologies possess the great potential to generate biocompatible scaffolds with controlled topology and high-resolution structural properties enabling more accurate design of 3D complex scaffolds for cell sheet delivery. Nevertheless, the maintenance of the native microstructure and composition of extracellular matrix during tissue decellularization is possible. Further research is suggested to optimize the promising decellularization
protocols and culturing conditions in order to improve the outcome of transplantation and future endeavors in the engineering of structurally complex tissues.
7. Appendix I

Table 7.1. Summary of characteristics of MS-5 and HCEC sheets cultured for 1 week and 2 weeks.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Detachment time (min)</th>
<th>Cell number (x10⁶)</th>
<th>Cell sheet thickness (µm)</th>
<th>Cell sheet diameter (mm)</th>
<th>Cell sheet area (cm²)</th>
<th>Cell sheet metabolic activity</th>
<th>Stratification</th>
<th>Paxillin</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS-5</td>
<td>1 week</td>
<td>10±3</td>
<td>0.8±0.1</td>
<td>17±3</td>
<td>12±3</td>
<td>1.1±0.6</td>
<td>88%</td>
<td>+</td>
</tr>
<tr>
<td>MS-5</td>
<td>2 weeks</td>
<td>10±3</td>
<td>4.1±0.4</td>
<td>45±5</td>
<td>22±3</td>
<td>3.8±1.1</td>
<td>84%</td>
<td>+</td>
</tr>
<tr>
<td>HCEC</td>
<td>1 week</td>
<td>Impossible</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HCEC</td>
<td>2 weeks</td>
<td>35±5</td>
<td>2.2±0.3</td>
<td>15±5</td>
<td>23±2</td>
<td>4.2±0.8</td>
<td>94%</td>
<td>+</td>
</tr>
</tbody>
</table>
8. Appendix II

List of Publications


Film Thickness Determines Cell Growth and Cell Sheet Detachment from Spin-Coated Poly(N-Isopropylacrylamide) Substrates

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ABSTRACT: Poly(N-isopropylacrylamide) (pNIPAm) is widely used to fabricate thermoresponsive surfaces for cell sheet detachment. Many complex and expensive techniques have been employed to produce pNIPAm substrates for cell culture. The spin-coating technique allows rapid fabrication of pNIPAm substrates with high reproducibility and uniformity. In this study, the dynamics of cell attachment, proliferation, and function on non-cross-linked spin-coated pNIPAm films of different thicknesses were investigated. The measurements of advancing contact angle revealed increasing contact angles with increasing film thickness. Results suggest that more hydrophilic 50 and 80 nm thin pNIPAm films are more preferable for cell sheet fabrication, whereas more hydrophobic 300 and 900 nm thick spin-coated pNIPAm films impede cell attachment. These changes in cell behavior were correlated with changes in thickness and hydration of pNIPAm films. The control of pNIPAm film thickness using the spin-coating technique offers an effective tool for cell sheet-based tissue engineering.

KEYWORDS: pNIPAm, spin-coating, film thickness, wettability, protein adsorption, cell sheet engineering

1. INTRODUCTION

A short-term goal of regenerative medicine is the development of functional tissue substitutes created from a patient’s own autologous cells that can be applied in clinical practice. Cell sheet transplantation provides a promising therapeutic approach for tissue regeneration because it does not cause inflammation and enables improved integration and attachment to a host tissue. Biomaterials play a significant role in the creation of synthetic cell culture substrates. Poly(N-isopropylacrylamide) (pNIPAm) is one of the most studied intelligent biomaterials widely used for cell sheet harvesting. The substrates when coated with pNIPAm or its copolymers enable the detachment of cells from culture dishes by temperature reduction below the lower critical solution temperature (LCST) and without using digestive reagents that destroy extracellular matrix assembly, intercellular connections, and focal adhesion complexes. The preserved ECM and developed extracellular matrix assembly, intercellular connections, and roughness, surface chemistry, pH, and temperature can be important for the adsorption of proteins and in the evaluation of material’s cell adhesion properties. Although the cell behaviors on covalently grafted pNIPAm brushes are well-understood, there are a limited number of studies that shown the dependency of cell behavior on the thickness of covalently grafted pNIPAm films. In particular, the pNIPAm films with thickness higher than 20 nm fabricated by electron beam irradiation impede cell attachment, and the optimal thickness was found to be between 15 and 20 nm. It was found that atom transfer radical polymerization (ATRP)-fabricated pNIPAm brushes with thickness between 20 and 45 nm are the most suitable for HepG2 cell adhesion and detachment. The key advantage of a spin-coating technique is the combination of reproducibility and versatility. The method allows very fast, gentle and nondeforming drying of polymer films with uniform surface which characteristics can be controlled very carefully. The technique provides a more inexpensive and convenient way to prepare thermoresponsive cell culture surface and can be deployed in many research laboratories.

The adhesion properties of pNIPAm films are closely related to protein adsorption. Considering the surface characteristics and conditions of surrounding environment, surface hydration, roughness, surface chemistry, pH, and temperature can be important for the adsorption of proteins and in the evaluation of material’s cell adhesion properties. Although the cell behaviors on covalently grafted pNIPAm brushes are well-understood, there are a limited number of studies that

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systematically investigated cellular adhesiveness of spin-coated pNIPAm films.

In the present study, we tested cell behavior on non-cross-linked pNIPAm films of different thicknesses. Cell response to pNIPAm films were analyzed in terms of mouse stromal (MS-S) cell attachment, growth, and detachment, metabolic activity, viability and proliferation rate, morphology, and cytoskeleton organization. The stromal cells including MS-S cells have been commonly used for cytotoxicity evaluation of biomaterials. Moreover, they provide a supportive environment for stem cells, including human hematopoietic stem/progenitor cells (HSPCs). The thickness of pNIPAm films was measured optically by lowering the temperature. No relevant detachment was observed on Thermanox control or thick pNIPAm films. The actin cytoskeleton is connected to the extracellular matrix via macromolecular complexes, such as focal adhesions. Paxillin, the major focal adhesion adaptor protein, binds several proteins that contribute to the organization of actin cytoskeleton and thus plays a crucial role in signaling network that can regulate cell adhesion and spreading. We observed that the attachment, growth, and detachment as well as metabolic activity of MS-S cells depend on the thickness of pNIPAm films. The thick pNIPAm films showed a lower cell attachment when compared to a Thermanox control and to thin pNIPAm films. The possibility to detach the cells in the form of a contiguous sheet was shown using thin pNIPAm films by lowering the temperature. No relevant detachment was observed on Thermanox control or thick pNIPAm films.

These spin-coated thin pNIPAm films can find application as intelligent surfaces for use in research, medical devices, and regenerative medicine. The results of present study may be important for chemists improving polymer properties, engineers optimizing the polymer production processes and biomedical scientists specializing in cell sheet-based tissue engineering.

2. EXPERIMENTAL SECTION

2.1. Materials.

The polymer pNIPAm (poly-N-isopropylacrylamide) with M<sub>n</sub> = 20 000–40 000 and anhydrous ethanol (200 proof, >99.5% assay) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Thermanox plastic 25 mm discs from NUNC (Naperville, IL, USA), the high quality fused silica glass discs 20 mm in diameter from UQG Optics Ltd. (Cambridge, UK), borosilicate cover glass slides 25 mm in diameter from VWR (Radnor, PA, USA). Plastic consumables were purchased from Sarstedt (Nürnberg, Germany). Dulbecco’s phosphate buffered saline (DPBS), Hanks balanced salt solution (HBSS) and Dulbecco’s modified Eagles medium (DMEM) were purchased from Lonza (Switzerland), and antibiotics (penicillin-streptomycin) and fetal bovine serum (FBS) were purchased from HyClone (Logan, UT, USA). The alamarBlue reagent, live/Dead viability/cytotoxicity kit, and Quanti-IT PicoGreen dsDNA assay kit were purchased from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA). Rabbit monoclonal antipaxillin antibody (ab32084) and Alexa Fluor 488 goat anti-rabbit secondary antibody (ab150077) were purchased from Abcam (Cambridge, UK). Phalloidin eFluor660 (50–6559–05) was purchased from Affymetrix (CA, USA). UltraCruz mounting medium was purchased from Santa Cruz Biotechnology (CA, USA). Heating dry bath from Torrey Pines Scientific (Carlsbad, CA, USA) was used for careful temperature control.

2.2. pNIPAm Film Preparation and Deposition.

Spin-coated pNIPAm films were fabricated by initially depositing a 150 μL aliquot of a 5, 10, 20,40 and 80 mg/mL ethanol pNIPAm solution onto a slowly spinning (150 rpm), substrate (Thermanox plastic discs or glass discs) for 9 s followed by rapid acceleration to 4000 rpm for 30s, on a Laurell Technologies WS-400B-256. The coated samples were slowly dried overnight in an ethanol saturated atmosphere and then left in a vacuum oven at 40 °C and 600 mbar for a minimum of 4 h to eliminate any residual solvent. Thermanox coverslips were used as the underlayer substrates for advancing contact angle measurements, cell morphology, qualitative cell assays (alamarBlue and PicoGreen) and cell detachment analysis. Clean and optically flat borosilicate cover glass slides were used for atomic force microscopy and fluorescence microscopy. The fused silica glass substrates were used for laser ablation and optical profilometry analysis.

2.3. Evaluation of pNIPAm Film Thickness.

An ArF excimer laser (ATL Atlex, Wermelskirchen, Germany) was used to ablate the selected areas on the thin pNIPAm film deposited on fused silica glass discs, 20 mm in diameter from UQG optics. The excimer laser operates at a wavelength of 193 nm with a pulse length of a few ns. Laser parameters included a pulse repetition frequency of 200 Hz at a fluence of 66 mJ/cm². A standard mask projection machining approach was used to shape the laser beam. An optical demagnification of 5× was employed to produce 9 periodically arranged ablated areas of approximately 400 μm × 400 μm.

Optical profilometry analysis was used to assess the thickness of pNIPAm films. All measurements were made in air. The thickness of pNIPAm films was measured using white light interferometry (Zygo New View 100) with an accuracy of 0.1 nm. The z-height distance between the remaining polymer and the underlying substrate was measured to accurate assess the thickness. Statistically relevant data was obtained by repeating all measurements three times, and scans of 5 randomly selected ablated windows were recorded on 3 different samples. The objective used for all measurements was a 20× Mirau, with zoom set at 0.5×.

2.4. Contact Angle Measurements.

Advancing contact angle measurements were performed on a home-built goniometer assembled on an optical rail from Newport Optics with opto-mechanical components from Newport Optics and Edmund Optics. DROPImage software marketed by Rame Hart and developed by P.K. Hansen was applied to determine contact angles.

pNIPAm-coated samples were placed in a temperature-controlled environmental chamber mounted on an adjustable tilt stage. Contact angles were taken at 40 °C, i.e. above the LCST of the pNIPAm polymer, and controlled using a therocouple attached to the stage surface. A drop of ultrapure water was deposited on the surface with an initial radius of about 3 mm. For the advancing contact angle experiments, a thin stainless steel needle (gauge 22) was inserted in the center of the drop from above. The volume of a drop was increased by pumping liquid into the drop using a syringe pump. The pumping speed was maintained at the rate of advancing to below 0.5 mm/min. The values of advancing contact angles were taken and averaged between approximately 450 and 500 s. The values of contact angles were measured in two different positions for each coverslip. Contact angle was also measured after thermal annealing at 120 °C for 2 h in a vacuum oven.

2.5. AFM Analysis of pNIPAm Film Surface Roughness.

AFM images were obtained in tapping mode in air using a Dimension 3, 100 AFM (Digital Instruments, Santa Barbara, CA, USA), and Veeco 1–10 Ohm-cm phosphorus (n) doped Si tips and a matrix of 512 × 512 data points along the x–y plane were analyzed in a single scan. Four 10 μm × 10 μm scans were recorded at a scan rate of 0.5 Hz on each pNIPAm film to ensure statistical accuracy. Atomic force microscopy (AFM) was used to assess the roughness of the deposited pNIPAm coatings using 10 μm × 10 μm scans. The roughness of the films was reported as root-mean-square (RMS) roughness values.

2.6. Cell Culture.

For cell culture experiments, pNIPAm films were sterilized under mild UV light for 2 h. The mouse (MS-S) stromal cell line were cultivated in DMEM, supplemented with 10% FBS, 1% penicillin streptomycin antibiotics and maintained in a humidified incubator at 37 °C and 5% CO₂.
2.7. Cell Activity Assays. For experimentation, MS-5 cells were seeded in triplicate at a density of 40,000 cells/cm² on pNIPAm-coated and Theranox bare tissue culture coverslips. The metabolic activity of MS-5 cells grown on pNIPAm films and Theranox controls was assessed using an alamarBlue assay 48 h after cell seeding. Total DNA content of MS-5 cells attached and grown on pNIPAm films and Theranox controls was quantified at 24 and 48 h by the QuantiT® PicoGreen dsDNA assay kit. Both assays were performed according to the manufacturer’s instructions. Cell numbers were obtained by calculation based on the cell DNA, using a calibration curve of total cell DNA versus known numbers of cells. AlamarBlue and PicoGreen fluorescence was measured using a Thermo Scientific Varioscan Flash Multimode plate reader.

A Live/Dead cell viability assay was used for cell viability analysis. Titration was performed for both calcine-AM and ethidium homodimer-1 to define optimal dye concentration according to the manufacture protocol. A solution of 1 μM Calcine-AM and 2 μM ethidium homodimer-1 in HBSS was mixed thoroughly, and then added to the cells for 20 min at 37 °C. The fluorescent staining was observed using an Olympus IX81 fluorescence microscope (Olympus) and images were captured using a DP72 CCD camera (Olympus). This experiment was repeated three times.

2.8. Immunofluorescent Staining. The MS-5 cells grown on pNIPAm films and control glass cover slides were fixed at 37 °C with 4% paraformaldehyde in DPBS for 10 min. Afterward they were washed three times in DPBS and then incubated with 3% goat normal serum for 60 min at 37 °C to block nonspecific binding. MS-5 cells were stained to detect actin cytoskeleton and the focal adhesion protein paxillin. Actin stress fibers were detected using Phalloidin eFluor660 at 1:500 dilution for 30 min at 37 °C. Immunostaining of paxillin was performed using rabbit monoclonal antipaxillin antibody at 1:1000 dilution of Alexa Fluor 488 nm goat antirabbit secondary antibody at 37 °C to block nonspecific binding. Double-stained images were superimposed with ImageJ software. At least five images were taken from each sample for analysis. The number of focal adhesions per cell was assessed using immunofluorescence images of paxillin and ImageJ software. The micrographs were magnified to the same size and a total of 10 cells were analyzed in each group. The absence of nonspecific binding was confirmed by the use of appropriate primary and secondary antibody controls.

2.9. Cell Detachment. MS-5 growth and detachment was microscopically observed using a Leica inverted-microscope (Leica, Solms, Germany) on either pNIPAm-coated or bare Theranox controls. The cells were rinsed with prewarmed HBSS to remove any traces of serum. Cold serum-free DMEM was added to cells and the samples were left on a digitally controlled thermal/cooling plate set to 4 °C. Micrographs of cells were taken every 10 min on a phase contrast microscope to monitor cell detachment.

2.10. Statistical Analysis. Statistical analysis was performed using Statistica 8.0 software. Values are expressed as mean ± standard error of the mean (SEM). Student t test were conducted to compare independent groups. Multiple comparisons were made using one-way ANOVA test. Statistical significance was defined as p-value <0.05.

3. RESULTS AND DISCUSSION

3.1. Physical Characterization. The thickness range measured from dry pNIPAm films is presented in Figure 1. The results are in agreement with our previously obtained thickness measurements.

Advancing contact angles of the coated films give the information regarding surface energy properties used for more detailed qualitative assessment of the wettability or hydrophobicity/hydrophilicity of film surface. The advancing contact angle for water on spin-coated pNIPAm films with different thicknesses are summarized in Table 1. It was shown earlier for thick pNIPAm films that although pNIPAm becomes hydrophobic over its LCST, the polymer matrix still contains a certain degree of water. The same results were obtained by Ryder and coauthors. Using fluorescence emission of 3-hydroxyflavone probes, they revealed that dry non-cross-linked pNIPAm films have significant water uptake capacity even above LCST when pNIPAm exists in a more hydrophilic and collapsed state. According to our results, 50 and 80 nm dry pNIPAm thin films were quite hydrophilic at 40 °C, while the contact angle of thicker pNIPAm films gradually increased at the same temperature (Figure 2). This implies that thinner pNIPAm films adsorbed water and become more hydrophilic with a decrease in thickness. Our data are corroborated by the results obtained in Muller-Buschbaum’s group. They recently showed that water can be accumulated by non-cross-linked spin-coated pNIPAm films with time and that pNIPAm films become more hydrophilic with decrease in thickness. This indicates that spin-coated non-cross-linked pNIPAm films showed a positive relationship between hydrophobicity and film thickness. Films with thickness below 300 nm show

Table 1. Average Advancing Water Contact Angle Measurements Taken at 40 °C on Spin-Coated pNIPAm Films before and after Thermal Annealing

<table>
<thead>
<tr>
<th>pNIPAm film thickness (nm)</th>
<th>water contact angle (deg)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>as-coated</td>
</tr>
<tr>
<td>50</td>
<td>49 ± 1</td>
</tr>
<tr>
<td>80</td>
<td>49 ± 1</td>
</tr>
<tr>
<td>120</td>
<td>52 ± 1</td>
</tr>
<tr>
<td>300</td>
<td>57 ± 2 “a”</td>
</tr>
<tr>
<td>900</td>
<td>60 ± 3 “a”</td>
</tr>
</tbody>
</table>

“Stick angle.

Figure 1. Polymer film thickness as a function of polymer concentration.

Figure 2. Dependence of contact angle on pNIPAm film thickness before and after thermal annealing at 120 °C under vacuum.

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advancing contact angle which stays constant across the sample (Figure 3A–D). However, a “sawtooth” pattern was observed for 300 and 900 nm-thick films in the advancing contact angle (Figure 3E, F). It is known as “stick–slip” behavior of a moving drop. Such behavior is the result of the advancing leading edge sticking to the ridge formed on the surface, which causes an increase in contact angle. While more liquid is pumped into the
drop, the drop front jumps to a new location, resulting in reduction in contact angle again. We name the angle just prior to the drop front slipping the “slip angle”. The angle after the drop has jumped to a new position is named the “stick angle”. Stick–slip behavior was previously examined and explained on

Table 2. Thickness and RMS Values of pNIPAm Films

<table>
<thead>
<tr>
<th>polymer concentration (%)</th>
<th>thickness of polymer films (nm)</th>
<th>RMS value (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>50</td>
<td>6.8 ± 2.2</td>
</tr>
<tr>
<td>1</td>
<td>80</td>
<td>4.4 ± 3.2</td>
</tr>
<tr>
<td>2</td>
<td>120</td>
<td>3.9 ± 2.6</td>
</tr>
<tr>
<td>4</td>
<td>300</td>
<td>7.3 ± 4.1</td>
</tr>
<tr>
<td>8</td>
<td>900</td>
<td>5.3 ± 3.2</td>
</tr>
</tbody>
</table>

Figure 3. Contact angles measured on (A) Thermanox control, (B) 50, (C) 80, (D) 120, (E) 300, and (F) 900 nm pNIPAm films at 40 °C.

Figure 4. Bright-field microscopy images of MS-5 cells cultured on (A) Thermanox control, (B) 50, (C) 80, (D) 120, (E) 300, and (F) 900 nm pNIPAm films. Initial cell-seeding density 40 000 cells/cm², incubation time 48 h, scale bar 100 μm.

Figure 5. Adhesion and proliferation of MS-5 grown on pNIPAm films of different thicknesses (50–900 nm). Initial cell-seeding density 40 000 cells/cm², incubation time 12, 24, and 48 h. Data are mean ± standard error of mean (SEM), * p < 0.05 compared with control.

Figure 6. Cell metabolic activity of MS-5 grown on pNIPAm films with different thickness after 48 h. Alamar Blue assay test results. Initial cell-seeding density 40 000 cells/cm², incubation time 48 h. Data are mean ± standard error of mean (SEM), * p < 0.05 compared with control.
pNIPAm thick solvent cast films where it was more pronounced.\textsuperscript{29} According to our results, such slip–stick behavior was more obvious on 900 nm thick coated films than on 300 nm thick films and was not observed on 50, 80, and 120 nm pNIPAm films, indicating thickness dependence. The reported contact angle for thick 300 and 900 nm pNIPAm films corresponds to stick contact angle, which is taken after the liquid drop reaches its equilibrium state.

As heat-treatment is widely employed in industrial processes for stabilization of material microstructure, the pNIPAm surface was analyzed after thermal annealing. The applied heat provides the energy that is needed to relieve internal stress which can occur during spin-coating and to order pNIPAm linear chains after spin-coating and thus to enhance the stability of pNIPAm polymer thin films. Contact angle measurement was repeated on pNIPAm films after the coated films were dried and then annealed for 2 h at 120 °C under vacuum. It was found that after annealing, the trend of surface wettability did not change and the stick–slip effect was still observed on 300 and 900 nm pNIPAm films (Figure 2 and Table 1). These results indicate that the spin-coating process allows the preparation of thin films with stable and uniform surface structure and that spin-coating technique itself does not influence pNIPAm film physicochemical properties such as wettability.

The values of root-mean-square (RMS) roughness analysis showed no significant differences between the groups and were comparable with flat noncoated glass slides (RMS 4.5 ± 2.0 nm) (Table 2). This indicates that all dry spin-coated pNIPAm films were assessed to be smooth and flat regardless of film thickness.

3.2. Cell Cultures on pNIPAm Films of Different Thicknesses. MS-5 cell attachment and proliferation were monitored for a period of 7 days and analyzed by microscopic examination, metabolic activity, viability assessment, and DNA content analysis.

According to microscopic observation, after 24 h, MS-5 cells cultured on Thermaconx control were already confluent and after 48 h, they grew in layers on top of each other. MS-5 cells grown on 50 and 80 nm pNIPAm films presented similar morphology. The cells were fully spread and had a large cell area with numerous well-defined and fully stretched pseudopodia, indicating a good cell attachment. In contrast, MS-5 cells on 120 nm did attach and proliferate but formed short rudimentary processes and did not display the fibroblast-like shape. MS-5 cells seeded on 300 and 900 nm pNIPAm films tended to grow in loosely attached clumps, did not flatten out, and generally had a rounded morphology (Figure 4).

The DNA content analysis confirmed significant variability in cell densities on spin-coated pNIPAm films of different thicknesses (Figure 5). During the first 12 h after cell seeding, the number of attached cells on 50 and 80 nm pNIPAm films was comparable to that on Thermaconx controls with no significant difference. The cells attached to 120 nm pNIPAm films but cell density was 38.3%, i.e., significantly less than on Thermaconx control. The cells seeded on 300 and 900 nm pNIPAm films loosely attached, and therefore the cell density was less than 10%. The DNA content after 12 and 48 h of incubation on 120 nm and particularly on 300 and 900 nm pNIPAm films were still significantly lower when compared with Thermaconx controls.

The decrease in proliferation rate of MS-5 cells that preferably formed clumps on 300 and 900 nm pNIPAm films was accompanied by decrease in metabolic activity that may be a consequence of poor cell attachment (Figure 6). It is interesting to note that although the MS-5 cells on 120 nm thick pNIPAm films displayed a slow proliferation rate, their metabolic activity still remained at a high level. This discrepancy could imply that although the cells remained in a semiquiescent state, they were attached, spread out, and able to uptake sufficient nutrients to meet the metabolic demands. This suggestion is supported by the study where Coller and coauthors demonstrated high metabolic rates in quiescent fibroblasts.\textsuperscript{33} The authors concluded that although quiescence signals often inactivate TOR kinase, resulting in reduced cell growth, it is not always associated with reduced metabolism. In contrast, MS-5 cells on thick 300 and 900 nm pNIPAm films were loosely attached and therefore, easily detached from the surface. As the fluorescence intensity of alamarBlue reagent is directly proportional to the cell number, the low metabolic activity on thick spin-coated pNIPAm films might be associated with small number of attached cells. The analysis of cell viability from the Live/Dead assay results did not reveal any significant difference.
di
different in cell survival among groups. The percentage of viable cells stained with calcein AM reached almost 100% on all tested samples, as indicated in Figure 7. However, there was a limitation, with performing a live/dead assay on 300 and 900 nm thick pNIPAm films because of the low number of adhered cells on the surface.

The changes in cell morphology were accompanied by changes in actin cytoskeleton organization (Figure 8A−F). The qualitative immunofluorescence analysis indicated that the cells grown on 50 and 80 nm pNIPAm surfaces exhibited a well-spread shape with long, thin filopodia and a well-organized pattern of numerous and straight actin stress fibers that is similar to the control group. The cells grown on the 120 nm pNIPAm surface had shorter filopodia with a smaller number of short actin filaments, and the intact actin stress fibers of cells seeded on 300 and 900 nm pNIPAm surfaces were mostly lost. Quantification of cells expressing paxillin shows that 50 and 80 nm pNIPAm films had similar number of paxillin-positive cells as the cells grown on bare Thermanox controls. In contrast, paxillin was poorly expressed in cells grown on 120 nm pNIPAm films, and cells on thick 300 and 900 nm pNIPAm films formed significantly fewer paxillin-positive focal adhesions as cell grew in loosely attached clumps (Figure 8G).

Therefore, the analysis of adhesion, proliferation, viability and metabolic activity, morphology, and cytoskeleton organization revealed that 50 and 80 nm pNIPAm films are the most compatible coatings to support cell growth and maintain cell function. The 120 nm pNIPAm surfaces did not affect MS-5 metabolic activity and viability, but significantly reduced the rate of cell growth, and 300 and 900 nm thick pNIPAm films were not suitable for long-term cell culture because of poor cell attachment.

Many surface characteristics, conditions, and components of surrounding environment influence cell adhesion and growth through protein adsorption process. Numerous studies have reported different cell behaviors on cell culture surfaces with different wettability, which are likely related to a different pattern, i.e., the type, quantity, and conformation of adsorbed proteins from serum-containing media. It is considered that due to the low water content on hydrophobic surfaces the

Figure 8. Immunofluorescence microscopy images of paxillin (green) and actin (red) staining of MS-5 cells cultured on (A) control slides, (B) 50, (C) 80, (D) 120, (E) 300, and (F) 900 nm pNIPAm films. (G) Statistical analysis of the number of paxillin-positive focal adhesions per cell in different groups. Initial cell-seeding density 40 000 cells/cm², incubation time 48 h, scale bar 50 μm. Data are mean ± standard error of mean (SEM), * p < 0.05 compared with control.

Figure 9. Illustration of the multicomponent protein layer adsorbed from serum-containing cell culture media on hydrophilic and hydrophobic surfaces of pNIPAm films with increased thickness and hydrophobicity. (A) Albumin adsorbs to the hydrophilic surface along with adhesive proteins allowing the latter to expose their cell-binding sites. (B) Albumin rapidly and firmly adsorbs to the hydrophobic surface, spreads and affects the adsorption of adhesive proteins in their active conformation. The cells adhere and spread over more hydrophilic surface while loosely attaching on more hydrophobic pNIPAm surface. Albumin is represented in red and adhesive proteins are in green.
latter rapidly interacts with the internal hydrophobic protein domains which attach, unfold from the core of the protein, and spread over the surface. On the other hand, hydrophilic surfaces tend to interact with charged and polar groups of protein’s surface, which attach but did not change their conformation, i.e. do not unfold and spread. It is well-known fact that albumin, which is a primary component of fetal bovine serum, readily adsorbs to the surface and resists cell attachment, whereas vitronectin or fibronectin are responsible for cell adhesion. It is necessary to note that protein molecules adsorb to the surface from multiprotein serum-containing media in a competitive mode according to their concentration, size and affinity to the surface. Hydrophobic surfaces tend to firmly adsorb larger and the most abundant proteins like albumin, which undergo structural rearrangements and modifications with a greater extent than on hydrophilic surfaces. Hydrophilic surfaces adsorb a lower quantity of proteins, but because of small conformational changes, albumin does not occupy the whole surface and allows lower concentrations of cell adhesion proteins to attach to the surface. Moreover, due to a low affinity for hydrophilic surfaces, albumin can be replaced with time by proteins with a higher affinity like fibronectin. Therefore, hydrophobicity and hydrophilicity of a surface can significantly alter cell behavior including cell orientation, morphology, and cytoskeleton arrangement. An interesting study performed by Kleinman et al. showed that nonadhesive protein albumin can maintain the exposure of active integrin-binding RGD cites in fibronectin. Such relationships between albumin and fibronectin increases cell adhesive activity of adhesion proteins on hydrophilic surface (Figure 9). Hence, when serum-containing media is added to cell culture surface, albumin adsorbs and dominates on hydrophobic surfaces, whereas it cooperates with fibronectin and other adhesive proteins, supporting their active conformation on hydrophilic surfaces.

In the case of the spin-coated pNIPAm films, the surface wettability is closely related to thickness. Muller-Buschbaum’s group recently described the mechanism of interfacial interaction of non-cross-linked pNIPAm films with water fabricated by spin-coating technique. pNIPAm films start to adsorb water in a one-dimensional manner in height and accumulate water molecules into accessible free space between linear pNIPAm chains. These studies that corroborate our findings also showed that pNIPAm films become more hydrophilic with decrease in thickness. We showed that the cells attached, rapidly proliferated, and reached confluency on the more hydrophilic 50 and 80 nm pNIPAm films with similar surface wettability, attached but did not spread on moderately hydrophilic 120 nm pNIPAm films and loosely attached and formed spheroid clusters on more hydrophobic 300 and 900 nm pNIPAm films. Our results are in accordance with the results of numerous studies that have shown that hydrophilic surfaces, in comparison with hydrophobic substrates, have higher levels of cell attachment, spreading, proliferation, and differentiation. Therefore, it is unlikely that cell attachment and growth is a single-valued function, but other factors including chemical and structural nature of material, the method of preparation, and the influence of wettability on conformational changes in adsorbed proteins should be considered.

3.3. Cell Detachment Analysis. To complete the analysis of cell behavior on spin-coated pNIPAm films with different thicknesses, we analyzed the detachment of MS-5 cells grown on pNIPAm films for 7 days. Qualitative image analysis demonstrated the typical pattern of cell detachment from pNIPAm films after the temperature decreased down to 4 °C. We observed that the cells detached from pNIPAm polymer films with different thicknesses in different ways. After the temperature was dropped, cells grown on Themanox control surfaces did not detach as expected, but the cells grown on pNIPAm 50 and 80 nm films detached as a contiguous cell sheets for 25–30 min (Figure 10). The results of the study suggest that more hydrophilic 50 and 80 nm thin pNIPAm films are more preferable for cell sheet fabrication while more hydrophobic 300 and 900 nm thick spin-coated pNIPAm films impede cell attachment. The control of the thickness of pNIPAm films fabricated by the spin-coating technique with high reproducibility offers an effective tool for cell sheet-based tissue engineering.

4. CONCLUSIONS

The thickness of polymer pNIPAm films fabricated by spin-coating is shown to influence the cellular attachment and spreading. The contact angle of pNIPAm films is gradually increased with increasing of film thickness. It is unlikely that this change in surface wettability is related to the process of film fabrication, because the annealing treatment of spin-coated pNIPAm films resulted in a similar trend in the contact angle values. Although the cells grow on spin-coated pNIPAm surfaces, there are significant changes in cell densities and perturbations in cellular cytoskeletal organization. In agreement with previous studies, 300 and 900 nm thick spin-coated pNIPAm films impede cell attachment. Although other spin-coated pNIPAm surfaces attach and release cells, 50 and 80 nm pNIPAm substrates would be more preferable for applications such as cell sheet engineering, because cell attachment, functions, and detachment as a contiguous cell sheet are more successful on thinner pNIPAm films. The reason might be due to changes in surface hydration with film thickness changes and relationships between pNIPAm film surface and multicomponent cell culture.
media containing proteins of different size, concentration, and affinity toward the surface.

This study is important for the assessment and design of thermoresponsive surfaces for cell-sheet-based tissue engineering using commercially available pNIPAm. The results suggest that it is possible to control the cell behavior on thermoresponsive surface by changing the thickness of non-cross-linked pNIPAm films and employing fairly fast and reproducible spin-coating technique. This should also be taken into account during the design of new pNIPAm-based thermoresponsive surfaces for biological applications, especially for cell sheet fabrication.

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Notes
The authors declare no competing financial interest.

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Novel biodegradable star-shaped polylactide scaffolds for bone regeneration fabricated by two-photon polymerization

Aim: To assess the properties of 3D biodegradable scaffolds fabricated from novel star-shaped poly(D,L-lactide) (SSL) materials for bone tissue regeneration. Materials & methods: The SSL polymer was synthesized using an optimized synthetic procedure and applied for scaffold fabrication by the two-photon polymerization technique. The osteogenic differentiation was controlled using human adipose-derived stem cells cultured for 28 days. The SSL scaffolds with or without murine MSCs were implanted into the cranial bone of C57/Bl6 mice. Results: The SSL scaffolds supported differentiation of human adipose-derived stem cells toward the osteogenic lineage in vitro. The SSL scaffolds with murine MSCs enhanced the mineralized tissue formation. Conclusion: The SSL scaffolds provide a beneficial microenvironment for the osteogenic MSCs' differentiation in vitro and support de novo bone formation in vivo.

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Keywords: MSCs • osteogenic differentiation • star-shaped polylactide • tissue engineering scaffolds • two-photon polymerization (2PP)

Bone defects in the head and face due to trauma or congenital abnormalities leave patients with reduced tissue function and render them psychologically scarred. In 2001, 37,732 children underwent surgery to repair birth defects and 24,298 patients required maxillofacial surgery for injuries to the face and jaw [1]. In total, the current market for craniofacial bone is estimated as $390 million for trauma alone, based on the overall market for trauma of $3 billion [2]. One of the future directions in medicine is associated with the development of patient-specific treatment approaches such as the creation of individualized pharmaceuticals [3] or surgical application of biodegradable scaffolds stimulating regeneration of the lost parts of tissues or organs [4]. Such scaffolds are often applied in combination with autologous stem cells [5,6]. A very promising approach for the creation of microstructured scaffolds from biodegradable polymers is the method of two-photon polymerization (2PP). With the accuracy down to a few hundreds of nanometers, the 2PP technique can ensure creation of a specific scaffold structure for the tissue defect repair, providing the required fixation in the area of injury. Recently, it has been shown that scaffolds based on cross-linked biodegradable polymers including copolymers of lactide and e-caprolactone are very promising for the future medical rapid prototyping [7,8]. Synthetic polymers represent the largest group of biodegradable polymers that can be produced under controlled conditions. Polylactic acid (PLA)-based copolymers are among the most commonly used synthetic polymers for bone tissue engineering [9].

In contrast to linear polylactide, star-shaped polylactide (SSL) materials have a lower viscosity and lower glass transition and melting temperatures. In addition, SSL have a lower crystallinity than linear poly-
lactide [10,11]. The above properties provide a better processability of SSL that, in turn, facilitates the construction of SSL-based 3D scaffolds. Furthermore, the mechanical properties of SSL-based materials can be easily controlled by changing the number and lengths of their arms, whereas the low polydispersity of the synthesized star-shapes allows for the construction of well-defined scaffolds with controlled pore sizes. In the present work, we suggest a new advanced approach for the synthesis of biodegradable star-shaped PLA-based polymers. The novel SSL polymer is used for fabrication of scaffolds by the 2PP technique. The SSL polymer allows for an excellent control over the mechanical properties of 3D scaffolds as well as the scaffold biofunctionalization with biologically active molecules enhancing the cell growth and proliferation. Integration of the high spatial resolution with the gradient structure and biofunctionalization of 3D scaffolds might be one of the most effective approaches in the regenerative medicine. The fabricated scaffolds have been biologically tested in vitro on their ability to support differentiation of mesenchymal stem cells (MSCs) toward the osteogenic lineage. A special characteristic of 2PP-fabricated scaffolds is that they exhibit fluorescence due to the presence of the photoinitiator in the polymer composition. And, this fact allowed the elaboration of a novel technique of fluorescence analysis to evaluate the scaffold degradation in vitro. The subsequent tests in vivo were aimed at the assessment of the bone-reconstructive potential of star-shaped PLA scaffolds upon their implantation into the cranial bone defect in mice.

Materials & methods

Photocurable polymer system synthesis

All chemicals were purchased from Sigma-Aldrich (Deisenhofen, Germany) unless otherwise indicated. D,L-lactide (98%) was twice recrystallized from toluene and then dried in vacuum at 55°C. Tin(II) 2-ethylhexanoate (Sn(Oct), 92.5–100%) was dried over CaH₂ and then distilled from CaH₂ under reduced pressure. Pentaerythritol (99%) was twice recrystallized from H₂O and then dried in vacuum at 80°C for 24 h. Pyridine (99.8%) was dried over KOH, distilled from CaH₂, Dichloromethane (anhydrous, ≥99.8%) and CDCl₃ (99.8%, Carl Roth, Karlsruhe) were dried over CaH₂ and then distilled from CaH₂ under argon atmosphere. Petroleum ether (40–60°C), methacryloyl chloride (97%), silica gel 60 (0.063–0.200 mm, Merck Chemicals GmbH, Darmstadt, Germany) were used as received.

All manipulations were carried out using standard Schlenk techniques under argon atmosphere. The synthetic protocol consists of three steps: synthesis of the SSL; SSL methacrylation; and photosensitive material preparation. These three steps are summarized in the following section.

Synthesis of SSL

SSL was synthesized by the ring-opening polymerization of D,L-lactide in bulk using Sn(Oct), as a catalyst and pentaerythritol as an initiator in a Schlenk tube equipped with a magnetic stirrer bar. As an example of a typical procedure for the synthesis of SSL, polymerization was carried out as follows ([D,L-lactide]/[initiator] = 20). The melt of pentaerythritol (0.23 g, 1.7 × 10⁻³ mol) in 5 g of D,L-lactide was prepared in a tube. Then the Schlenk reactor was charged by the catalyst solution (1 M) in toluene 0.56 ml (5.6 × 10⁻⁴ mol). After removing dichloromethane under the vacuum conditions, the pentaerythritol melt in D,L-lactide was added to the reactor. Then, the reaction vessel was immersed into an oil bath preheated to 130°C for 15 min for polymerization to give the desired poly(D,L-lactide). Yield: 99%; Mₘ (size exclusion chromatography [SEC]) = 4530 g mol⁻¹; Mₚ (NMR) = 2600 g mol⁻¹; Mₚ/Mₘ = 1.21. ¹H NMR (CDCl₃): δ (ppm) = 1.40–1.70 (m, Hc); 4.15 (m, Hₐ); 4.32 (m, Hd); 5.05–5.20 (m, Hc). See Figure 1 for assignments.

Star-shaped methacrylate functionalized poly(D,L-lactide)

The reaction of star-shaped hydroxyl-terminated poly(D,L-lactide) with methacryloyl chloride in the presence of pyridine was carried out in a 50 ml Schlenk tube equipped with a magnetic stirrer bar under argon atmosphere. SSL (5 g) was dissolved in dichloromethane (35 ml) and then pyridine (0.31 ml, 3.8 × 10⁻³ mol) was added. After that, the reaction vessel was cooled to 0°C and methacryloyl chloride (0.37 ml, 3.8 × 10⁻³ mol) was slowly added to the reactor. The reaction was allowed to warm up to room temperature and stirred for 24 h. The resulting products were cooled down for 12 h at -30°C (to precipitate the pyridinium salt), then filtered, purified by column chromatography using silica gel, and finally CH₂Cl₂ was evaporated with a rotary evaporator to give the desired star-shaped methacrylate-functionalized poly(D,L-lactide). Yield: 92%; Mₘ (SEC) = 4870 g mol⁻¹; Mₚ (NMR) = 2800 g/mol; Mₚ/Mₘ = 1.16. ¹H NMR (CDCl₃): δ (ppm) = 1.40–1.70 (m, Hc); 1.95–2.00 (s, Hₑ); 4.20 (m, Hₐ). See Figure 1 for assignments.

Star-shaped polymer characterization

¹H NMR (400 MHz) spectra were recorded in CDCl₃ at 25°C with a Bruker AC-400 spectrometer calibrated relative to the residual solvents resonances. Size exclusion chromatography (SEC) was performed using an
Agilent 1200 apparatus with a Nucleogel GPC LM-5, 300/7.7 column and one precolumn (PL gel 5 μm guard) thermostated at 30°C. The detection was achieved with a differential refractometer. Tetrahydrofuran (THF) was eluted at a flow rate of 1.0 ml/min. The calculation of the molar mass and polydispersity was based on polystyrene standards (Polymer Labs, Germany).

2PP fabrication of star-shape polylactide scaffolds

A liquid solution was prepared by dissolving one unit of the star-shaped methacrylate-functionalized poly(D,L-lactide) in double the volume of dimethyl chloride. To obtain a photoactive material, 1 wt% of 4,4’-bis(dimethylamino)benzophenone photoinitiator was added to the solution and mixed for 2 h. The scaffolds were produced using a Ti:sapphire femtosecond laser system (Chameleon, Coherent, Germany), which delivers 150-fs pulses at 80 MHz repetition rate. The experimental setup is similar to that previously described in [12].

Scanning electron microscopy (SEM)

The 2PP fabricated SSL scaffolds were examined with a Quanta 400F scanning electron microscope (FEI Company, OR, USA), which was equipped with EDX spectrometry (EDAX EDS System and Genesis Software, EDAX, Inc., NY, USA).

Mechanical characterization of SSL scaffolds

The mechanical characterization of the 2PP-fabricated SSL scaffolds was conducted in the same way as described in [12], using a nanohardness measurement module with a Berkovich tip for the ‘INTEGRA-Therma’ nanolaboratory and the NanoScan (Closed JSC “NT-MDT”, Russia) software. Two hundred indents with a constant load of 0.2 N (the standard deviation was 0.01%) and an indenting time of 10 s were applied to the scaffold surface, enabling immersion of the probe to a depth of 240–300 nm. Based on the change in the immersion depth, the local values of the Young’s modulus (E) and of microhardness (H) of the investigated sample could be calculated.

Cell culture for in vitro experiments

All cell culture reagents were purchased from Sigma-Aldrich (Deisenhofen, Germany), unless otherwise stated. Human adipose-derived stem cells (hASCs) were isolated and cultured as previously described [13]. Subculturing was performed before hASCs reached confluence. Passages 6–7 were used for the SSL scaffold seeding experiments. The scaffolds were sterilized under UV light for 30 min prior to the cell seeding. Each scaffold was seeded with 10^5 cells/cm^2 in a 48-well cell culture plate. The SSL scaffolds from the differentiation group were placed in an osteogenic induction medium consisting of DMEM supplemented with 10% FBS, 1 μM dexamethasone, 50 μM ascorbate-2-phosphate and 10 mM β-glycerophosphate. The scaffolds in the control group were cultured in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin only. This experiment was carried out over the period of 28 days. The differentiation and control media were changed every second day. The scaffolds with cells were analyzed using an AxioImager A1.m microscope (Carl Zeiss, Oberkochen, Germany) equipped with an AxioCamICc1 camera (AxioVision Rel. 4.8 software) and a Leica DM IL LED Fluo (Germany) inverted fluorescence microscope.

Analysis of SSL scaffold cytotoxicity

The potential cytotoxicity of the SSL scaffolds was quantitatively analyzed using the lactate dehydrogenase (LDH) assay. This method estimates LDH leakage into the culture medium, caused by cell membrane damage [14]. hASCs were seeded onto the scaffolds at a density of 10^5 cells/cm^2 and incubated for 24 h. LDH released into the supernatant was detected by the LDH activity assay (OPS Diagnostics, NJ, USA). A Tecan
Infinite M200Pro microplate reader with the Tecan i-controlTM software (Tecan, Crailsheim, Germany) was used to analyze the LDH turnover at a 492 nm wavelength. As a 100% positive control of cell damage (maximum LDH release), the cells were treated with 1% Triton-X100. The spontaneous LDH release from the equivalent number of cells cultured on a tissue culture plastic served as a comparative negative control. The calcein AM/propidium iodide-based live/dead staining was used to visualize live and dead cells on the SSL scaffold 24 h post seeding. The cell nuclei were stained with Hoechst 33342.

Immunohistochemical & histochemical staining
After 28 days of osteogenic and control culture, the cells on the SSL scaffolds were stained by a two-step indirect method. First, the scaffolds were fixed in 4% paraformaldehyde. A 2% bovine serum albumin (BSA/ PBS) solution was used to block nonspecific antibody binding, thereafter, primary antibodies (diluted in 0.3% Triton X-100/PBS solution) against osteocalcin 1:100 (mouse monoclonal IgG2a, clone C8, Santa Cruz, Heidelberg, Germany) were applied overnight at 4°C. After several washing steps, a horseradish peroxidase-conjugated goat anti-rabbit/goat anti-mouse IgG (H + L) secondary antibody (1:100, Dianova, Hamburg, Germany) was added to the SSL scaffolds for 1 h. The peroxidase staining was visualized by incubation with 3-amino-9-ethyl-carbazole substrate in a sodium acetate buffer (0.1 mol/l, pH 5.2) containing hydrogen peroxide. Calcium deposits were detected by staining with 2% Alizarin Red S (pH 4.2).

Cell culture for in vivo experiments
MSCs were isolated from the bone marrow of male 4-week-old C57/Bl6 mice. The MSC suspension was centrifuged, mixed with the growth medium (Mesenchyl™ MSC Basal Medium (Mouse), stimulatory supplements, STEMCELL Technologies, Canada), 0.58 mg/ml L-glutamine (PanEco, Moscow, Russia) and 0.58 mg/ml L-glutamine (PanEco, Moscow, Russia) and plated on culture flasks. After 2 days, nonadherent cells were removed and the monolayers of adherent cells were cultured under the standard conditions (37°C, 5% CO2, saturation humidity). Before cell seeding, the SSL scaffolds were sterilized with 70% ethanol and then exposed to UV for 3 h. Then, the scaffolds were individually seeded with MSCs (2 × 105 cells/scaffold) in the third passage and cultured for 3 days prior to implantation.

Surgical procedure
In vivo experiments were performed on male C57/Bl6 mice. The mice were anesthetized with Zoletil (80 mg/kg). Four-millimeter-diameter critical-sized defects were created in the cranial bone of each animal using a dental bur. Immediately after the generation of these defects, the SSL scaffolds with and without (control) MSCs were implanted into the defect sites. All in vivo experiments were approved by the Ethics committee of the Nizhny Novgorod State Medical Academy (Nizhny Novgorod, Russia).

Fluorescence analysis of SSL scaffold degradation in vivo
The fluorescence analysis of the SSL scaffolds’ degradation was performed using the ImageJ 1.43u software (NIH, USA). In the fluorescence images imported in ImageJ 1.43u, the area of each defect with the implanted scaffold was identified, and the ‘Integrated density’ parameter, which shows the integral value of the signal intensity, was calculated. This parameter can be used as a criterion for evaluation of the scaffold biodegradation according to the level of its fluorescence. The area of the scaffold and the proportion of the newly formed bone were evaluated with the ‘Area’ parameter.

Analysis of bone formation after SSL scaffold implantation into mice
The cranial bones were harvested 5 or 10 weeks after the implantation. To evaluate osteogenesis, the harvested bones with scaffolds were stained with Alizarin Red S (Sigma Aldrich, Deisenhofen, Germany) for the verification of calcium deposits. Light microscopy imaging was performed using an Axio Zoom V16 (Carl Zeiss, Germany) stereo zoom microscope. For the histological analysis, the harvested bones were fixed in a 10% formalin solution and decalcified with nitric acid for 2 weeks. Then, the samples were dehydrated and embedded into paraffin wax. Cross-sections were cut using a Leica SM 2000 (Jena, Germany) microtome and stained with Van Gieson picrofuchsin.

Statistical analysis
Ten SSL scaffolds were cultured in the osteogenic conditions and ten SSL scaffolds were cultured in the control conditions for in vitro experiments. For LDH-based cytotoxicity test, the supernatant from 20 seeded scaffolds was used. For in vivo experiments, the mean values (M) and standard deviations (σ) were calculated for three mice in each group. The Student’s t-test was used to compare the data (p ≤ 0.05 was considered statistically significant).

Results
Star-shaped methacrylate-terminated oligo(D,L-lactide)s molecules with four arms were synthesized according to the synthesis strategy depicted in Figure 1.
This strategy consists of Sn(Oct)$_2$-catalyzed bulk ring-opening polymerization of the D,L-lactide using pentaerythritol as a tetrafunctional initiator, followed by end-capping of the resulting SSL with methacryloyl chloride. The bioresorbable star-shaped polymers were obtained with a high yield (>90%) and featured a low molecular weight ($\text{M}_n \sim 2800$ g mol$^{-1}$) and a rather narrow molecular weight distribution ($\text{M}_w/\text{M}_n < 1.2$). The structure and purity of the synthesized compounds were confirmed by $^1$H NMR spectroscopy (see Materials & methods section for details). Although a similar methodology has previously been used for preparation of (meth)acrylate-functionalized SSLs [15–19], we have optimized the existing synthetic procedure. First of all, the reaction time to obtain hydroxyl-terminated SSL with a quantitative yield has been significantly reduced in our case from 3–48 h [15,17–19] to 15 min (step (i) in Figure 1). In addition, we demonstrate that isolation and purification of the SSL before its reaction with methacryloyl chloride is not necessary. Therefore, the synthesis of hydroxyl-terminated SSL (step (i) in Figure 1) and its functionalization (step (ii) in Figure 1) can be carried out in one and the same reactor. These improvements make the procedure for preparation of functionalized SSL much simpler and more cost-efficient.

The 2PP technique was used to create 3D porous scaffold structures, shaped as hexagons. The scaffolds were composed of two layers of hollow cylinder arrays in a hexagonal arrangement (Figure 2). The second layer of cylinders was fabricated with $130 \mu$m X and Y offsets to the previous layer of cylinders. The mechanical characteristics of the resulting structures were evaluated using the nanoindentation method. It was shown...
Figure 3. Analysis of the star-shaped poly(D,L-lactide) scaffolds cytotoxicity via qualitative Live/Dead staining and lactate dehydrogenase assay 24 h post seeding. (A) The uniform distribution of cell nuclei indicates that the scaffold is well-populated with vital hASCs; propidium iodide staining (right image, red fluorescence) indicates only few dead cells on the SSL scaffold. (B) hASCs on the scaffolds show the degree of LDH release comparable to the spontaneous LDH release from the cells seeded simultaneously on a tissue culture plastic (Control 1), both values compared with maximum LDH release from lysed cells (Control 2). (C) Combination of the Hoechst (cell nuclei) and Calcein (live cells)/Propidium Iodide (dead cells) channels (the lack of red zones indicates that all cells are alive). The scale bars are 75 μm for all images.

hASC: Human adipose-derived stem cell; LDH: Lactate dehydrogenase; SSL: Star-shaped poly(D,L-lactide).
Figure 4. Alizarin Red S (A–C) and osteocalcin (D & E) staining of the SSL scaffolds on the day 28 of in vitro differentiation. (A) Alizarin Red S staining was performed directly in the culture wells showing that the cells cultured in the osteogenic conditions deposited mineralized matrix both on the scaffold and in the whole well area around the scaffold. hASCs on the SSL scaffolds cultured in the control conditions without the osteogenic stimulation deposited mineralized matrix only on the SSL scaffold, and the area around the scaffold is minimally Alizarin Red-positive. (B & C) A higher magnification view of the Alizarin Red stained scaffolds cultured in the osteogenic and control conditions, respectively. (D & E) Osteocalcin expression on the day 28 of in vitro differentiation. The scale bars are 1 cm (A) and 75 μm (B–D), respectively.

hASC: Human adipose-derived stem cell; SSL: Star-shaped poly(D,L-lactide).

that for the fabricated structures, the local values of E and H were 4.11 GPa (with the root-mean-square deviation of 14%) and 0.36 GPa (with the root-mean-square deviation of 9%), respectively (Figure 2B).

hASCs showed a high affinity to the SSL scaffolds upon seeding. The majority of seeded cells could attach to the scaffolds and uniformly populate their whole surface (Figure 3A). Although the scaffolds have a strong fluorescence in all channels, fluorescent live/dead staining 24 h post seeding allowed discrimination of the very few dead cells on the SSL scaffolds. For the quantitative assessment of scaffold cytotoxicity, the LDH assay was also carried out 24 h after the scaffold seeding. hASCs on the scaffolds showed the degree of LDH release comparable to the spontaneous LDH release from the cells seeded simultaneously on a tissue culture plastic (Figure 3B). These results indicate the noncytotoxic character of the SSL scaffolds.
**In vitro osteogenic differentiation of hMSCs on the SSL scaffolds**

Alizarin Red S staining was performed directly in the culture wells, showing that the cells cultured in the osteogenic conditions had deposited mineralized matrix both on the scaffolds and in the whole well area around the scaffold (Figure 4A). hASCs on the SSL scaffolds, cultured in the control conditions without the osteogenic stimulation, deposited more mineralized matrix only on the SSL scaffold and minimally in the area around the scaffold, as shown by the Alizarin Red-positive. The analysis of osteocalcin expression was used to confirm the osteogenic differentiation of hASCs on the SSL scaffolds. Immunohistochemical staining was performed on the day 28 of in vitro differentiation (Figure 4D & E). Although hASCs on the scaffolds cultured in the osteogenic conditions demonstrated a remarkably stronger osteocalcin expression, the cells cultured in the control conditions were also osteocalcin-positive.

**Assessment of the SSL scaffolds biodegradation in mice according to the level of fluorescence**

After the formation of 4 mm defects in the skulls of mice and implantation of scaffolds into the defects, their area was assessed using their fluorescence (Figure 5). Immediately after the implantation into mice, the SSL scaffolds represented strongly fluorescent structures with clear borders (Figure 5A & B). After 5 weeks, the level of the scaffold fluorescence decreased by nearly a half of its initial value for both groups (+MSCs and -MSCs). The area of the implanted SSL scaffolds was also decreased to 84.2 ± 4.3% and 75.3 ± 3.1% for +MSCs scaffolds and -MSCs scaffolds, respectively (Figure 5C & D & Table 1). By 10 weeks, the area of the -MSCs scaffolds remained almost unchanged as compared with the data from the 5th week (Figure 5C & E & Table 1). The level of fluorescence decreased by 8%. In contrast, the area and the level of the fluorescence signal from the +MSCs scaffold had decreased by three-times compared with the week 5 (Figure 5F & Table 1).

**Assessment of the scaffold mineralization**

The harvested bones with the SSL scaffolds were stained with Alizarin Red S to assess the degree of mineralization (Figure 6). Alizarin Red S staining localized the bone tissue formation to the areas of mineralization. Five weeks after the defect formation and scaffold implantation, in the MSC(-) group, we observed the formation of small mineralized regions on one side of the defect, though the major part of each defect with the scaffold remained mineralized.
tion-free (Figure 6A & Table 2). The defects repaired with MSCs(+) scaffolds were filled with mineralized regions to 86% (Figure 6B & Table 2). By 10 weeks, the MSCs(-) scaffolds were filled by 50% with mineralized tissue. Calcium deposits formation was observed at one side (Figure 6C & Table 2). The bone defects repaired with MSCs(+) SSL scaffolds were observed as almost completely mineralized areas by week 10 (Figure 6D & Table 2).

The histological images show that, by week 5, the sections still have a large amount of undegraded scaffold material (Figure 7A & B). However, in the MSCs(+) SSL implants, the scaffold material is present as more fractured and smaller pieces interlaced with connective tissue (Figure 7B). A small number of connective tissue fibers were present and individual blood vessels were also observed.

By week 10, the scaffold material in the MSCs(-) and MSCs(+) implants was still present in a significant amount (Figure 7C & D). Almost the whole volume of the MSCs(-) and MSCs(+) implants was filled with a coarse-fibered connective tissue. Here again, the MSCs(+) scaffolds were more segmented by connective tissue fibers, which evidences a better infiltration of the reconstructive tissue.

Connective tissue is usually widely present in the embryogenesis and in the early postnatal histogenesis of skeleton bones, and, as a rule, is replaced by lamellar bone tissue in the course of regeneration [20].

Discussion
In the field of a patient-specific restorative treatment, bone tissue engineering is the most promising approach, which can be actively adopted into the medical practice in the near future. A bone tissue engineered construct fabricated by laser structuring methods consists of a 3D scaffold, which serves as a substructure for autologous stem cells. The structural characteristics of such implantable scaffolds play an important role in the new bone tissue formation. The scaffold properties depend on the type of applied biomaterial and fabrication process. Different types of biomaterials, such as metals, ceramics, glass, chemically synthesized polymers, natural polymers and combinations of these materials (composites) have been intensively studied [21–24]. The optimal requirements for scaffolds in bone tissue engineering have been reviewed, including the structural properties [25], aspects of degradation [23,26], mechanical properties [27] and combinations of scaffolds with cells [28].

Star-shaped copolymers are the most promising precursors for the laser-based fabrication of 3-D scaffolds, because they allow variation of the scaffold’s mechanical properties [15]. Changes in the length of each specific ray of such star-shaped polymers can modify the surface morphology of the structures obtained by the laser-induced formation [16]. Furthermore, the creation of homogenous structures based on star-shaped polymers does not require application of any extra cross-linking agents, due to the fact that the polymer structure has four unsaturated groups [29,30]. The optimized method described in this work has been shown to provide both the complete conversion of monomers within 15 min and the methacrylation reaction without any extra purification of the SSL. As a result, the synthesis efficiency and the resulting product purity are enhanced, which are significant parameters for biomedical materials. The 3D scaffolds based on synthesized SSL were fabricated by the method of 2PP. Recently, it has been demonstrated that human mesenchymal stem cells (hMSCs) cultured on the 2PP-produced scaffolds can differentiate toward the osteogenic lineage without any chemical osteogenic stimulation. It has been shown that there is an optimum in the scaffold’s mechanical properties for increased mineralization of differentiated hMSCs on the scaffolds [12]. It is a well-known fact that effective replacement of bone defects and initiation of spontaneous osteogenic differentiation (if stem cells are used) require a scaffold with a high tensile strength close to the lower limit of the Young’s moduli.
Figure 7. Van Gieson stained histological sections of the star-shaped poly(D,L-lactide) scaffolds. (A) MSC(-) scaffolds after 5 weeks, (B) MSC(+) scaffolds after 5 weeks, (C) MSC(-) scaffolds after 10 weeks, (D) MSC(+) scaffolds after 10 weeks. The scale bars are 100 μm for all images.

C: Coarse-fibered connective tissue; MSC: Mesenchymal stem cell; S: Scaffold; V: Blood vessel.

Restoration of the normal shape, architecture and function of the bone tissue is a special task in tissue engineering, and the success of such operations in eliminating defects, as well as during the period of formation of a new bone, depends on a great number of factors [37]. The structural characteristics of implantable scaffolds play an important role in the new tissue formation. Application of biodegradable polymers allows engineering of scaffolds that can undergo significant structural remodeling during the formation of new tissues. That is why one of the most important criteria is the rate of scaffold biodegradation [38]. There have been many studies in which the degradation of scaffolds has been evaluated in vitro [26,39–41]. But there is only a small number of studies offering noninvasive methods of controlling the rate of degradation in vivo [42]. The study by Zhang Y. et al. showed that the rate of biodegradation could be assessed by fluorescent agents cross-linked to the scaffold structure. During the 2PP scaffold fabrication, due to the mechanism of photo-induced degradation of the initiator, the luminescent nontoxic fragments are included into the resulting scaffold structure. Therefore, such scaffolds have an intense fluorescence, which provides an effective and long-lasting method of assessing the rate of the scaffold degradation and its replacement by the native tissue in vivo.

The 2PP produced SSL scaffolds have demonstrated an excellent biocompatibility in vitro and in vivo. Upon implantation, no obvious necrosis, inflammatory responses or fibrous membrane formation have been found in the histological slices from each of the experimental groups.

Table 2. Assessment of the mineralization percentage according to the Alizarin Red S stained area.

<table>
<thead>
<tr>
<th>Time after implantation (weeks)</th>
<th>-MSCs</th>
<th>+MSCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>35.5 ± 5.1</td>
<td>86.5 ± 3.9†</td>
</tr>
<tr>
<td>10</td>
<td>65.8 ± 4.9</td>
<td>92.3 ± 4.8†</td>
</tr>
</tbody>
</table>

†p ≤ 0.05 compared with the ‘MSC(-) group’, each number represents M ± σ (n = 3).
The microenvironment (43). The 3D microarchitecture and the high value of the Young’s modulus of the fabricated SSL scaffolds could better support both stimulated and spontaneous osteogenic differentiation of MSCs in vitro and in vivo.

Proliferation of osteoblasts followed by formation of matrix and its mineralization is known to be important for the process of bone formation [44]. Mineralization of the bone tissue, that is, deposition of inorganic substances, in particular, calcium phosphates, is another criterion of bone formation at the sites of defects. It has been shown that, to achieve this effect, autologous stem cells (MSCs) can be used, which, in the process of in vitro development, differentiate toward osteoblasts and start matrix mineralization. It has also been shown that the level of mineralization at the sites of defects with scaffolds strongly depends on the presence of inoculated cells [28,45]. In the control implanted scaffolds, without inoculated cells, the levels of mineralization achieved 65.8% by the week 10, while the scaffolds preseeded with MSCs demonstrated a significantly higher percentage of mineralization of 92.3% by the week 10 (Figure 6 & Table 2). Our results demonstrated that both groups of scaffolds (with and without seeded cells) had significant percentage of mineralization by the 10th week. These values are several times higher than the percentage of mineralization obtained on other samples without inoculated cells [45].

The applied biomimetic 3D architecture of the 2PP-fabricated SSL scaffolds provides excellent conditions for cell infiltration into the scaffold, accelerating the degradation process and mimicking the natural mineralized bone microenvironment.

Conclusion
Biodegradable scaffolds with a 3D microscopic architecture have been fabricated from a novel SSL copolymer by applying the 2PP technique. It has been demonstrated that the fabricated SSL scaffolds are able to provide a beneficial microenvironment for the osteo-

Executive summary

Background
• Synthetic biodegradable star-shaped polylactide (SSL) materials allow the construction of 3D scaffolds with an easily controllable microstructure and mechanical properties for biomedical implant application and bone regeneration.
• Two-photon polymerization (2PP) technique is a promising method for 3D scaffold fabrication with a high accuracy, enabling modeling the cellular interaction and tissue organization with a precise structural resolution.

Materials & methods
• The 2PP-fabricated scaffolds shaped as hexagons were prepared from synthesized star-shaped methacrylate functionalized poly(D,L-lactide) and examined for their nanostructure with a scanning electron microscope and for their mechanical properties using a Berkovich indenter.
• The 2PP-fabricated scaffolds were loaded with human adipose-derived stem cells in vitro. The potential cytotoxicity of the SSL scaffolds was quantitatively analyzed using the LDH assay. Osteogenic differentiation in vitro was controlled after 28 days using Alizarin Red S staining and analysis of osteocalcin expression.
• Murine MSCs from bone marrow were loaded into the SSL scaffolds and implanted into the cranial bone of male C57/Bl6 mice in vivo.
• The monitoring of the SSL scaffold biodegradation and the degree of scaffold mineralization was performed in 5 and 10 weeks after implantation in mice, using the scaffold autofluorescence and Alizarin Red S staining, respectively.

Results
• The optimized method of the SSL polymer synthesis described in the current study allowed the complete conversion of monomers and the methacrylation reaction resulting in a high-yield one-pot synthesis and enhanced purity of the final product.
• The 2PP-fabricated SSL scaffolds showed the high value of Young’s modulus (>4 GPa) with densely cross-linked structures and a high spatial resolution.
• The SSL scaffolds preseeded with MSCs better supported both stimulated and spontaneous osteogenic differentiation of MSCs in vitro and in vivo and demonstrated a significantly higher percentage of mineralization by the week 10 after implantation.
• The SSL scaffolds showed an excellent in vivo biocompatibility with no obvious necrosis, inflammatory responses and fibrous membrane formation.

Conclusion
• The novel SSL copolymer in combination with the cutting-edge 2PP technique allowed production of scaffolds with a 3D microarchitecture.
• Biodegradable and biocompatible 2PP-fabricated SSL scaffolds preseeded with MSCs provided a beneficial microenvironment for osteogenesis and bone regeneration in vitro and in vivo.
Regenerative differentiation of MSCs in vitro and support de novo bone formation in vivo, which shows them as very promising 3D microstructured implants for bone regeneration applications.

Financial & competing interests disclosure
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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research
The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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Novel biodegradable SSL scaffolds for bone regeneration fabricated by two-photon polymerization  

Research Article

• Analyzes how laser fabrication parameters and powder layer thickness influence the morphology and mechanical properties of selective laser sintering-made scaffolds by using a volume energy density function.
• Developed an in situ method of fabrication of fluorescently labeled potentially injectable hydrogel to assess the biodegradation rate of the scaffolds in vivo.
• Developed an in situ method of fabrication of fluorescently labeled potentially injectable hydrogel to assess the biodegradation rate of the scaffolds in vivo.
• Developed an in situ method of fabrication of fluorescently labeled potentially injectable hydrogel to assess the biodegradation rate of the scaffolds in vivo.
Natural and Synthetic Materials for Self-Renewal, Long-Term Maintenance, and Differentiation of Induced Pluripotent Stem Cells

Nina A. Dzhoyashvili, Sanbing Shen, and Yury A. Rochev

Induced pluripotent stem cells (iPSCs) have attracted considerable attention from the public, clinicians, and scientists since their discovery in 2006, and raised huge expectations for regenerative medicine. One of the distinctive features of iPSCs is their propensity to differentiate into the cells of three germ lines in vitro and in vivo. The human iPSCs can be used to study the mechanisms underlying a disease and to monitor the disease progression, for testing drugs in vitro, and for cell therapy, avoiding many ethical and immunologic concerns. This technology offers the potential to take an individual approach to each patient and allows a more accurate diagnosis and specific treatment. However, there are several obstacles that impede the use of iPSCs. The derivation of fully reprogrammed iPSCs is expensive, time-consuming, and demands meticulous attention to many details. The use of biomaterials could increase the efficacy and safety while decreasing the cost of tissue engineering. The choice of a substrate utilized for iPSC culture is also important because cell-substrate contacts influence cellular behavior such as self-renewal, expansion, and differentiation. This Progress Report aims to summarize the advantages and drawbacks of natural and synthetic biomaterials, and to evaluate their role for maintenance and differentiation of iPSCs.

1. Introduction

Age-related diseases including ischemic heart disease, Parkinson’s disease, osteoarthritis and osteoporosis could become a top cause of disability by the year 2020.[1,2] Current treatments are largely to help ease and manage the symptoms. The main reason for the low-efficiency of current medicine is the limited regenerative abilities of adult tissue, especially, in aged and diseased patients. Current advances in the understanding of stem cell biology offer new and promising approaches for the restoration of damaged tissues. However, senescence and disease-associated decline of autologous stem cells such as the widely used mesenchymal stem cells (MSCs) from elderly and diseased patients may restrict their efficacy in clinical practice.[3,4] Reprograming somatic cells to iPSCs may represent a new vein in stem cell research, and has shown the stem cell rejuvenation and improvement in their proliferation and differentiation.[5] The iPSC era has been initiated by the integration a new method involved the ectopic expression of four transcription factors in somatic cells.[6] These four factors, Oct4, Sox2, Klf4/Lin28, and c-Myc/Nanog were introduced into somatic cells using retroviral or lentiviral systems to induce reprogramming to ESC-like pluripotent state. Further investigation of self-renewal and differentiation mechanisms of iPSCs in vitro revealed a similar network of cell-intrinsic signals that regulate stem cell behavior in tissues at different stages of life.[7,8] Understanding of the biology of iPSCs may provide a clinically relevant tool to improve our knowledge regarding disease pathogenesis and ways to prevent their progression.

Yet despite the largely positive results, there are some concerns which relate to iPSC safety and efficacy and require further optimization before the application of iPSC cell technology to clinically relevant problems. The standard reprogramming protocols widely utilize viruses and chemicals to generate iPSCs. However, the use of viral vectors for cellular reprogramming raises issues about their clinical safety due to a high risk of genetic alterations, chemically induced mutations and, therefore, teratoma formation.[9] Patients’ death from viral-based gene therapy in the first clinical trials raised many questions concerning the safety of viral gene delivery system.[10–13] At the same time, non-viral transfection methodologies including Lipofectamine 2000 (Invitrogen) and FuGENE HD (Roche) show low transfection efficacy and biocompatibility and high cost for large scale application.
Next, the generation of iPSCs is now commonly based on iPSC co-culture with feeder mouse embryonic fibroblasts (MEFs). These cells support the pluripotency and proliferative potential of iPSCs. However, the use of animal cells presents a risk of transmitting pathogens to human stem cells that may be associated with immune complications. To avoid these problems, extracellular matrices instead of fibroblast feeder can provide the attachment and expansion of iPSCs. Several commercial substrates have been successfully tested for iPSC culture and these include Matrigel (Corning Life Sciences, USA), Geltrex (Life Technologies, USA), MesenCult-SF Attachment Substrate (Stemcell Technologies, France) and Cultrex Basement Membrane Extract (Trevigen Inc, USA) which,[14–17] They are produced from Engelbreth-Holm-Swarm mouse sarcoma which contains numerous growth factors, inhibitors, major proteins of basement membrane including laminin, collagen IV, entactin, and heparin sulfate proteoglycan as well as a broad variety of other unknown proteins. However, animal-derived substances can cause problems related to immunogenicity in addition to microbial and viral contamination that limit their clinical applications. The concentration of growth factors, inhibitors, and other proteins in Geltrex or Matrigel is difficult to control. It impedes the optimization of many protocols and reduces the efficiency and reproducibility of results. Therefore, because of safety issues and problems related to batch-to-batch variability as well as to potential immunogenicity, only well-defined substrate components should be applied. The generation of human iPSCs without the use of virus and animal derived products is a major challenge facing clinical research today.[18] One strategy to circumvent such limitations is the development of biomaterial-based platform for iPSC generation and culture (Figure 1).

A wide range of biomaterials has been developed for tissue engineering. For the purposes of this report, materials will be classified as natural and synthetic. The different materials and their applications for long-term maintenance and expansion are discussed in Section 2. This information along with the relevant references is listed (Table 1), and the advantages and drawbacks of each type are summarized (Table 2). This report also highlights in Section 3 how biomaterials can support iPSC differentiation into ectodermal, mesodermal, and endodermal lineages in vitro and in vivo (Table 3 and 4). Regulation of iPSC expansion and differentiation by substrate stiffness, surface topography, and dimensional structure of biomaterials are discussed in Section 4. Additionally, the way in which integrin-biomaterial interaction can affect iPSC behavior is analyzed. Overall, the current report seeks to summarize main recent information on using different biomaterials for iPSC self-renewal, expansion, and differentiation.

2. From Non-Defined to Defined Conditions for iPSC Self-Renewal and Maintenance: Natural and/or Synthetic Substrates and Culture Medium

2.1. Natural Biomaterials

Due to high biocompatibility, good biodegradability and availability, natural substrates have found a wide application for tissue engineering. Extracellular matrix (ECM) proteins (such
as fibronectin, vitronectin, and collagen) can provide a necessary niche-like microenvironment in vitro and avoid the use of animal-derived products. In addition, natural biomaterials better maintain cell adhesion and growth than that of synthetic polymers. Although natural substrates can increase the attachment and growth of iPSCs, there are still limitations. The peptide-modified surfaces cannot be reused because peptides are degraded by metalloproteases secreted by the cultured cells. As a result of degradation, more culture substrates will be needed and this again increases the costs of the polymer and, thus, may hinder successful clinical applications. Additionally, to ensure clinical applicability, culture substrates should be compatible with common sterilization techniques such as e-beam and gamma-radiation. However, large-scale radiation sterilization can degrade proteins. It is also important to mention that the production of recombinant ECM proteins is still costly. Among the various types of natural biomaterials, hydrogels stand out as a promising substrate for iPSC culture. Because of their high biocompatibility and similarity with tissue components of the body, hydrogels can create a tissue-like three-dimensional microenvironment suitable for cell guidance and for controlling stem cell differentiation. This important property made them ideal biomaterials for tissue engineering, wound dressing, soft tissue replacement and many more applications. Matrigel is the most common hydrogel for iPSC culture. It was shown in many studies that Matrigel can support iPS attachment, growth and differentiation toward cardiomyocytes, skeletal muscle cells, neural cells, hepatocytes, and intestinal tissue. However, Matrigel is an animal-derived product and, therefore, the high risk of disease transmission significantly restricts its use for human tissue regeneration. Hyaluronan (also called hyaluronic acid or HA) hydrogels have been widely used for tissue engineering as a biomaterial which architecture, mechanics, and degradation can be well controlled. Interestingly, MEFs, which are used as a feeder-layer for iPSC culture, produce large amounts of HA. However, a significant disadvantage of hyaluronic acid is that cells do not adhere to its surface due to the highly negative charge and hydrophilicity. Hence, HA hydrogels have to be modified with other materials to promote iPSC adhesion and growth. Natural adhesive factors such as gelatin, fibronectin, vitronectin, laminin, E-cadherin, and collagen are used to optimize iPSC growth in vitro. Liu et al. successfully tested commercial hyaluronan-based hydrogel HyStem-C for growth and maintenance of human iPSCs. The study presented gelatin incorporated hyaluronan hydrogel which was free or covalently bound with fibronectin (Fn). The hydrogel was tested for mouse embryonic stem cells (ESCs). As with human iPSCs, mouse ESCs showed no significant differences in adhesion, growth rate, morphology and expression of pluripotency markers between hyaluronan-modified hydrogels and Matrigel. The same results were confirmed in human iPSCs, as there was no any difference in growth rate or pluripotency markers expression between modified hyaluronan hydrogels and Matrigel. In particular, during long-term passaging on hyaluronan hydrogels, human iPSCs demonstrated positive expression of pluripotency markers confirmed by RT-PCR and flow cytometry analysis and formed embryoid bodies in vitro. In addition, the karyotype of human iPSCs grown on hyaluronan hydrogels after long-term cultivation was normal. These results were obtained after multiple passaging of iPSCs in MEF-conditioned medium. As it is now under active discussion, to successfully bring stem cell–based technology closer to clinical practice, a fully defined xeno-free culture system is necessary.

Figure 1. The advantages of iPSCs for biomaterial-based tissue engineering and regenerative medicine.
### Table 1. Natural and synthetic materials for iPSC cell attachment and maintenance in vitro and in vivo.

<table>
<thead>
<tr>
<th>Primary substrate</th>
<th>Characteristics</th>
<th>Composition with other polymers</th>
<th>Tested material</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geltrex, Matrigel, Cell Matrix Basement Membrane Gel</td>
<td>Cell adhesive due to laminin, collagen IV, entactin and heparin sulfate proteoglycan</td>
<td>Tissue culture plastic &amp; wide range of synthetic polymers</td>
<td>2D coated dish</td>
<td>[14–17,19–24]</td>
</tr>
<tr>
<td>Hyaluronan</td>
<td>Creates niche-like microenvironment, prevents cell adhesion, biodegradable, weak mechanical properties</td>
<td>Fibronectin, gelatin</td>
<td>2D/3D HyStem-C hydrogel-based system</td>
<td>[25,31]</td>
</tr>
<tr>
<td>Gelatin</td>
<td>Supports cell spreading and adhesion, tissue integrity, biodegradable, weak mechanical properties</td>
<td>Hyaluronan, fibronectin</td>
<td>2D gelatin coated dish</td>
<td>[30]</td>
</tr>
<tr>
<td>Vitronectin</td>
<td></td>
<td>PVA, hyaluronan, POEGMA, acrylate</td>
<td>3D PVA/VN nanofibers</td>
<td>[31]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2D poly(OEGMA-co-HEMA) / VN coated dish</td>
<td>[32]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2D Synthemax dishes</td>
<td>[33]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2D VN-N and VN-NC coated dish</td>
<td>[26]</td>
</tr>
<tr>
<td>Laminin</td>
<td></td>
<td></td>
<td>2D E8 fragment of laminin coated dish</td>
<td>[27]</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Non-adhesive, biodegradable, non-cytotoxic</td>
<td>Hyaluronan, vitronectin</td>
<td>2D laminin-511 coated dish</td>
<td>[28,75]</td>
</tr>
<tr>
<td>Cellulose</td>
<td></td>
<td></td>
<td>2D E cadherin/IgG Fc coated dish</td>
<td>[29]</td>
</tr>
<tr>
<td>Cellulose</td>
<td></td>
<td></td>
<td>3D nanofibrillar cellulose hydrogel</td>
<td>[87]</td>
</tr>
<tr>
<td>PNaAMPS</td>
<td>Biodegradable, strong mechanical properties, low cell attachment and spreading</td>
<td>Hyaluronan, vitronectin</td>
<td>3D PVA/HA/VN nanofibers</td>
<td>[31]</td>
</tr>
<tr>
<td>PVA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POEGMA</td>
<td>Low protein adsorption, low cell attachment and spreading</td>
<td>Vitronectin</td>
<td>2D poly(OEGMA-co-HEMA) / VN coated dish</td>
<td>[32]</td>
</tr>
<tr>
<td>PMEDSAH</td>
<td>Good adhesion properties, enabling heparin-like binding of growth factors and self-renewal, can be subjected to long-term storage and sterilization, supporting xeno-free conditions, reproducible</td>
<td></td>
<td>2D PMEDSAH coated dish</td>
<td>[40–42]</td>
</tr>
<tr>
<td>pNIPAM</td>
<td>Good cell adhesion, cell growth and spreading depend on method of preparation, biodegradable</td>
<td>Poly(ethylene glycol)</td>
<td>3D pNIPAAm-PEG hydrogel</td>
<td>[39]</td>
</tr>
<tr>
<td>Graphene and graphene oxide</td>
<td>Biocompatible, good cell adhesion and growth</td>
<td></td>
<td>2D graphene and graphene oxide coated dish</td>
<td>[92,93]</td>
</tr>
</tbody>
</table>

PNaAMPS: poly(2-acrylamido-2-methyl-propane sulfonic acid sodium salt); PVA: polyvinyl alcohol; POEGMA: poly[oligo(ethylene glycol)] methacrylate; PMEDSAH: poly[2-(methacryloyloxy)ethyl dimethyl(3-sulfopropyl) ammonium hydroxide]; pNIPAM: poly[N-isopropylacrylamide]; pNIPAAm-PEG: poly[N-isopropylacrylamide]-co-poly(ethylene glycol) hydrogel; VN: vitronectin

Although the xeno-free medium turned out not to support the attachment of human iPSCs, the addition of fibronectin to hyaluronan-modified hydrogels was able to significantly improve cell attachment to 24-well HyStem-C +Fn pre-coated plates in the presence of xeno-free medium so that the result was comparable to that on Matrigel. It is interesting to note that this result could not be reproduced in 6-well HyStem-C +Fn pre-coated plates. However, this inconsistency might be caused by the difference in hydrogel coating density/thickness between 24-well and 6-well plates. Therefore, the study showed that fibronectin can be an effective material in promoting human iPSC attachment. Biomaterial-based cell culture system should
be further developed to make coatings for cell culture surfaces more effective, safer, and less expensive.

Chen et al. investigated the culture system where all reagents including chemically defined E8 cell culture media and vitronectin-coated culture surface were non-animal and well qualifying. [26] The wild type vitronectin (vitronectin-WT) was compared with three variants truncated at the N-terminus (vitronectin-N), C-terminus (vitronectin-C) or both (vitronectin-NC) as coating materials for human ESCs and human iPSCs. It occurred that N-terminal/C-terminal truncated and N-terminal truncated vitronectin supported human ESC attachment and survival better than wild-type vitronectin, but less efficiently than Matrigel did in E8 medium. However, the addition of ROCK inhibitor or blebbistatin produced a positive effect and supported initial hESC survival and cloning as effectively as Matrigel did. As vitronectin-NC successfully maintained human ESCs attachment and growth, this substrate was tested for human iPSCs. It was shown that vitronectin-NC promoted the growth of human fibroblasts in E8 medium and improved the derivation efficiency and growth of iPSCs for more than 25 passages. Importantly, human iPSCs did not show any signs of karyotypic abnormalities and maintained the ability to differentiate into all germ lineages. Several other groups demonstrated that short fragments derived from laminin also supported adhesion, survival, self-renewal and differentiation of human iPS. Miyazaki et al. showed that recombinant E8

Table 2. Overview of advantages and disadvantages of various natural and synthetic materials.

<table>
<thead>
<tr>
<th>Type of materials</th>
<th>Materials</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthetic</td>
<td>CPC, PCL, TCP, PHB, PLLA, PVA, POEGMA, PMEDSAH, pNIPAm, PEG</td>
<td>✓ Easily fabricated with desired porosity, mechanical properties and degradation time</td>
<td>• Difficulty in homogenous cell seeding</td>
</tr>
<tr>
<td></td>
<td></td>
<td>✓ High reproducibility</td>
<td>• Lack of cell control over scaffold thickness</td>
</tr>
<tr>
<td></td>
<td></td>
<td>✓ Low immunogenicity</td>
<td>• Highly porous scaffolds can have weak mechanical properties</td>
</tr>
<tr>
<td></td>
<td></td>
<td>✓ Long shelf-life</td>
<td>• Small pore size scaffold has low cell infiltration and integration with host tissue after implantation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>✓ Easy to sterilize</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Matrigel, alginate, chitin, chitosan, gelatin, silk fibroin, collagen, fibronectin, hyaluronan, laminin, E-cadherin, vitronectin</td>
<td>✓ More relevant to cellular microenvironment</td>
<td>• May provoke inflammatory/autoimmune reactions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>✓ Better maintained cell adhesion, growth and differentiation</td>
<td>• Low mechanical properties</td>
</tr>
<tr>
<td></td>
<td></td>
<td>✓ Biodegradable</td>
<td>• Difficult to sterilize</td>
</tr>
<tr>
<td></td>
<td></td>
<td>✓ Easily available</td>
<td>• Short shelf-life</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>High cost</td>
</tr>
</tbody>
</table>

Table 3. Synthetic biomaterials for iPS cell differentiation in vitro and in vivo.

<table>
<thead>
<tr>
<th>Primary substrate</th>
<th>Composition with other polymers</th>
<th>Tested material</th>
<th>Application for tissue regeneration</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium phosphate (CPC)</td>
<td>CPC scaffold</td>
<td>Osteoblasts</td>
<td>[61]</td>
<td></td>
</tr>
<tr>
<td>Polycaprolactone (PCL)</td>
<td>Ceramic tricalciumphospate (TCP)</td>
<td>PCL-TCP-Hyaluronan scaffold</td>
<td>Osteoblasts</td>
<td>[57]</td>
</tr>
<tr>
<td>Polylactic acid (PLA)</td>
<td>Poly(b-hydroxybutyrate) (PHB)</td>
<td>PCL-PHB scaffold</td>
<td>Neurons</td>
<td>[48]</td>
</tr>
<tr>
<td>Polyglycolic acid (PGA)</td>
<td>Polyl-lactic acid (PCL)</td>
<td>PLLA-PCL scaffold</td>
<td>Neurons and Schwann-like cells</td>
<td>[49]</td>
</tr>
<tr>
<td>Polyamide polymers</td>
<td>Electrospun nanofibers</td>
<td>Endothelial cells/Blood vessels</td>
<td>[63]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Smooth muscle cells/Blood vessels</td>
<td>[64]</td>
<td></td>
</tr>
</tbody>
</table>
fragments from laminin-511 and laminin-332 (laminin-E8s) in the presence of chemically defined mTeSR1 medium promoted even greater adhesion of human iPSCs or human ESCs than Matrigel and full length laminin did. It is important to mention that usually complete dissociated human pluripotent cells underwent extensive cell death and increased risk of karyotypic abnormalities. In contrast to Matrigel, full length laminin and fibronectin, the laminin-E8s substrate supported the formation of cell-cell contacts and highly dense colonies from single pluripotent cells and had significantly higher adhesive properties. The flow cytometric analysis and RT-PCR confirmed the high expression of pluripotent markers and the low expression of differentiated markers. Therefore, the study revealed that a fully defined feeder-free culture system including short fragments laminin-E8s and defined xeno-free medium allowed the long-term culture of hiPSCs or hESCs. This approach enables to remove the burden during transition from research to clinical applications. The results of this study were recently confirmed and improved by Nakagawa et al. who developed the feeder-free system for hiPSC and hESC expansion using an E8 fragment of recombinant laminin-511. As the authors claimed, they used more efficient serum-free StemFit medium that supported hiPSC and hESC adhesion better than mTeSR1 medium did.

Duncan’s lab published the results of the study in which they grew human ESCs and iPSCs on plates coated with a fusion protein consisting of E-cadherin and the IgG Fc domain using serum-free mTeSR1 medium. E-cadherin is an essential adhesion molecule, which is also a characteristic of undifferentiated human pluripotent stem cells. First, they confirmed that human ESCs grown on hE-cad-Fc-coated culture surface and Matrigel were morphologically similar during long-term culture. Both hE-cad-Fc-coated culture dishes and Matrigel supported hESC proliferation at similar rates, and the karyotype analysis did not reveal any chromosome abnormalities and this should be considered as an advantage. There were no differences in the expression of pluripotency markers between these two groups. Interestingly, human ESC pluripotency was shown in MEF-conditioned medium and confirmed in serum-free mTeSR1 medium. As hE-cad-Fc-coated culture surface supported pluripotency of hESCs, this was also tested for human iPSC growth and expansion. Human iPSCs adhered and formed ES-like colonies. They expressed pluripotency markers during long-term culture that was also confirmed in serum-free medium. Moreover, the iPSCs formed teratoma after their implantation in mice. The investigators concluded that both human iPSCs and human ESCs could be maintained in completely defined culture conditions onto hE-cad-Fc-coated surface instead of Matrigel. However, further analysis is needed to advance the understanding of molecular mechanisms which explains the role of E-cadherin in controlling pluripotency as well as in the differentiation of iPSCs.

### 2.2. Synthetic Biomaterials

It is still impossible to define the ideal materials for iPSC. Synthetic biomaterials provide an alternative to natural ones.
These materials offer many advantages including reproducibility, the ability to control the mechanical properties and degradation rate. The mechanical properties of a scaffold can influence the iPSC differentiation. The ability to regulate the degradation rate is an important advantage that synthetic scaffolds have over natural biomaterials that may improve the results of iPSC transplantation in vivo. However, most of synthetic polymers have difficulty in cell attachment and homogeneous seeding. In 3D synthetic scaffolds, there is a lack of cell control over scaffold thickness. Other limitations include the biocompatibility and suitability for transplantation in vivo. Additionally, the material and its byproducts can trigger an immune response.

Yang et al. investigated the potential of three different scaffolds: a gelatin coated polystyrene (Gelatin-PS); a neutral hydrogel poly (N,N-dimethylacrylamide) (PDMAAm); and a negatively charged hydrogel poly(2-acrylamido-2-methyl-propane sulfonic acid sodium salt) (PNaAMPS), for their ability to maintain mouse iPSCs over a period of two-month subculture. [30] The passed iPSCs on the hydrogels and the Gelatin-PS scaffold showed a similar or increased gene expression of undifferentiated markers compared to that of input cells. They also maintained long-term self-renewal and formed teratomas in SCID mice with derivatives of all three germ layers. Although mouse iPSCs kept pluripotency on all scaffolds, mouse iPSCs on PNaAMPS hydrogel showed the highest proliferation rate in vitro and formed teratoma more efficiently than either PDMAAam hydrogel or Gelatin-PS scaffold. Additionally, mouse iPSCs subcultured on PNaAMPS hydrogel showed higher levels of SSEA-1 and alkaline phosphatase. Comparing these three scaffolds, the PNaAMPS hydrogel also demonstrated simpler operation of cell expansion than the PDMAAm hydrogel and Gelatin-PS scaffold. The authors concluded that PNaAMPS hydrogel was an excellent feeder-free scaffold because of its higher efficiency in long-term expansion of a large number of undifferentiated mouse iPSCs in vitro, simplicity of operation and low cost. However, these data were obtained in mice, and it is difficult to predict whether these results could be directly applicable to humans.

Recently, Deng et al. developed polyvinyl alcohol/hyaluronan polysaccharide nanofibers (PVA/HA) using electrostatic spinning. [31] Synthetic PVA nanofibers were modulated with ECM components such as hyaluronan and vitronectin because vitronectin supports cell attachment and growth, and hyaluronic acid significantly improves cell proliferation and migration. PVA nanofibers were made from a water-soluble synthetic polymer which is widely used due to its excellent hydrophilicity, high biocompatibility, and sound mechanical properties. Therefore, the authors hypothesized that the incorporation of these two components of ECM could support iPSC culture. The PVA decorated with HA and vitronectin nanofibers were compared with Matrigel-coated PVA/HA and the pure Matrigel-coated culture dishes. They showed that human iPSCs were not able to attach and grow on bare PVA nanofiber surface. The iPSC viability and growth were compared then on vitronectin-decorated PVA/HA surfaces and on Matrigel-coated PVA/HA nanofibers at different time points. Interestingly, there was no significant difference in cell viability between PVA nanofibers with different content of HA coated with Matrigel. Meanwhile, nanofibers prepared from PVA80/HA20 (weight ratios) were found to provide a higher viability of human iPSCs than that of PVA90/HA10 nanofibers during all periods of time in culture. Moreover, the viability of cells on vitronectin-decorated PVA80/HA20 nanofibers was comparable with PVA/HA/Matrigel nanofibers. Importantly, it was shown that the amount of decorated vitronectin increased with the increase of HA content. Therefore, the cell viability did not depend on HA contents on PVA/HA – Matrigel-coated nanofibers, but improved on vitronectin-decorated PVA nanofibers with higher HA content, that might be caused by the increase of vitronectin immobilization. The second main finding of the study is that vitronectin -decorated nanofibers supported typical iPSC-like morphology colonies. In particular, iPSC colonies consisted of many tightly packed cells and had defined colony borders. For this reason, it was concluded that vitronectin-decorated PVA80/HA20 nanofibers could replace Matrigel and support iPSC growth with a similar proliferation rate and viability efficacy. Additionally, as these results were obtained during iPSC culture in chemically defined serum-free mTeSR1 medium, they may be important in terms of developing of xeno-free conditions for human iPSC culture. This approach would help to decrease the risk of immunological reactions or prevent viral and microbial contamination. Interestingly, this study also showed that human iPSCs seeded onto PVA/HA/vitronectin nanofibers expressed a much lower level of pluripotent markers than cells onto Matrigel-coated nanofibers and culture dishes did. This finding could mean that the use of 3D PVA nanofibers in combination with a high concentration of HA and vitronectin might not be sufficient to support iPSCs in an undifferentiated state. Considering that PVA is used for bone tissue engineering, PVA/HA/vitronectin nanofibers would seem appropriate candidates for bone tissue regeneration. However, such hypothesis needs further investigation.

In another study, the same research group investigated the role of different concentrations of a short fragment of vitronectin peptide tethered to poly(oligoethylene glycol)] methacrylate polymer for iPSC culture. [32] It was found that the concentration of vitronectin significantly influenced the attachment, proliferation and the morphology of iPSC colonies. Interestingly, not only did the adhesion of iPSC colonies depend on the peptide concentrations but the morphology of iPSCs was also influenced by functional peptide density and distribution. For example, at low vitronectin concentrations (0.25–0.5 mM) only a small number of iPSC colonies with many differentiated cells attached and their morphology was highly variable. However, the higher peptide concentration (0.75, 1. and 1.25 mM) significantly increased the attachment of iPSC colonies with typical iPSC-like morphology comparable to that of Matrigel. Moreover, the analysis of short-term iPSC culture (1–2 days) revealed that iPSCs proliferated even faster on a peptide-decorated polymer surface than on Matrigel. The long-term culture of iPSC colonies also showed that the attached cells proliferated and continuously maintained iPSTypical undifferentiated morphology. Analysis of pluripotent markers (Nanog, Oct-4, SSEA-3 and SSEA-4) by RT-PCR and immunofluorescence staining confirmed their pluripotent characteristics throughout more than 10 passages. Therefore, this demonstrated that the polymer surface decorated with
even a short fragment of vitronectin peptide was sufficient to support the growth of iPSCs in an undifferentiated state in both a short-term and long-term period of culture. It is important to highlight that, despite the fact that Matrigel contains more ECM proteins and growth factors, the deposition of only one vitronectin-derived fragment was sufficient for iPSCs to successfully grow and proliferate in an undifferentiated state. In addition, these results were obtained in commercially available serum-free, chemically defined mTeSR1 medium that provides a fully defined xeno-free system for iPSC culture. In the light of these findings, it would also be interesting to assess the differentiation potential of iPSCs on vitronectin-decorated poly(OEGMA-co-HEMA) polymer surface and their ability to support a differentiated state in the peptide-decorated polymer in vivo.

Jin et al. investigated Synthemax (Corning), an acrylate polymer modified with amino-containing peptides, for human iPSC attachment and expansion in the presence of chemically defined medium mTeSR1. The Synthemax coating is a commercial product based on the vitronectin-derived Arg-Gly-Asp (RGD) motif. The polymer was compared with routinely used Matrigel. It was found that Synthemax supports the attachment and spreading of iPSC colonies which have typical iPSC-like morphology. In particular, they expressed a high level of pluripotency markers confirmed by RT-PCR analysis (OCT4) and immunofluorescent staining (OCT4, SSEA4). The analysis of cell morphology showed that cells connected by denser actin filaments on the Synthemax surface expressed more zyxin. Zyxin is significantly upregulated during cell spreading and proliferation and is inversely correlated to differentiation. This means that it may support iPSC attachment and proliferation on the Synthemax surface. The expression of another cytoskeleton protein vinculin which is recruited to the focal contacts was decreased; but, its distribution in iPSC was similar on both Synthemes and Matrigel surfaces. Synthema coating was shown to support long-term self-renewal and differentiation of iPSCs into all three germ layers as well as lineage-specific differentiation in a defined medium. In particular, the authors observed that Synthema surface supported human iPSCs in an undifferentiated state for more than ten passages. However, despite these encouraging results, Synthema is now the only commercially available synthetic stem cell culture platform, and its high cost significantly hinders access and wide use.

The cells grown on regular tissue cultureware usually require enzymatic digestion to passage them. This method has some disadvantages. The use of enzymes for cell dissociation can affect iPSC attachment. Many of these enzymes are derived from animals, and this carries risk for virus and disease transmission that does not meet the xeno-free standards of Good Manufacturing Practice (GMP). In addition, enzymes degrade synthesized ECM and cell-cell contacts, which may decrease cell survival and promote apoptosis. In this regard, thermoresponsive polymers could be an alternative and attractive approach for passaging iPSCs. Thermoresponsive polymers are synthetic, biocompatible and enable cell harvest during a mild process for cells by simply changing temperature in the range of 4 °C to 37 °C. The thermoresponsive hydrogels are mostly synthesized from poly (N-isopropylacrylamide) (pNIPAM). pNIPAM undergoes a transition above and below the lower critical solution temperature (LCST) at about 32 °C. Above the LCST (i.e., at the cell culture temperature of 37 °C), the pNIPAM-treated surfaces are more hydrophobic, so the cells adhere and proliferate. Below the LCST, the pNIPAM-treated surfaces rapidly hydrate, and the cells spontaneously detach from pNIPAM-coated surface. Therefore, by regulating only the temperature, intact single cells as well as whole cell sheets can be rapidly recovered from pNIPAM coated dishes (Figure 2). Recently, Lei et al. presented a culture system based on the thermoresponsive poly(N-isopropylacrylamide)-co-poly(ethylene glycol) (pNIPAM-PEG) hydrogel for long-term expansion and differentiation of human ESCs and iPSCs derived from mesenchymal stem cells (human iPSCs) and dermal fibroblasts (human iPS-Fib).[39] The authors compared 2D and 3D systems with respect to cell attachment and expansion and found that 3D-thermoresponsive scaffold showed higher cell expansion. They also assessed the influence of different substrates on human iPSC-MSC growth, and showed that none of the non-thermoresponsive materials (alginate, agarose and hyaluronic acid) could achieve the levels of expansion observed with pNIPAAm-PEG hydrogel. In addition, it was shown that the use of xeno-free E8 medium with 4day treatment of ROCK inhibitor significantly improved cell growth. The long-term expansion revealed some differences in growth rates among hiPSC-MSC (10-fold over 4 d), hiPSC-Fib (20-fold over 5 d) and hESCs (sevenfold over 4 d). However, after long-term culture, 95% of all cells expressed pluripotency markers Nanog and Oct4 and maintained their pluripotency for up to 60 passages. Each line could undergo embryoid body differentiation in vitro and formed teratomas with three germ layers in vivo. Considering the value of human pluripotent stem cells for biomedical applications, the cells from 3D hydrogel were also able to differentiate into neural lineage, contracting spheroids and endoderm in respective induction media. Therefore, pNIPAM-PEG thermoresponsive hydrogel enabled the long-term growth, proliferation and self-renewal of undifferentiated human iPSC-MSCs, human iPSC-Fib, and human ESC in 3D conditions. The analysis of growth kinetics of pluripotent cells did not reveal any differences in the expansion rate, pluripotency marker expression, or karyotype between the first and tenth passages. However, there was some difference in growth rates among the different human pluripotent cell lines. A synthetic polymer poly[2-(methacryloyloxy)-ethyl dimethyl (3-sulfopropyl) ammonium hydroxide] (PMEDSAH) was previously used to coat dishes for long culture and self-renewal of human embryonic stem cells (ESCs).[40,41] Villa-Diaz et al. also showed that iPSCs can attach and continuously proliferate on PMEDSAH in an undifferentiated state and yet will be capable of generating functional MSCs in vitro and bone formation in vivo. The iPSC-derived MSCs were positive for mesenchymal cell surface markers (CD166+, CD105+, CD73+, CD44+) and showed low expression of pluripotency markers (Oct4, Nanog, Sox-2). Among other advantages, the polymer is fully defined and can be reproducibly fabricated and subjected to long-term storage and sterilization. Importantly, the karyotype analysis did not reveal any genetic abnormalities in iPSC-MSCs.
PROGRESS REPORT

3. iPSC–Biomaterial Interactions for In Vitro and In Vivo Tissue Engineering

3.1. Neural Engineering

The increased interest in the use of human iPSCs for clinical application is mainly explained by their ability to differentiate into any cell type of the three embryonic germ layers. Biomaterials have been shown to possess a potential to make a significant impact on MSC attachment and differentiation towards neuronal lineage.43,44 However, application of biomaterials for iPSC differentiation towards neuron-like cells has not yet been thoroughly investigated. Khayyatan et al. presented a study in which they analyzed the effect of physical, chemical and mechanical properties of 3D collagen scaffolds on growth and neuronal differentiation of human iPSC-derived neural progenitors (hiPSC-NPs).45 In particular, scaffolds were prepared from different collagen concentrations and at freezing temperatures to compare iPSC-NP behavior depending on pore size, porosity and mechanical properties. The viability of cells was increased with time on all types of scaffolds. The results revealed that while the lower freezing temperature caused the decrease of pore size of scaffolds, this did not significantly affect their porosity. It might be thought that smaller pore size could hamper cell migration inside the scaffolds, in particular, scaffolds fabricated at −196 °C had oriented pores, and scaffolds made at −20 °C had circular pores. This conclusion may have a value for tissue engineering, as analysis of iPSC-NPs differentiation confirmed that the cells cultured on the scaffolds made at −80 °C and −196 °C have the parallel arrangement similar to that of neuronal processes. Previously, the same research group showed that hESC-derived neural tubes cultured in collagen scaffolds organized typical neurite processes that were oriented toward each other and formed synapses.46 In the current study, hiPSC-NPs on the surfaces of the collagen scaffolds (−80 °C, 1%) expressed neuron-specific class III beta-tubulin marker after four weeks of differentiation. However, neuronal differentiation of hiPSC-NPs was observed only for scaffolds fabricated from 1% collagen at −80 °C. To prove the effect of pore orientation on neuronal differentiation, further analysis with different pore orientations may give more detailed information. Another important result of the study showed that the coating of collagen scaffolds with laminin can significantly improve hiPSC-NPs attachment, proliferation and infiltration. Considering all these results, it would also be interesting to know how collagen scaffolds fabricated from different collagen concentrations and low freezing temperatures with and without laminin coating can support long-term expansion and hiPSC-NP differentiation into neuronal-like cells.

It was previously shown that poly(β-hydroxybutyrate) (PHB) could support attachment and proliferation of adult Schwann cells.47 The heparinized binary scaffolds composed of polycaprolactone (PCL) and PHB accelerated neuronal differentiation and reduced the number of phenotypic iPSCs after cultivation.48 PCL scaffolds were also used to evaluate the ability of 3D conduits to support the growth of mouse iPSC-derived

Figure 2. Principle of 2D- and 3D-thermoresponsive constructs for iPS cell sheet-based tissue engineering.
neurospheres. In particular, the outer mesh of the constructs was composed of synthetic polymer poly(γ-L-lactide) (PLLA) multifilament fibers, while the inner layer was composed of a PLLA and PCL porous sponge in equal ratios. The tubular scaffold was elastic and flexible. Importantly, the inner layer had a honeycomb structure with a pore size suitable for cells. iPSC-derived neurospheres showed good attachment to the inner layer of PLLA/PCL scaffolds and, moreover, the ability to migrate into the inner porous sponge. The immunofluorescence staining and histological analysis confirmed the expression of markers of primitive neurons (anti-βIII tubulin), Schwann cell-like (anti-S-100) and glial (anti-glial fibrillary acidic protein, GFAP) cells. The benefit of the study was that the use of conduits consisted of two different structures allowed fabrication of the constructs that were strong enough to maintain the tubular structure, but also flexible enough to handle without difficulties during the operation. However, these promising results should be followed up with studies to prove the safety, efficacy in terms of the long-term effect of neuron regeneration after implantation and suitability for human iPSC-derived nerve conduits in vitro and in vivo. It is also important to exclude the risk of teratoma formation. Different combinations of natural hydrogels are used for neuronal differentiation of iPSCs. Kuo et al. revealed that alginate-chitosan-gelatin scaffolds with NGF improve the adhesion and viability of iPSCs as well as material-driven differentiation of iPSCs into neuron-like cells. Alginate is widely used in biomaterial and tissue engineering applications due to its biocompatibility, abundance, low cost and mild crosslinking mechanism. Moreover, a highly attractive quality of alginate is its ability to rapidly form hydrogels. Although chitosan does not support cell adhesion on its own, its cationic chains can complex with negatively charged glycosaminoglycans such as heparin. In doing so, chitosan imparts the ability to bind heparin-binding biomolecules such as NGF. Another hydrogel composed of alginate and poly γ-glutamic acid with surface NGF showed efficient neuronal differentiation of iPSCs. The effect was tested by morphological analysis which confirmed neuron-like morphology of differentiated iPSCs. Moreover, neuronal-lineage differentiation was induced and supported with the surface NGF. Immunohistological staining showed the increase in intensity of neurological marker beta III tubulin and the decrease in intensity of pluripotency marker SSEA-1 that indicates the differentiation of iPSCs toward neurons. Another advantage of this construct is that the alginate/poly γ-glutamic acid hydrogel was rapidly degraded because of its high hydrophilicity. Gelatin can be used to support iPSC differentiation towards neuron-like cells. Kuo et al. also presented scaffold comprising chitin, chitosan, and gelatin and compared the two different geometries of a scaffold. Scaffolds with pore geometry of inverted colloidal crystals (ICC) and freeform constructs influenced the attachment and differentiation of iPSCs in different ways. Although the adhesion of iPSCs was found to be more efficient on freeform constructs, the viability of iPSCs was higher on the ICC constructs. The results also demonstrated that the differentiation of iPSCs toward neurons in the ICC construct was faster than that in the freeform construct. Moreover, phenotypic characteristics of iPSCs were better preserved in the freeform scaffolds and the quantity of neuronal marker was higher in the ICC construct. This indicates that iPSCs cultured on complex chitin-chitosan-gelatin ICC scaffolds can be an effective tool for neuronal regeneration.

3.2. Cartilage and Bone Tissue Engineering

As we previously discussed iPS cells with acquired rejuvenated state can be generated from somatic cells of elderly diseased patients with diabetes. Wei et al. successfully reprogrammed human osteoarthritic chondrocytes (OCS) into iPSCs and then stimulated chondrocyte differentiation onto alginate matrix. OC-derived iPSCs were transduced with TGF-β and co-cultured with chondrocytes in an alginate matrix. It was previously shown that human ESCs in co-culture with human adult chondrocytes significantly improved their chondrogenic differentiation potential. The biomaterial in conjunction with chondrocytes create native cartilage microenvironment and might promote further cartilage differentiation because of, first, secreted autocrine and paracrine factors and, second, direct physical cell–cell contact. This was shown to be true as the expression of the cartilage marker genes for collagen II, aggrecan and cartilage oligomeric matrix protein (COMP) was significantly higher in the combined group of iPSCs/TGF-β1/alginate/chondrocytes than in the respective control cultures including iPSCs/alginate, iPSCs/alginate/chondrocytes and iPSCs/TGF-β1/alginate. However, the gene expression was still lower than that in the positive control group of chondrocytes. Interestingly, the expression of the VEGF gene which is up-regulated during cartilage degeneration was decreased in the combined group. In vivo experiments confirmed that more new cartilage was formed in the co-culture of TGF-β1-transfected iPSCs with chondrocytes in alginate matrix.

Another interesting approach involves MSC derivation from iPSCs. Kuhn et al. demonstrated the derivation of MSC-like cells from human iPSCs and ESCs onto fibrillar collagen substrate that can potentially play a supporting role and promote MSC generation. This study showed that both human iPSCs and ESCs singly plated on fibrillar collagen matrix acquired mesodermal characteristics including fibroblast-like spindle-shaped morphology, high expression of MSC surface markers, and a lack or low expression of hematopoietic markers. The cells were singly dissociated before plating to increase the differentiation yield. Meanwhile, the same cell lines seeded on tissue culture treated polystyrene in the absence of collagen did not generate MSC-like cells. The tri-lineage osteogenic, chondrogenic, and adipogenic differentiation capacity was confirmed by gene expression analysis and histological examination. This technique, based on the fibrillar collagen coating, could be applied for the cartilage and bone regeneration. However, additional in vivo studies are needed to fully confirm the efficacy and safety of this approach.

Bone and cartilage tissue transplantation and reconstructive surgery is now an important issue in clinical medicine due to the high prevalence of musculoskeletal disorders in the human population. Clinically demanded bone tissue engineering could be achieved by iPSC differentiation into osteoblasts and improved with biomaterials. This study demonstrated the ability of mouse iPSCs seeded in gelatin sponge to differentiate into functional osteoblasts in vitro and in vivo.
Because of their similarity to bone tissue, calcium phosphate cements (CPCs) are biocompatible, resorbable, osteoconductive and injectable. Several CPCs have been approved for clinical use, such as cranioplasty, bone augmentation, implant fixation and fracture. Recently, Tang et al. showed the potential of human iPSC-derived MSCs seeded on calcium phosphate cement (CPC) scaffold for bone engineering. The cells displayed good viability after attachment on CPC scaffold. Importantly, iPSC-MSCs seeded on CPC scaffold exhibited typical MSC characteristics. This was demonstrated by the analysis of immunophenotype and the ability to differentiate into adipocytes, chondrocytes and osteoblasts. In addition, iPSC-derived MSCs differentiate toward osteogenic lineage as confirmed by high gene expression of osteogenic markers and the mineral synthesis in osteogenic medium when compared with the control medium. The study showed that the CPC construct could successfully support mineral synthesis because of good attachment, proliferation and differentiation of the MSCs. Liu et al. have reported the effect of human iPSC-MSCs modified with bone morphogenetic protein 2 (BMP2) gene and seeded onto CPC scaffold biofunctionalized with RGD for bone regeneration. The BMP2-modified iPSC-MSCs are considered to improve bone regeneration as BMP-2 is able to stimulate osteogenesis. The study showed that BMP-2-modified iPSC-MSCs successfully attached and underwent osteogenic differentiation. Although there was no significant difference in the viability, cell growth, growth kinetics and percentages of cells on the different cycle stages between BMP2-iPS-MSCs and control iPS-MSCs, BMP2-modified iPSC-MSCs were more efficient in osteogenic differentiation and in the production of mineral components on RGD- CPC scaffolds. These results were confirmed by higher expression of collagen type 1alpha 1, osteocalcin and quantification of mineralization in the BMP2-iPSC-MSC group. Therefore, the results revealed that the CPC scaffold is suitable for human iPSC delivery for bone regeneration because of its excellent osteoconductivity and bone-replacement capability. However, poor mechanical properties are still the main disadvantage of CPC that significantly limit its clinical application. The increase of mechanical properties leads to the decrease of the pore size of CPC scaffold that impedes the fast bone ingrowth and the CPC bioresorption layer by layer. This feature is still the main drawback of the CPC usage in tissue engineering.

3.3. Smooth Muscle, Cardiac and Vascular Tissue Engineering

A similar approach as was used to engineer iPSC-derived neurospheres was applied to engineer vascular grafts from iPSC-derived vascular cells. The scaffold was fabricated from flat non-woven porous polyglycolic acid (PGA) mesh and a copolymer sealant solution of ε-caprolactone and 1-lactide which created a sealed tube with a porous structure. The differentiated cells expressed endothelial markers (VEGF, PECAM, and E-cadherin) and decreased the expression of pluripotency marker SSEA-1. The cell sheet was harvested using the thermoresponsive polymer and then wrapped around the scaffold. This technique significantly improved the cell attachment to the scaffold. After implantation, the endothelialization was
confirmed by histological analysis. Importantly, there was no indication of thrombosis, aneurysm formation, graft lesion, or calcification. Moreover, no iPSCs were found in the implants at 10 weeks. Since one case of teratoma formation was detected at 10 weeks, it is important to assess the safety issues of vascular grafts in the design of future studies. It is also needed to evaluate the maturity of vascular constructs in terms of mechanical (burst strength, compliance, elastic recoil) and biological (vasoactivity, selective permeability) functions.

Sundaram et al. recently developed a new system for vascular graft engineering. Human iPSCs were differentiated into mesenchymal progenitor cells and cultured on PGA mesh over a silicon tube in a pulsatile bioreactor for 8 weeks. Under these conditions, hiPSC-derived MSCs acquired early smooth muscle cell markers including \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA), SM22\( \alpha \) and calponin, but were negative for the mature marker smooth muscle myosin heavy chain. Importantly, the vessel walls were negative for osteochondrogenic markers collagen 2 and aggrecan. The results of positive expression for both \( \alpha \)-SMA and calponin were observed by immunostaining and confirmed by Western blotting. These data may claim the differentiation of hiPSC-derived MSCs towards SMCs in engineered vascular graft. Additionally, the graft walls were tested for extracellular matrix deposition that confirmed the presence of collagen I, collagen II and fibronectin. However, they were negative for elastin. Mechanical tests confirmed mechanical strength properties that were half those of native veins. It should be noted that the main problem for successful clinical application of vascular grafts is related to incomplete endothelialization and thrombosis. Therefore, further optimization of vascular grafts requires the development of a functional endothelial layer.

Xie et al. investigated the growth of iPSC-derived smooth muscle cells (SMC). Synthetic polymer poly (L-lactide) (PLLA) is widely used in tissue engineering because it is a type of nontoxic and biodegradable material approved in many studies with nerve cells. Mouse iPSCs were seeded on 3D macro-porous nanofibrous (NF) PLLA scaffolds. To increase iPSC differentiation into mesodermal lineage, the iPSCs were cultured in the presence of retinoid acid (RA). It was shown that PLLA scaffold was able to support iPSC differentiation into smooth muscle cells. It was presumed that 3D PLLA scaffolds could create the niche-like microenvironment with architecture that was similar to the spatial structure of ECM in smooth muscles. The 3D-nanofibrous scaffolds were able to support iPSC-derived SMC phenotype and growth which was demonstrated by high expression of SMC markers (MyoCD, smoothelin, SM22\( \alpha \), and SM-MHC) and low level of pluripotency markers. Immunological analysis confirmed this data and showed highly positive \( \alpha \)-SMA expression. Considering these results, it was concluded that 3D-nanofibrous PLLA scaffolds could be an efficient substrate for the maintenance of iPSC-derived SMCs. The study showed that implanted cell-seeded scaffolds in the presence of RA expressed high level of SMC marker MyoCD and SM22\( \alpha \). However, the protocol needs to be amended to minimize residual pluripotent cells in 3D constructs to avoid the risk of tumor formation. Another concern may be addressed to dedifferentiation or the differentiation of the transplanted iPSC-derived SMCs into other lineages which can be promoted and supported by the local tissue environment after implantation.

In addition to these points, the protocol should be validated for its application to human iPSCs. In another approach, this study could be improved by the investigation of the role of growth factors which could enhance and maintain iPSC differentiation toward SMCs in SMC growth.

The gelatin-coated plates have been used efficiently to induce cardiac cell differentiation of mouse iPSCs. The process of differentiation was confirmed by the gene expression of specific cardiac markers. Moreover, iPSC-derived cardiomyocytes demonstrated typical myofibrillar organization. Immunocytochemistry confirmed the expression of sarcomeric \( \alpha \)-actinin and Tnni proteins. Importantly, the generated cardiomyocytes have functional L-type Ca2+ channels, which are required for cardiac contraction, as well as K+ and Na+ channels. Moreover, to assess the efficacy of cardiac differentiation, contracting areas in embryoid bodies were compared between mouse iPSCs and mouse ESCs. The results revealed a higher amount of contracting EBs from iPSC (44.8%) than from ESCs (33.3%) at the late passages. In addition, cardiovascular gene expression was higher in iPSCs compared to ESCs. Therefore, the authors concluded that iPSCs on gelatin-coated plates can differentiate into contracting and functional cardiomyocytes with efficiency even higher than that of ESCs.

### 3.4. Liver and Pancreatic Tissue Engineering

Iwamuro et al. used gelatin-coated plates for the differentiation of iPSCs towards hepatocytes. First, embryonic bodies were induced from mouse iPSCs and then transferred onto a gelatin surface. To support differentiation into endoderm lineage, the cells were cultured initially in the presence of activin A and basic fibroblast growth factor (bFGF), and then treated with hepatocyte growth factor (HGF) to generate hepatocytes. The results from functional analysis revealed increased albumin production and ammonia removal by the iPSC-derived hepatocyte-like cells (iPSC-Heps). Moreover, the intensified expression levels of the hepatocyte gene (carbamylphosphate synthetase, CPS, phosphoenolpyruvate carboxykinase 1, PCK 1, and transferrin, Trf) on gelatin-based hydrogel confirmed the hepatic differentiation of iPSCs. However, the differentiation toward a mature hepatocyte was not uniform as the expression of \( \alpha \)-Fetoprotein (AFP), which is present in endoderm but is not expressed in mature hepatic cells, was also detected. This study used 2D-gelatin coated dishes to generate hepatocytes. Yamazoe et al. compared the effect of electrospun 3D synthetic nanofibers (sNF) made from polyamide polymers with traditional 2D culture dishes coated with collagen, fibronectin, gelatin and Matrigel. First, they confirmed that mouse ESCs seeded on sNF matrix were able to differentiate into the hepatocyte-like cells. In particular, they found the decrease in the expression of pluripotent marker Oct3/4 and the increase in the expression of mesodermal marker Gsc, endoderm markers Sox17 and Foxa2, and hepatic markers alpha-fetoprotein and albumin. The presence of albumin and fetoprotein proteins was also confirmed by immunocytochemical analysis. The functional assays confirmed glycogen synthesis in the cytoplasm of differentiated cells. Importantly, these results were obtained not only in mouse ESCs but also confirmed in human ESCs and human
iPSCs. Considering the effect of 3D-scaling, the cells cultured on sNF matrix showed higher levels of hepatocyte-specific proteins such as serine peptidase inhibitor a1 (Serpina1), albumin 1 (Alb 1), and transthyretin (TTR). The authors presumed that this system could be used and further developed for liver tissue engineering.

Progress in the engineering of different tissues that are generated from pluripotent stem cells is quite variable. There has been more success in the derivation of neuronal cells, chondrocytes and osteoblasts than in any other tissues. The derivation of human insulin-producing cells in cell culture from induced pluripotent stem cells remains a big challenge. There are few successful studies devoted to the differentiation of human iPSCs and ESCs into insulin-like cells. Such studies mainly focused on the development of protocols using different transfection and differentiation agents in vitro. However, one more problem which impeded the development of pancreatic tissue engineering is the low success of islet transplantation due to host immune response. This should also be resolved before their pre-clinical tests. This obstacle can be overcome by the development of a biomaterial-based encapsulation system. Alginate encapsulation of human ESC-derived islet like cells was tested by Richardson et al. They showed that a chemically inert, biocompatible and non-degradable alginate can be used successfully for human ESCs differentiation. Being encapsulated, human ESCs differentiated more efficiently than the same cells did on traditional 2D culture dishes. The mature state was confirmed by up-regulation of insulin gene expression and the presence of c-peptide confirmed by immunostaining analysis. Further study should provide information regarding the feasibility and efficiency of this alginate-based system for human iPSC-derived pancreatic cells generation and differentiation.

3.5. Epithelial Tissue Engineering

Despite the high biocompatibility of extracellular matrix proteins such as the above mentioned alginate, collagen, or fibronectin, these nevertheless possess low mechanical properties and must be used in combination with scaffolds. However, the attempts to use conventional matrices produced from synthetic polymers or acellular and non-degradable alginate did not show long-term effects that were stable or efficient. Silk fibroin, another natural polymer, has been clinically approved and used for sutures. The range of its properties including biocompatibility, slow degradability and strong mechanical qualities makes it attractive for use in tissue engineering. It was shown that silk scaffolds coated with collagen type I or V1 or fibronectin successfully support iPSC attachment and differentiation toward both urothelial and smooth muscle cells. The silk fibroin aqueous solutions were prepared from Bombyx mori silkworm cocoons. The silk scaffolds were produced by a gel spinning process and compared between smooth, compact multi-laminates and rough, porous lamellae-like sheets. To create a natural tissue-like microenvironment, the scaffolds were coated by ECM proteins. Collagen I surrounds smooth muscle bundles and nerves; collagen IV is included in the basement membrane and fibronectin encompasses smooth muscle fibers and vessel walls. The best results were obtained on porous silk scaffolds consisting of lamellar-like sheets and coated with fibronectin. It is important to mention that porous silk scaffolds showed better attachment than compact scaffolds did, and the results were significantly improved after coating with fibronectin. However, the ECM coating did not have an improved effect on the cell attachment to compact silk scaffolds. Therefore, the combination of silk porous scaffold and coating with fibronectin can significantly increase iPSC cell attachment and differentiation towards both urothelial and smooth muscle cells. Thus, the current studies provided valuable knowledge of different synthetic and natural substrates and their ability to support iPSC differentiation in vitro and after implantation in vivo. This valuable information is important for a deeper understanding of how chemical substrates and mechanical properties provoke and support iPSC differentiation. Yet another recent study reported that spider silk can be used successfully for long-term human iPSC and ESC expansion. As mentioned above, silk is an attractive biomaterial for tissue engineering because of its excellent mechanical properties and biocompatibility. Moreover, the recombinant small spider silk protein 4RepCT can be easily produced in Escherichia coli. In the current study, different substrates were prepared from genetically fused 4RepCT and short fragments of binding peptides. Moreover, these biomaterials were used to prepare two forms: 2D films and 3D foams and fibers. It was found that only Matrigel and PQVTRGDVFTM (from vitronectin)-silk-based substrate supported a human ESC colony formation. Further analysis revealed that different human ESC and iPSC lines formed colonies on the silk-based substrate. This also supported pluripotency marker expression (Oct 4 and Nanog) at high levels in vitro and form teratomas in vivo. It is important to note that pluripotent cells differentiated towards neuroectoderm on 2D films as well as in 3D mesh and foam. However, to better assess the potential of spider silk for tissue engineering, further in vivo studies with 2D surface and 3D scaffolds are required.

4. Controlling iPSC Self-Renewal and Differentiation: The Role of Signaling Molecules and Engineering Microenvironment

As discussed above, iPSC and ESC attachment, expansion and differentiation requires extracellular matrix proteins such as laminin, collagen and fibronectin. These ligands contact with specific cell-surface receptors including integrins. Integrins are heterodimeric surface glycoproteins that comprise different combinations of α subunits and β subunits. Although recent studies have obtained information regarding integrins that characterize human ESCs and human iPSCs, there remains some controversy because of the difference in inter-line variation and culture methods. Despite this, more information of the specific integrin pattern for pluripotent and differentiated states is important to understand the mechanisms of iPSC attachment, growth and differentiation. Integrins are related to specific molecular pathways such as P13K-Akt and Mek/Erk which play an important role in human ESC survival. For example, the P13K-Akt pathway is involved in human ESC
survival after their dissociation. Furthermore, PI3K-Akt activity maintains self-renewal in pluripotent stem cells via suppression of the Raf/Mek/Erk and canonical Wnt signaling pathways.\textsuperscript{[74]} The comparison of different types of laminin isoforms showed that laminin 511 can maintain self-renewal of iPSCs with higher efficiency than Matrigel.\textsuperscript{[75]} In contrast, the contact area of cells on laminin-111 was smaller than that on Matrigel, and the adhesion of iPSCs to laminin-411 was even lower than that with the cells plated on poly-D-lysine. The study revealed that laminin-511 could be more important for survival and self-renewal of iPSCs in vitro than other isoforms of laminin such as laminin-111 and laminin 411. In its turn, another study showed that iPSCs can be differentiated and supported toward hepatocyte-like and cholangiocyte-like cells on human laminin 111.\textsuperscript{[76]} Considering the mechanisms of iPSC attachment to different substrates, the analysis of integrin expression showed that undifferentiated human iPSCs express α5, α6, αv, β1, and β5 integrin subunits,\textsuperscript{[75]} and α6- and β1- integrins were the most important for the attachment of undifferentiated iPSCs to human laminin-511. Meanwhile, human iPSC-derived hepatoblast-like cells also indicated high levels of α6- and β1- integrins as well as α1-integrin expression which could be responsible for the attachment to human laminin-111.\textsuperscript{[76]} The role of α6β1 integrin in human ESC attachment was also confirmed in the previously discussed study. Using blocking antibody against a cell membrane protein, it was possible to completely prevent human iPSC and ESC adhesion to laminin E8 fragments.\textsuperscript{[32]} As the Corning Synthemax surface\textsuperscript{[33]} contained the RGD motif, it was found that the blocking of αvβ3 integrin significantly inhibited the attachment of human iPSCs. The same integrin was required for human iPSC attachment to vitronectin.\textsuperscript{[77]} The major subunit β1/integrin regulates pluripotent cell attachment to collagen and gelatin (degraded collagen). Suh et al. showed the role of α6β1 integrin in collagen I-mediated self-renewal of mouse iPS C,\textsuperscript{[78]} while Li et al. confirmed α6β1 integrin participation in mouse ESC migration via collagen IV.\textsuperscript{[79]} It was previously shown that neuronal differentiation of pluripotent stem cells can be controlled by the interaction between laminin and α6β1-integrin, vitronectin and αβ1, αβ3, αβ5-integrins.\textsuperscript{[80]} αβ1 and α6β1 integrins can efficiently support early mesodermal differentiation of human ESCs.\textsuperscript{[81]} At the same time, fibronectin/α5β1 and vitronectin/αvβ5 contacts are important for endodermal differentiation (Figure 3).\textsuperscript{[82]} Substrate elasticity has a potent influence on the cell fate. Several studies have shown how the substrate mechanical properties could regulate iPSC self-renewal and expansion. Fujita et al. revealed that, after reprogramming of mouse embryonic fibroblasts, the cells expressed a higher level of pluripotency genes Nanog and Oct3/4 on soft polyacrylamide gels coated with gelatin than rigid plastic plates did.\textsuperscript{[83]} The same results were obtained for adult human dermal fibroblasts. The authors suggest that regulating the substrate stiffness can enhance the efficiency of iPSC generation. Previously, Tanaka et al. also showed that soft substrate supports self-renewal and pluripotency of mouse ESCs.\textsuperscript{[84]} By contrast, Kiessling argued that stiff substrates are more appropriate for effective long-term expansion and self-renewal of iPSCs.\textsuperscript{[85]} The substrate mechanical properties could regulate specific pathways linked to pluripotency. The authors suggested that the stiff substrate promotes transcriptional activators YAP (Yes-associated protein) and TAZ (transcriptional coactivator with PDZ-binding motif), which in turn upregulate pluripotency genes. Interesting results were reported from the studies in which the influence of matrix stiffness on the development of tissue-like structures was reported. It is well known that cardiomyocytes (CMs) do not expand and lose their sarcomere integrity on traditional cell culture plastic.

![Figure 3. Integrin-mediated interaction between pluripotent stem cells and culture surface.](www.MaterialsViews.com)
This substrate does not display cell-binding groups, and they have mechanical properties that are different from those of natural tissues. In this regard, the use of materials adapted to the stiffness of cardiac tissue could facilitate the culture of iPSC-derived CMs. It was shown in another study that hydrogels from polyacrylamide allow improvement of the long-term culture of iPSC-derived CMs. The best cell attachment was found on the 2.5-dioxopyrrolidin-1-yl-6-acrylamidohexanoyl crosslinking compound which showed monolayers of synchronized beating CMs. In contrast, sulfosuccinimidyl-6-(4-azido-2-nitrophenylamino) hexanoate (Sulfo-SANPAH) and acrylic-acid-N-hydroxysuccinimide-ester (NHS-acrylate) hydrogels did not support cell attachment. The role of a crosslinker is to allow the covalently binding of different ECM proteins. Therefore, the flexibility of polyacrylamide and the ability to bind adhesion proteins allows the control of the stiffness of the substrate. It was possible to mimic the stiffness of the native CM niche that consequently promoted cell attachment and contractility. Another interesting study investigated the role of flexible 3D-environment for long-term iPSC culture. The pluripotent cells were cultured in the natural plant-derived nanofibrillar cellulose (NFC) hydrogel. The pluripotency of iPSCs was proven by the expression of main pluripotency genes, in vitro flexible 3D-environment for long-term iPSC culture. The pluripotency of iPSCs was proven by the expression of main pluripotency genes, in vitro embryonic body formation as well as in vivo teratoma formation.

The topographical effect of cell culture surface could also contribute significantly to the generation and growth of iPSCs. 3-D topography of niches regulates stem cell self-renewal and differentiation in vivo. Stem cell signaling and fate might be more tightly controlled by mimicking the mechanical forces and physicochemical cues of stem cell niches in combination with soluble and surface-bound cytokines and proteins of extracellular matrix. Li et al. showed that iPSC generation can be enhanced if mouse or human reprogrammed fibroblasts are seeded onto poly(dimethylsiloxane) (PDMS) polymer substrates with parallel microgrooves or onto poly l-lactic acid or poly(l-lactide-co-caprolactone) nanofiber scaffolds with aligned fiber orientation. The researchers presumed that these substrates change the cell morphology that altered specific epigenetic status, such as histone acetylation and methylation, and promoted a mesenchymal-to-epithelial transition in somatic cells. However, the way in which mechanical signals act during reprogramming is not fully understood. The results of the study indicated that regulators of histone methylation such as WD repeat domain 5, a subunit of H3 methyltransferase, and HDAC2, histone deacetylase 2, might be a key mediator of mechanotransduction. In addition, the cytoskeleton might be involved in the reprogramming. The morphological changes significantly correlated with an elongation of the nucleus and could induce epigenetic modifications. The study was conducted on primary fibroblasts, but it is still unclear whether substrate-dependent cell reprogramming is a general phenomenon and how surface properties affect the reprogramming from other types of cells. Having considered that mechanical properties and the pattern of culture surfaces may influence the derivation of iPSCs, graphene with its honeycomb structure and unique physical, chemical and mechanical properties have been actively used in different areas of biotechnology. This material possesses good biocompatibility at low concentrations, great mechanical strength, and the capability to create graphene-based 3D porous macrostructures for tissue engineering. Substrates coated with graphene (G) and graphene oxide (GO) have facilitated the culture of several mammalian cells including NIH-3T3 fibroblast and A549 cell line. In addition, G-coated substrates have recently been shown to accelerate the osteogenic differentiation of human MSCs. Chen et al. showed that graphene and graphene oxide are biocompatible with murine iPSCs and enable cell adhesion and proliferation, and GO enables more favorable iPSC adherence and proliferation than G. Interestingly, G and GO supported iPS colonies in an undifferentiated state, but the pattern of Oct-4 and Nanog expression was different. Their expression declined on the control glass and GO at day 9, but remained high on G. Comparative analysis between groups revealed that the highest level of pluripotency gene expression was on the G surface. Moreover, G substrate failed to support the expression of differentiation markers in comparison with GO or glass surface. RT-PCR analysis revealed sharp up-regulation of endodermal specific markers GATA4 and Ihh on all substrates, in particular, GO surface. However, the expression of ectoderm marker Fgf 5 was found only in the beginning of differentiation and Nestin was up-regulated at day 9, there was no significant difference in their level of expression among glass, G and GO surfaces. The same pattern of expression was found for mesodermal markers. The mesodermal marker gene expression was higher at day 5 and then decreased, while Bmp 4 was increased until day 9. However, iPSCs grown on all three substrates differentiated towards mesodermal lineage with equal efficiency. These results allowed the conclusion that G surface may be more appropriate for the maintenance of iPSC pluripotency, while GO substrate may be used for endodermal differentiation. Despite the thorough analysis of iPSC attachment, proliferation, the pluripotency maintenance and differentiation into three lineages, the mechanism of graphene-associated self-renewal and differentiation of iPSCs remains unknown. In support of this study, Yoo et al. recently showed that graphene substrates can improve the generation of mouse iPSCs by inducing epigenetic changes associated with reprogramming. The advantage of this study is that the authors conducted thorough analysis of pluripotency state of iPSCs colonies between control glass and graphene-based substrates. The analysis of iPSC colony morphology and undifferentiated state after staining for alkaline phosphatase revealed a significantly greater number of iPSC colonies on graphene substrates than on the control. The quantitative assessment using FACS analysis for Oct4-GFP-positive iPSCs and quantitative RT-PCR for Oct4, Nanog, Sox2 and Esrrb showed that the number of Oct4-positive cells and the level of pluripotency gene expression were also significantly higher on graphene-coated substrate. These results were confirmed by immunostaining of pluripotency markers such as Oct4 and Sox2. Moreover, the authors investigated not only the potential of iPSC colonies to maintain their pluripotent properties, but also confirmed the capability of cells to differentiate into three germ layers on graphene-based substrate using immunostaining, RT-PCR and teratoma formation assay. According to the study, graphene did not affect epithelial-to-mesenchymal transition which plays a critical role in tumor progression and metastasis diseases. However, there is some evidence that the induction of mesenchymal-to-epithelial transition during reprogramming is related to epigenetic
modifications and metastatic process. Additional experiments are necessary to test the safety of graphene for cell culture in terms of cytotoxicity and genotoxicity because of the prolonged cell culture on graphene substrate in combination with high expression of pluripotency genes. It was shown that graphene nanoplatelets have genotoxic effects on human MSCs through DNA fragmentation and chromosomal aberrations, even at low concentration of 0, 1 µg/mL. Considering the high probability of genetic abnormalities, karyotype analysis of iPSCs grown on graphene should be performed to assess the risk of genetic instability and tumorigenesis.

Thinking of the dimensional structure of material, Vallier compared 2D and 3D matrices for iPSC growth and differentiation towards hepatocytes. It turns out that 3D collagen matrices significantly increase the differentiation of iPS-derived hepatocytes compared to that of 2D substrate. In a more recent study, Cui et al. found similar results for human ESCs. In particular, the authors successfully differentiated human ESCs towards a definitive endoderm in a monolayer culture and then transferred them into a 3D Algimatrix scaffold. The study confirmed that 3D structures significantly improve the efficiency of hepatocyte differentiation. It was suggested that 3D structure could be more preferable for iPSC differentiation. Electrospinning is a widely used method for 3D scaffold fabrication. The microstructure has high surface area to volume ratios that can significantly improve cell attachment when compared to that of a homogenous flat surface. To develop the optimized protocol for iPSC generation, Cordie et al. used the electrospinning method to prepare aligned and randomly oriented nanofibers from polylactic acid (PLA), polycaprolactone (PCL), thermoplastic polyurethane (TPU) and polypropylene carbonate (PPC). It was presumed that electrospun nanofibers could mimic the structure of ECM and, therefore, enhance reprogramming efficiency. A similar approach was tested by Liu et al. who investigated the effect of 3D-PCL electrospun nanofibrous scaffolds on the chondrogenic differentiation in vitro and in vivo. It was shown that all four prepared scaffolds were hydrophobic. Therefore, to improve cell attachment, nanofibers were pre-coated with Matrigel. A similar result was obtained in the second study, but in this case the gelatin PCL were electrospun together. Both studies confirmed that most iPSC cells attached and grew well on the surface of all scaffolds. The nanofibers coated with Matrigel were also tested for reprogramming efficiency. iPSC colonies were generated on all substrates and were positive for pluripotency markers (Nanog, Oct 4, Sox2, SSEA-4, and TRA-1–60). In their turn, PCL/gelatin scaffolds were successfully used for chondrogenic differentiation of iPSC in vitro and showed a stronger effect in chondrogenesis and articular cartilage repair in vivo than scaffolds without cells and control group. Combining these results, it may be presumed that PCL nanofibrous scaffolds prepared with electrospinning method and covered with Matrigel or mixed with gelatin nanofibers could be efficiently used for iPSC generation and chondrogenic differentiation respectively. However, further studies are needed to optimize this protocol to avoid the use of animal-derived ECM, such as Matrigel. It would be also interesting to test how the other three fabricated nanofibers support iPSC differentiation in vitro and in vivo.

5. The Outlook: Overcoming Obstacles, Reducing Risk

Although the numerous studies with iPSCs have brought a sense of optimism, many formidable obstacles remain before the technology can be brought to clinics. First, there are several technical aspects which need to be improved in relation to the safety and reproducibility of iPSC generation from adult somatic cells. In particular, the use of viral vectors can lead to the possible mobilization of the vector by endogenous viruses in the genomes of patients and insertional mutagenesis leading to cancer. In addition to this, it is also important to understand how modified substrates influence the quality and genomic stability of iPSCs. Second, there is a need for an animal protein-free culture system that can replace products such as Matrigel. Again, this is an issue of safety, relating to the high risk of disease transmission. The caveats to using Matrigel derived from the mouse Englebreth-Holm-Swarm tumor are the difficulties in regulating production using Good Manufacturing Practices due to the animal source of the substrate. Therefore, well-defined xeno-free substrates for iPSC culture remain a major hurdle for their clinical application. Third, the mechanism of iPSC action is not yet fully understood. The bulk of evidence about iPSCs came from studying cells in vitro. Nevertheless, the understanding of molecular signals between cells in situ requires more sophisticated experiments. Composite biomaterials which are presented in the current review provide new possibilities for resolving these problems. They may provide controlling reprogramming and long-term self-renewal in vitro as well as iPSC differentiation in vitro and in vivo. The material produced from synthetic polymers and human recombinant peptides may bypass the use of animal-derived products. And last, but no means at least, smart biomaterials can be utilized to create the artificial microenvironment that may mimic injured tissue and, thereby, create the model of disease in vitro. Without any doubt, further development of novel smart biomaterial systems is necessary to eventually turn iPSC-based tissue engineering into a clinical reality. It is important to consider, as a final remark in this report, the solutions to the obstacles in the way of bringing iPSC therapy to clinical medicine will need to be both widely available and of low cost. These relate more to the logistics of cell manufacturing, supply and delivery.

6. Conclusion

In summary, striking achievements in tissue regeneration have been made. iPSCs are currently involved in the development of new biomedical technology that might be more effective than ever before in the regeneration of damaged tissue. However, there are still formidable fundamental, technical and logistical obstacles which demand clear understanding of iPSC biology and therapy. The assembly of different sciences from cellular biology and medicine to biomaterial-based engineering open a new way for regenerative medicine.

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