Linköping Studies in Science and Technology

Dissertations. No. 1686

Multifunctional Biomimetic Scaffolds Tailored for Cardiac Regeneration

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Linköping 2015

Cover: Scanning Electron Microscopy image of a mesenchymal cell on polycaprolactone fibers.

During the course of the research presented in this thesis, Abeni Wickham, was enrolled in Forum Scientium, a multidisciplinary graduate school at Linköping University.

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ISBN: 978-91-7519-021-1

ISSN: 0345-7524

Linköping Studies in Science and Technology. Dissertations. No. 1686

Electronic publication: <http://www.ep.liu.se>

Printed in Sweden by LiU-Tryck, 2015

*To the **Aili** group*

Colophon

This thesis is not only the summation of 5 years of research, but also a material manifestation of my happiness.

It has not been straightforward and I have had numerous failures, disappointments and doubts. These have always been positive moments, as each difficult moment gave me the chance to choose a different path to still achieve my research goals.

I have spent the past few years fighting for what I love the most; answering the questions that I ask myself every day. I have been lucky to have two supervisors, Daniel and Bo who pushed me to be better scientifically and family/friends who kept me grounded.

During this work, I found who I really am; this thesis is a representation of me.

Abstract

Nature has had millions of years to perfect the structural components of the human body, but has also produced the dysfunctions that result in the cancers and diseases, which ruin that perfection. Congenital heart defects, and myocardial infarction lead to scarring that remodels heart muscle, decreasing the contractility of the heart, with profound consequences for the host. Regenerative medicine is the study of strategies to return diseased body parts to their evolutionarily optimum structure.

Cells alone cannot develop into functional tissue, as they require mechanical support and chemical signals from the extracellular matrix in order to play the correct role in the body. In order to imitate the process of tissue formation optimized by nature, scaffolds are developed as the architectural support for tissue regeneration. To mimic the elasticity and strength seen in the heart muscle is one of the major scientific conundrums of our time. The development of new multifunctional materials for scaffolds is an accepted solution for repairing failing heart muscle. In this thesis I accept the notion that endogenous cardiac cells can play a major role in addressing this problem, if we can attract them to the site of defect or injury and make them proliferate. I then proceed to show how improving on a commonly used synthetic polymer was used to develop two new biomaterials.

Polycaprolactone (PCL) fibers and sheets were studied for their ability to adsorb proteins based on their surface energies. We found that although the wettability of the PCL might be similar to positive controls for cell attachment, the large differences in surface energies may account for the increased serum protein adsorption and limit cell adhesion. The effect of fiber morphology was then investigated with respect to proliferation of mesenchymal stem cells and cardiac progenitor cells. PCL was also mechanically enhanced with thiophene conjugated single walled carbon nanotubes (T-CNT); where small concentrations of the T-CNT allowed for a 2.5 fold increase in the percentage of elongation, while retaining the proliferation profile of the cardiac progenitor cells. Although PCL is a well-known implant material, the ability to attract and adhere cardiac cells was limited. Therefore we sought to develop new biomaterials with fiber morphologies similar to the muscle fiber of the heart, but with surface energies similar to positive controls for cell attachment. Poly[2,3-bis-(3-

octyloxyphenyl)quinoxaline-5,8-diyl-alt-thiophene-2,5-diyl] (TQ1) was then explored as a ribbon fiber and compared to collagen with embryonic cardiac cells, *in vitro*, and then implanted into rats for *in vivo* long term evaluations. The cardiac cells had a preferential adhesion to the TQ1 fibers, and *in vivo* the fibers attracted more blood vessels and regrew functional tissue compared to the collagen controls. TQ1 fibers had the added ability to emit light in the near infrared region, which would allow for consistent tracking of the material. Although this material offered the morphological preference for the cardiac cells, it does not degrade nor did it offer electrical conductivity. The heart muscle is an electrically active muscle. The dead tissue that is formed in the ischemic area loses its ability to transfer the electrical signals. Hence, I have then developed collagen fibrous materials with silver nanowires to help store and inject charges that would be generated during the contraction of the heart muscle. The silver nanowires served to help carry charges whilst providing resistance to bacterial growth on the material. The collagen/silver nanowires composites were mechanically apt for the culture of embryonic cardiac cells.

This thesis promotes the idea that morphology and favorable surface energies can help to attract and retain cardiac cells. It also shows the ability to manipulate materials to provide different functionalities, without losing their biocompatibility. I have added one new highly functional biomaterial to the list of materials capable of mimicking the extracellular matrix. I have also shown that small additions of metallic nanowires to a collagen matrix can increase its ability to conduct electrical signals, whilst retaining the mechanical properties that cardiac cells prefer. With the help of doctors and biomedical scientists, the materials of this thesis will hopefully translate to help patients have a better quality of life after heart injury.

Populärvetenskaplig sammanfattning

De strukturella och molekylära komponenterna i den mänskliga kroppen har utvecklats och förfinats under miljontals år. Dessvärre har detta inte förhindrat att sjukdomstillstånd, patogener eller trauma kan få kroppens vävnader och organ att irreversibelt förlora sin funktion. Vanligt är till exempel att medfödda hjärtfel eller hjärtinfarkt kan leda till nekros i hjärtmuskulaturen. Idag finns ingen tillfredställande behandling som återställer det skadade hjärtats funktion och dessa tillstånd är därför ofta dödliga. Inom regenerativ medicin strävar man efter att utveckla material och metoder som kan hjälpa och stimulera kroppens förmåga att läka eller ersätta skadad och förlorad vävnad. Celler kan på egen hand inte bilda funktionell vävnad utan de är beroende av både det strukturella stöd och de biokemiska signaler som tillhandahålls av den extracellulära matrisen (ECM). För att kunna härma den process som leder till nybildning av vävnad är det därför kritiskt att utveckla och studera ECM-liknande material. De unika mekaniska och elektrokemiska egenskaperna hos hjärtmuskulaturen gör denna vävnadstyp speciellt intrikat att efterlikna med syntetiska biomaterial.

Den här avhandlingen utgår från antagandet att endogena hjärtceller kan attraheras till det skadade området och stimulera återbildandet av vävnaden om rätt förutsättningar tillhandahålls. Avhandlingen beskriver både nya egenskaper och förbättringar av ett redan etablerat biomaterial samt två helt nya material. Fibrer och filmer av polykaprolakton (PCL) studerades med avseende på inverkan av vätbarhet och ytenergi på proteinadsorption. PCL har en vätbarhet som är jämförbar med material som uppvisar god celladhesion. Dock noterades stora skillnader i ytenergi mellan PCL och positiva kontroller vilket kan förklara den betydligt lägre graden av proteinadsorption och cell adhesion på PCL. Vidare studerades betydelsen av PCL fiberdiameter på proliferation av mesenchymala stamceller och hjärtprogenitorceller. Dessutom tillverkades en komposit mellan PCL och tiofen-modifierade kolnanorör (T-CNT) som uppvisade förbättrade mekaniska egenskaper men vidhållen förmåga att stimulera proliferation av hjärtprogenitorceller. Trots att PCL är ett vanligt förekommande implantatmaterial är dess förmåga att attrahera och stimulera adhesion av hjärtceller begränsad. Därför undersöktes möjligheten att tillverka material med en fiber morfologi som efterliknar

hjärtmuskulaturens och som uppvisar en ytenergi som stimulerar cell adhesion. Som del i detta studerades polymeren poly[2,3-bis-(3-octyloxyphenyl)quinoxaline-5,8-diyl-alt-thiophene-2,5-diyl] (TQ1) med avseende på proliferation av embryonala hjärtceller in vitro. Dessutom genomfördes en långtidsstudie av biokompatibilitet för TQ1 i råtta. Kardiomyocyter uppvisade god adhesion till materialet och dessutom stimulerades inväxt av blodkärl och nybildning av vävnad på implantatet i större utsträckning än för kontrollen. TQ1 är dessutom fluorescent och emitterar i det när-infraröda området vilket gjorde det möjligt att optiskt studera materialet i vävnaden. Trots flera fördelaktiga egenskaper så noterades ingen nedbrytning av materialet och det var heller inte elektriskt ledande, vilket är nödvändigt för att bilda fungerande hjärtvävnad. Därför utvecklades och studerades en nanokomposit bestående av kollagenfibrer och silvernanostrådar, där nanostrådarna bidrog till att ge materialet mycket goda laddningsinjektions- och laddningslagringsegenskaper. Dessutom inhiberade silvernanostrådarna påväxt av både Gram-positiva och negativa bakterier. Materialet uppvisade också fördelaktiga mekaniska egenskaper och var ett bra substrat för embryonala hjärtceller.

List of Publications

- I** Wickham A, Islam MM, Mondal D, Phopase J, Sadhu V, Tamás É, Poliseti N, Richter-Dahlfors A, Liedberg B, Griffith M, “Polycaprolactone -thiophene Conjugated Carbon Nanotube Meshes as Scaffolds for Cardiac Progenitor Cells”, *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, **2014**, 102, 1553-61

Contribution: Abeni Wickham (AW) developed the equipment, protocols for the materials, did the wettability measurements, and data interpretation. AW wrote the submitted manuscript with May Griffith.

- II** Wickham A, Koppal S, Dånmark S, Aili D, de Muinck E. Tentative title: “Influence of Polycaprolactone Scaffold Topography on Progenitor and Mesenchymal Cell Proliferation”, **2015**, *Submitted*.

Contribution: AW made the samples, did the surface energy studies, and collated the data. AW prepared the manuscript with Daniel Aili (DA) and Staffan Dånmark (SD).

- III** Wickham A, Sjölander D, Bergström G, Wang E, Rajendran V, Hildesjö C, Skoglund K, Nilsson K P R, Aili D, “Near-Infrared Emitting and Pro-Angiogenic Electrospun Conjugated Polymer scaffold for Optical Biomaterial Tracking”, *Advanced Functional Materials*, **2015**, In press.

Contribution: AW fabricated all the materials and designed the study with DA. AW interpreted most of the results and prepared the submitted manuscript with DA.

- IV** Wickham A, Vagin M, Kahlaf H, Bertazzo S, Hodder P, Dånmark S, Bengtsson T, Altimiras J, Aili D, “Collagen-Silvernanowire Nanocomposites as Electrically Active and Antibacterial Scaffolds for Embryonic Cardiac Cell Proliferation”, **2015**, *Submitted*.

Contribution: AW designed the study, prepared the samples, performed cell isolations, biocompatibility studies, SEM/EDS, and data interpretation. AW prepared the submitted manuscript with DA.

Papers not included in thesis

E. Alarcon, K. Udekwi, M. Skog, N. Poliseti, N. L. Pacioni, K. G. Stampelcoskie, M. Gonzalez-Béjar, A. Wickham, A. Richter-Dahlfors, M. Griffith, and J. C. Scaiano. The biocompatibility and antibacterial properties of collagen-stabilized, photochemically prepared silver nanoparticles. *Biomaterials*. June **2012**, 33, 4947-56

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Conference contributions

A. Wickham, M. Bolin, N. Polsetti, E. Jager, B. Liedberg, M. Berggren, M. Griffith. Conductive Biomaterials for Cardiac Tissue Engineering. *International Conference on Materials for Advanced Technologies*, **2011**, Singapore.

A. Wickham, D. Aili, F. Ajallouian, B. Liedberg, M. Griffith. Collagen Nanofiber Self-Assembly Does Not Require Telopeptides. *Colloids and Nanomedicine*, **2012**, Amsterdam, Netherlands.

Functionalized Scaffolds for Myocardial Regeneration. *Materials for Tomorrow*, **2012** Göteborg, Sweden.

Wickham A, Sjölander D, Bergström G, Wang E, Rajendran V, Hildesjö C, Skoglund K, Nilsson K P R, Aili D. Near-Infrared Emitting Electrospun Conjugated Polymer Scaffold for Non-Invasive Biomaterial Tracking and Tissue Regeneration. *Fourth International Conference on Multifunctional, Hybrid and Nanomaterials*, **2015**, Sitges, Spain.

Wickham A, Vagin M, Kahlaf H, Bertazzo S, Hodder P, Danmark S, Bengtsson T, Altimiras J, Aili D. Collagen-Silvernanowire Nanocomposites as Electrically Active and Antibacterial Scaffolds for Embryonic Cardiac Cell Proliferation, *Gordon Research Conference: Collagen*, **2015**, New Hampshire, U.S.A.

Wickham A, Sjölander D, Bergström G, Wang E, Rajendran V, Hildesjö C, Skoglund K, Nilsson K P R, Aili D. Near-Infrared Emitting and Pro-Angiogenic Electrospun Conjugated Polymer scaffold for Optical Biomaterial Tracking. *Gordon Research Conference: Biomaterials*, **2015**, Girona, Spain.

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Abbreviations

AgNP	Silver Nanoparticles
AgNW	Silver Nanowires
ASTM	American Society for Testing and Materials
AuNP	Gold Nanoparticles
C2C12	Mouse Myoblast Cell line
CNT	Carbon Nanotubes
CPC	Cardiac Progenitor Cells
DSC	Differential Scanning Calorimetry
ECCM	Embryonic Chicken Cardiomyocytes
ECM	Extracellular matrix
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
EDS	Energy-dispersive X-ray Spectroscopy
EHD	Electrohydrodynamic Atomization
GFP	Green Fluorescent Protein
ISO	International Organization of Standardization
LOX	Lysyl oxidase
MI	Myocardial Infarction
MSC	Mesenchymal Stem Cells
MTS	5-[3-(carboxymethoxy)phenyl]-3-(4,5-dimethyl-2-thiazolyl)-2-(4-sulfo-phenyl)-2H-tetrazolium inner salt)
NHS	N-hydroxysulfosuccinimide
PC	Plastic compression
PEDOT	Poly(3,4 ethylenedioxythiophene)
PEDOT-S	Poly(3,4 ethylenedioxythiophene) alkoxysulfonate
PCL	Polycaprolactone
SEM	Scanning Electron Microscopy
TCP	Tissue Culture Plate
TQ1	Poly[2,3-bis-(3-octyloxyphenyl)quinoxaline-5,8-diyl-alt-thiophene-2,5-diyl]
WST-1	sodium 5-(2,4-disulfophenyl)-2-(4-iodophenyl)-3-(4-nitrophenyl)-2H-tetrazolium inner salt)

“No progress without paradox”

-John Wheeler

Excerpt from The Strangest Man by Graham Farmelo

Preface

There were seemingly paradoxically important phases to my PhD studies, and this will be reflected in the papers. The top down versus the bottom up approach to scientific studies has always been at the farthest sides of the scientific continuum. This thesis hopefully will show the importance of both. It will also show that the initial identification of the problem and the usage of fundamental scientific studies, offers one of the best approaches to tackle clinical need and scientific curiosity ethically and efficiently. The almost five years of scientific work presented here, are at the very beginning, the naiveté of a young scientist, whose aim to make a cardiac patch as fast as possible hit a wall of fundamental questions by year two. As the work progresses the thesis shows the evolution of materials to suit the regeneration of cardiac tissue, in particular, with some descriptions of nerve regeneration.

Understanding the biological importance of structure that begets function of the cardiac tissue led to the migration from synthetic materials to being able to model collagen fibril formation and crosslinking in the lab. The ambition has been to design materials that could be easily carried through to the clinics and to understand their fundamental properties. This thesis tries to explore the fundamental basis of each material. This understanding is then transferred to engineering a structural component with multiple functionalities; something that evolution has already perfected but is hard to mimic.

It is my hope that this thesis surmounts the bias assumed in the top down and bottom up approaches. It is hoped that the use of both approaches are seen as complementary and equal towards the betterment of livelihoods of the human form.

Leaving my family and all that I knew in 2010 to come to Sweden in pursuit of my life goals is one of the most daring things I have done. Swedes have taught me to be humble, to be kind and to be grateful for the life that I live. Through troubled times there were many people who helped me keep to my goals and helped through this journey, this is my acknowledgement of them.

To my supervisors: **Daniel Aili**, for being my champion when I needed it the most, for pushing me to learn, to do and to stay true to finding the outmost truth in my work; **Bo Liedberg** for encouraging me to continue working, being a mentor for my personal development within the sciences; I am very grateful for both of you. To **Thomas Ederth**, thank you for your consistent honesty and incredible support from day one. I am extraordinarily indebted to **Bo Thunér**, for helping in setting up new instruments, and making my crazy ideas into functional equipment. I am grateful for **Stefan Klintström**, for helping me integrate into IFM and Forum and all the help with other transitions. I am also grateful to Tito Scaiano, Kajsa Uvdal, Karin Enander, and Caroline Brommesson for emotional and professional support over the years. To my group mates: Erik, Robert, Staffan, Christopher, Camilla, Mårten, Petter and extended group mates: Ranjith, Sandeep, Miraz, Mattias, Anna, Andreas, Jaywant, Viji, and Mohammad, you guys made each day coming to work very lively. I am also grateful to have met Olle Inganäs, for all the scientific discussions and Ingemar Lundström for always having a positive smiling face in the corridor.

I am sincerely grateful for the co-authors on each paper, and every collaborator that I have had over the years. Especially Jordi, Gunnar, Peter N, Ergang, Mikhail, Ebo, and Eva T. To Chyan-Jang and Kim M, Klas Udekwu, and Hanna, I am very happy that I got to work and learn from you. Along with Tobias E, Emma, Fredrik Björefors, and Maria B. To TA instruments, especially Claus, Els, and Peter, I am very grateful for all your emails, phone calls and meetings. Thank you to Pia, Anna Maria, Malin, Therese and Karin for translating important Swedish documents, and helping me through the admin. I am very grateful for Louise G, for administrative and personal support. Also to Göran and Per J for being my PhD mentors and Thomas Lingefelt for introducing me to instruments. I am also thankful for Tomas and Lisa at LiU tryck for all their help with

printing posters and this thesis.

To the wonder women: Elaine, Zhafira, Cecilia, Lia, Anna K, Ziafei, Feng-I, and Hung-Hsun, I am so glad I met you and I would not be this happy without our lunches/dinners/messages of support through the years. Also to the post-doc boys who kept me scientifically sharp, while having a blast: Luigi, Kristofer, Christian U, Axel F, Koen, and Sushanth, along with Karin M, Leif, Jonas, Zheng, Anders, Fredrick B, Per, Sara, Madhan, and all those in Forum Scientium, I have learned a lot from you and I am glad we got to meet, even if it was just to say hello. To all my friends, Laura U, Teresa, Larhonde, Pascal, Rafael, Shazia, Malaika, Per, Marcelle, Thomas, Tamisha K, Xu-Bin, Linnéa A, Joel, Henrik, Jonathan, Klas, and Elin, thanks for the support and especially all the good times.

I am extraordinarily grateful for my dad, **Iheatu**, for reminding me of the importance of integrity and love every week when I called home. I am so grateful that I was never alone because of him and those phone calls. Addie, CJ, and Aunt Lorraine, being with you with all the hugs and laughter, gave me the strength to come back each year and work even harder, and for that I am eternally grateful. To my mother, Sheranne, thank you for your thoughts and prayers during my roughest times.

Lastly to **Robert**, you have seen me at my worse, and have always pushed me to better and to stay focus on my goals. I am truly grateful for your patience and encouragement the past three years.

Abeni Wickham
July 2015
Linkoping, Sweden

“We cannot take for granted that the future will be better, and that means
we need to work to create it today.”

-Peter Thiel
Zero to One

Chapter 1. Introduction

Cancer, congenital defects, injury, and any other ailments that will result in the loss of tissue function or complete loss of the entire tissue, have deleterious effects to people's lives. In the European Union, some 67,000 people were still waiting for new organs.¹ Surgeons and material scientists developed the field of tissue engineering/regenerative medicine to find new ways to make organs without waiting for someone to lose theirs. The light out of the severe shortage of donor organs is the creation of organ mimics. However, every tissue is different and will require specific materials to replicate the complex structure and function of the native tissue.

An organ that is of particular concern is the heart. In the United States, more than 2150 Americans die each day from cardiovascular disease.² In the instance of myocardial ischemia, the loss of blood supply to the left ventricle due to blockage of the coronary artery creates a cascade of events that leads to a defunct heart muscle. Myocardial infarction is the death of cardiac cells and subsequently the death of the surrounding tissue caused by the loss of blood supply.³ In the U.S, this event caused a disease burden of about 7.5 million cardiovascular operations and in-patient procedures in 2010 alone.⁴ Apart from preventative strategic plans, there is still a need to offer solutions to mend the necrosed heart. Understanding the make up of the healthy heart muscle and function helps to engineer solutions to deal with the infarcted heart muscle.

Figure 1. The anatomy and position of the heart within the chest cavity. Adapted from ⁵ Copyright © 2005. With permission from Springer Science and Business Media.

1.1 Engineering a healthy myocardium

The heart is made of muscles that pumps blood from tissues through to the lungs and back again, Figure 1.⁵ The cardiac muscle consists of individual cells linked together by intercalated discs, which separate each muscle but serve to link each cardiac cell into a functional syncytium.⁶ Intercalated discs are composed of desmosomes that hold differing cell membranes together, adherens that anchor actin, and gap junctions that transmit electrical and chemical stimuli between the cells.⁷ The electrical signals from the sinoatrial node located in the upper right atria of the heart spreads through the syncytium such that the muscle contracts and relaxes synchronously.⁶ The myogenic property of cardiac cells, i.e. contraction arising from the cardiomyocytes rather than from innervation, enables the organ to be transplanted.⁶ The main cell populations of the functioning heart are fibroblasts, myocytes, endothelial and vascular smooth muscle

cells.⁸ After embryogenesis, the cardiomyocytes become binucleated, where subsequent growth of the mammalian heart arises from enlarging of the cardiomyocytes rather than division.⁹ Surrounding the cells, the cardiac extracellular matrix is mostly made of glycoprotein, glycosaminoglycans, and proteoglycans.¹⁰ Collagen is one of the main structural constituents of extracellular matrices of most tissues. It plays an important role in the myocardium allowing cardiomyocytes to elongate and be structurally supported for their pulsatile functions.^{11,12}

During myocardial infarction (MI), the ischemic area of the myocardium becomes heterogeneously necrotic, and then eventually fibrotic or scarred, resulting in the loss of about 20% of the left ventricle function.^{13,14} The remodeling that leads to left ventricle hypertrophy, affects the fibrillar collagen structure and crosslinking, where there is a loss of the normal collagen structure already 24 hours after the event.¹⁵ Figure 2 shows the changes in a rat myocardium at day 1 and day 28 after infarct. As the cells die, the tissue remodels to accommodate a similar pressure and volume of blood, but in doing so the myocardium undergoes hypertrophic remodeling and becomes thin.¹⁶ This is the hearts way of dealing with and repairing the heart muscle. The change in the extracellular matrix also leaves little to no signals for any surrounding cells other than inflammatory cells, to repopulate the area.^{17,18}

Figure 2. Transvers image of rat infarct heart, a) day 1, b) day 28. Copyright © 2007. Adapted from ¹⁹. With permission from the American Physiological Society.

The regeneration of a functioning myocardium through cell proliferation and differentiation is limited. For cardiomyocytes, fewer than 50 % are exchanged in a human's lifetime.^{20,21} Therefore changes in the surrounding tissue that would lead to cell death results in a permanent change in the cell populations.²² Evidence for cardiac stem cells has emerged in the past few years, where cardiac progenitor cells expressing stem cell markers have been located within the myocardium.²³ The finding that other stem cell populations, like bone marrow derived stem cells and mesenchymal stem cells can differentiate into cardiomyocytes has prompted studies on the influence of these cells when directly injected into a defunct rat myocardium and were found to increase the functionality of the left ventricle post-MI.^{24,25} There have been many cell injection therapy clinical trials in the past few years using, e.g. different stem cells, and smooth muscle cells.²⁶ The effect on regenerating the whole ischemic area is yet to be seen, as most cell engraftment occurs at injection site rather than throughout the scar.²⁷ Cell therapy of CD34⁺ cells into non-ischemic dilated cardiomyopathy patients, still resulted in a 6% increase in left ventricle stroke volume but with no difference in sudden cardiac death rates during treatment from the control group.²⁸ This modest progress in stem cell injection therapy is attributed to the harsh proinflammatory environment of the scarred myocardium, and the stiff substrate of increased collagen deposition and crosslinking that is incapable of allowing full stem cell engraftment.^{16,29,30} Before the introduction of stem cell injections into the myocardium, surgeons have been and still are replacing the scarred myocardium completely with the Dor or left ventricle remodeling procedure. In this procedure the scar tissue is resected and a material is used to reclaim the injured myocardium together to form a new artificial muscle, Figure 3.^{13,31}

Current materials for repair of the muscle rely on animal sources, like the bovine pericardium, or synthetics like, Dacron (polyethylene terephthalate). These materials offer temporary relief to the heart but do not offer a permanent, autologous, regenerative solution. Since most of the field is moving away from unsustainable devices and materials, such as porcine or bovine sourced, it is also important to develop a scalable, reproducible, and sustainable material to replace those currently used in these procedures.

Figure 3. Left ventricle restoration or Dor procedure, a) endocardial resection showing fibrous and normal tissue, b) endoventricular suturing and balloon used to check the diastolic volume, c) introduction and fastening of the cardiac patch, d) folding of rest of the tissue. Adapted from¹³. Copyright © 1992 Vincent J. Dor. With permission from John Wiley & Sons.

Materials suitable for replacing the bovine pericardium used in the Dor procedure, must have multiple functionalities that can create an environment for stem cells, endogenous or exogenous, to proliferate and differentiate into cardiomyocytes (Figure 4).

Figure 4. Biomaterial functionalities necessary to develop a functional myocardium.

1.2 The Biomaterial

The first known example of humans replicating body parts was discovered in 1931 when archeologist found Mayan skulls with nacre teeth perfectly integrated into the jawbone.³² Nacre, is an osteoinductive organic/inorganic composite of calcium carbonate and proteins, which naturally occurs in pearl producing mollusks.^{32,33} Nacre materials evidently have been used from the Mayan years, and now it is further engineered for the regeneration of bones in maxillary defects.³⁴

Dentistry is not the only field to benefit from the modern human ingenuity. In fact, most surgical fields have benefited from innovative surgeons, e.g. Vítězslav Chlumský who used several materials; from wax to silver plates, for hip arthroplasty.³⁵ Even today, Amerindians use the heads of large black ants to close wounds, a more drastic approach to wound healing.³⁶ These materials; nacre, wax, plaster of Paris, wood, et cetera, are called biomaterials, once they are engineered to interact with a biological system.

The early clinical need and first conceptual understanding of a biomaterial opted for a replacement only material that was inert, with no adverse effects on the immune system. This was also driven by the development of new manufacturing methods of materials during the industrial revolution. Screws used for bone fixation is one of the earliest examples, where in 1886 the first surgery is reported with such screws by German surgeon, Hansmann.³⁷ The development of stainless steel components for bone fixation in the late 1930s was a very progressive step, as it is still in use for this purpose.^{38,39}

Most materials that are used in clinics are still within the generation of biomaterials that are inert and developed to reduce adverse responses in the microenvironment to maintain the biomaterial stability.⁴⁰ Examples of 'newer' biomaterials approved for the clinics are listed in Table 1 along with function. Although these are new additions to the clinics they are not new materials. They are small steps in engineering materials that are already in use.

Table 1. Food and Drug Association approved medical devices from 2010-2014 those of which are being improved in tissue engineering and regenerative medicine research.

Device	Material	Use
ProGEL™ (2010)	Human serum albumin and polyethelene glycol	Sealing air leaks in lung
Ethicon™ OMNEX™ (2010)	2-octyl cyanoacrylate (2-OCA) and butyl lactoyl cyanoacrylate (BLCA).	Blood clotting sealant
Belotero Balance (2011)	Hyaluronic acid gel	Injected into facial tissue to smooth wrinkles
Gel-One® (2011)	Hyaluronate hydrogel from chicken comb in PBS	Osteoarthritis in the knee joint
AcrySof® Toric Intraocular Lens (2011)	Copolymer of phenylethyl acrylate and phenylethyl methacrylate, crosslinked with butanediol diacrylate	Restore sight after cataract surgery
EXOSEAL Vascular Closure Device (2011)	Polyglycolic acid foam	Close femoral artery wounds
Solesta® (2011)	Dextran and sodium hyaluronate	Fecal incontinence, injectable gel
LeGoo® (2011)	Triblock (ABA) of polyethele oxide polypropylene oxide (PEO-PPO-PEO)	Temperature sensitive reversible glue for short term occlusion
Edwards SAPIEN Transcatheter Heart Valve (2011)	Bovine tissue with stainless steel mesh frame and polyester wrapping	Heart valve replacement
Juvéderm Voluma XC (2013)	Streptococcus equi produced crosslinked hyaluronic acid lidocaine	Temporary facial filler
Perclose ProGlide Suture-Mediated Closure System (2013)	Polypropylene	Suture
VASCADE Vascular Closure System (2013)	Bovine collagen patch	Occlusion in major arteries (femoral)
Bellafill PMMA Collagen Dermal Filler (2014)	Polymethylmethacrylate microspheres and bovine collagen	Facial filler
Freedom SOLO Stentless Heart Valve (2014)	Bovine pericardial graft	Heart valve

As our understanding of wound healing, scarring and tissue regeneration has increased; the possibilities to meet clinical needs with new biomaterials with increasing functionalities have been investigated. Biomaterials are often categorized based on their proposed interactions with the biological system.⁴⁰ As described by Hench and Polak in 2002, the 1st generation biomaterial sought to be mechanical similar to the tissue that should be replaced, and to not be rejected. Examples include the cobalt chromium, or titanium hip implants, that are still used today. The 2nd generation biomaterials further sought to induce some tissue responses; like the hydroxyapatite coating for the same titanium and cobalt chromium hip implants. The 3rd generation biomaterial sought to elicit gene specific responses from the cells. Most of these materials are engineered to degrade and to have regenerated tissue in its place, and is patient specific. The 3rd generation biomaterial can also enroll the use of computed tomography to rapidly scan a defect, and combined with solid free form techniques, produces materials that are structurally designed to fit a specific defect.⁴¹ The 4th generation of biomaterials, so called “smart biomaterial,” imitates the seeming simplicity of nature to form the functional mimics of the extracellular matrix (ECM).⁴² These materials however, tend to use complex molecules and structures to accentuate and accelerate the patients own regeneration capacity.⁴³ The materials in this thesis are aimed to fall in the 3rd and 4th generation biomaterials. Developing new biomaterials for regenerative medicine brings engineering to the cusp of translation and fundamental studies. This deliberate interchange of fundamental and clinical knowledge, lends itself to the restoration of tissue with the use of cells and scaffolds offering similar or the same cues for regeneration, as if nature evolved them itself.

Simpler organs, with small subsets of cell types have been successfully engineered in the past years. The engineered bladder is one example of which a completely tissue engineered construct has been successfully implanted in a few patients for several years.⁴⁴ Another example is the skin, where many materials that function to repair and regenerated after injury have been developed.⁴⁵ However, for more complex organs, more innovative uses of materials become necessary. The development of biologically active scaffolds that interact with the host tissues to induce regeneration of damaged organs has become the strategy of choice in regenerative medicine, as it is now recognized that stem cells or progenitor cells alone are unable to reconstitute anatomical sites ablated by

disease, trauma or surgical intervention. Biologically active materials can provide microenvironments or templates that facilitate differentiation of stem or progenitor cells, and can potentially be designed to allow for repair and regeneration of specific target organs. Several of the most critical environmental conditions are set up by the ECM, which provide both mechanical support and biochemical cues that affect the cells, in several ways. While the use of extracted animal ECM proteins, like collagen, may create an acceptable microenvironment, potential contamination with viruses, prions, unknown proteins and other macromolecules raise issues about disease transmission, and batch-to-batch reproducibility. On the other hand, biopolymers, like collagen, and cellulose, although considered complex systems of variable outcomes⁴⁶, can be made more reproducibly by standardizing the sources. The increasing use of recombinant technologies for collagen and other biopolymers makes it possible to sustainably produce and develop similar materials to the native tissue⁴⁷ without the possibility of contamination.

“Great science emerges out of great contradictions”
-Siddhartha Mukherjee
The Emperor of all Maladies: A biography of Cancer

Chapter 2. Biomaterial Surface Properties and Topographical Effects

The extracellular matrix (ECM) is a fibrous hydrated network of proteins and other macromolecules found in the interstitial spaces of tissues. Between cells, the ECM is unified with the cytoskeleton by integrins on the cell surface. Therefore changes to the ECM will affect downstream chemical cues that dictate differentiation, proliferation and general tissue responses.⁴⁸ Tissue variations arise from differences in the composition and arrangement of the ECM constituents. For instance, the collagen in the myocardium forms 1- 10 μm wave like fibers connected by large diameter collagen fibers, and constitutes about 10% of the total mass, whilst in bone, collagen constitutes 90% of the solid mass and is a complex arrangement of collagen fibers and hydroxyapatite nanocrystals.^{49,50}

In vitro, materials with different chemical compositions and topography can also induce different levels of cellular responses, ranging from cell spreading dynamics to regulation of protein expression.⁵¹ The native ECM contains molecular information encoded in the amino acid sequence of the protein elements of the matrix. Shorter ECM-related peptide sequences have been isolated and identified to target and bind specific cells receptor, triggering signaling pathways that affect adhesion, differentiation, proliferation, apoptosis and morphogenesis. The profusely studied RGD (Arginine-glycine-aspartic acid) peptide, for example, was found to be the crucial component for cell adhesion in fibronectin.⁵² Although this is taken for granted *in vivo*, attracting cells to adhere to non-ECM materials is a requirement for a successful 3rd and 4th generation biomaterial. Materials that do not express cell receptor binding moieties, can either be modified with small peptide sequences containing the RGD peptide sequence⁵³, or exploit protein adsorption at the interfaces. The adsorbed proteins can in turn provide the appropriate cues for cell adhesion. Factors such as surface free energy and topography

of a material affects the protein adsorption process, which will thus change the material interaction with the biological environment.

2.1 Surface Energy

Structural proteins in the extracellular matrix encode for binding sites to which cells are able to attach.^{54,55} The domains interact with cellular receptors, integrins, and help to align the cells in either the direction of differentiation or proliferation.⁵⁶ Materials that lack inherent recognition sites for cell adhesion can utilize the fact that protein adsorption effectively alters the surface and thus the biointerface, which can prompt cues for cell adhesion. The surface chemistry of the biomaterial has a major effect on protein adsorption, which in turn affects cell integrin interactions.⁵⁷ Protein adsorption on biomaterials is a complex phenomenon that occurs through many interactions: electrostatic, hydrophobic interactions, hydrogen bonding, and van der Waals forces. In addition to the composition and chemistry of the surface, the surrounding environment, such as the ionic strength and pH, also dictates protein adsorption kinetics, albeit such factors are fairly constant under *in vivo* conditions. Protein adsorption onto foreign surfaces also displays a complex pattern of exchange and replacement reaction known as the “Vroman Effect”.⁵⁸

A rough idea about the properties of the biomaterials surface can be obtained by measuring the ability of liquids to wet the surface. The wettability of a material can be measured by water contact angles using the sessile drop technique, as shown in Figure 5. The contact angle (θ) provides an approximation of where on the hydrophobic (low affinity for water) or hydrophilic (high affinity for water) continuum a material is. Wettability is a general term used for how well a surface allows a liquid to spread and interact with the surface. The surface of a material is considered wetting when the water contact angle is less than 90° (hydrophilic), and non wetting when it is larger than 90° (more hydrophobic).⁵⁹

Figure 5. Schematic of the upper and lower ends wettability continuum. Hydrophobic surfaces have large water contact angles ($\theta > 90^\circ$), while more hydrophilic surface will have smaller angles ($\theta < 90^\circ$).

Water contact angles have been extensively used as a general method to characterize biomaterials. Habitually, the more wetting a material, the more likely the material is to be biocompatible.⁶⁰⁻⁶² This analysis is simplistic as both hydrophobic (Teflon) and hydrophilic (polyethyleneglycol) show very different effects on protein adhesion and both can be considered biocompatible. Depending on the surface chemistry, a hydrophilic surface could bind water molecules tightly, and proteins in an aqueous solution would not be able to displace the water molecules so as to bind to the surface. This is seen, e.g. with polyethylene glycol-like surfaces.⁶³ These hydrophilic polymers are hence considered antifouling surfaces because of this.

One can generally assume hydrophobicity for surfaces displaying molecules with aliphatic tails, such as methyl groups (CH_3), whereas surfaces with hydrophilicity for molecules having charged and polar tails, such as carboxylic (COOH) and hydroxyl (OH) side groups respectively. Serum albumin and fibrinogen adsorb onto self-assembled monolayers of both CH_3 and OH terminated thiols on gold coated glass surfaces, although the rate of serum albumin adsorption was faster on the CH_3 terminated surface.⁶⁴ Nonetheless, serum albumin is also more tightly bound to hydrophobic surfaces.⁶⁵ In order to create an entropy gain in aqueous systems, hydrophobic molecules aggregate together to reduce the number of hydrogen bonds broken from the nearing water molecules. The hydrophobic domains in the serum proteins interact with the hydrophobic surface of the materials forming strong hydrophobic-hydrophobic interactions. As a result, adhesion proteins, fibronectin/vitronectin, produced by the cells cannot easily displace the serum albumin,

and cell attachment can thus be limited.⁶⁶ Wettability can also be affected by surface roughness. For instance, by changing the surface roughness and molecular conformation of the temperature sensitive polymers, one can achieve drastic changes from super hydrophobicity to hydrophilicity.⁶⁷

Non-specific adsorption of proteins on biomaterial scaffolds is a complicated process where differences in adsorption pattern and conformational changes of adsorbed proteins on different materials cannot be solely explained by water contact angles. However, measuring the advancing and receding contact angles of water and other polar solvents on the material, and applying the model developed by Good, van OSS and Chaudhury (GvOC), the critical surface tension, or surface energy can be calculated.⁶⁸ From this model, the dispersive Lifshitz-Van der Waals (γ^{LW}) and polar Lewis acid (γ^+) and base (γ^-) components are obtained, which can provide a more detailed understanding about the surface properties and the affect on protein adsorption and cell-material interactions. Static contact angles and the calculated surface energy components of the thiophene/quinoxaline (TQ1), and polycaprolactone (PCL) polymers studied in this thesis are highlighted in Table 2, along with corresponding data for tissue culture plate (TCP), usually made from plasma treated polystyrene.

Table 2. Static contact angle measurements of polar and non-polar solvents used for surface energy calculations employing the GvOC model.

Sample	Static contact angle (°)			γ^{total}	γ^{LW}	γ^+	γ^-
	Water	DI ^a	EG ^b				
TQ1 Film	96.8± 3.7	47.4±1.7	67.2±3.6	29.2	29.2	0	0
PCL Film	72.43±1.9	72.0± 1.0	58.3±2.6	23.8	20.1	0.3	11.5
TCP ⁶⁹	65.9±4.3	41.4±4.3	35.9±1.7	44.0± 4.5	38.9±2.2	0.4±0.3	13.7±4.6

^aDiiodomethane, ^bEthylene glycol, surface energy (γ), dispersive (LW), (+) Lewis Acid, (-) Lewis base components.

In **Paper II**, changes to the PCL topography decreased the surface concentration of adsorbed proteins but increased the number of cells found on the scaffolds. TQ1 and PCL show fairly large differences in contact angles (Table 2). The surface energy of TQ1 is a result of the dispersive components, with no polar contributions. PCL shows pronounced Lewis base (electron donating) properties because of its ester bonds. TCP also shows basic properties due to its ability to partly donate pi-electrons. The differences in surface energy of TQ1, PCL and TCP highlight the striking effect the material interface has on the ability of cells to adhere and proliferate on these different materials. Embryonic cardiomyocytes, for instance, grew on the walls of the tissue culture plate and minimally on the PCL fibers. Whereas on the TQ1 polymer, after day 1 the same cells grew onto the fibers and the tissue culture plate, showing no preferential adhesion. One of the first studies of electrospun polymers for tissue engineering employed PCL fibers pre-coated with collagen to allow mesenchymal stem cells to penetrate and form multilayers of cells after 4 weeks of culture.⁷⁰ Adsorption of proteins can thus significantly improve cell adhesion of a rather modest cell adhesion material like PCL. Scaffold topography and fiber morphology, however, are at least equally important as the surface chemistry of the material, and can have a large influence on cell adhesion as shown in **Paper II**.

2.2 Physical Cues

The topography of a biomaterial, i.e. structural features such as porosity and fiber dimensions, can have large effects on biological interactions. The size of spheres, for example, regardless of chemical composition, greatly affects the foreign body responses and integration of the materials *in vivo*, where spheres 1.5 mm in diameter were shown to be best to avoid fibrosis.⁷¹ Cells can also be heavily affected by the mechanical properties of the scaffold e.g. osteoblasts require stiffer materials (150MPa), whereas embryonic cardiomyocytes require softer materials (modulus 1kPa) for regeneration of bone and cardiac tissue respectively.^{72,73} As the materials aim to mimic the ECM that surrounds the cells, especially in the heart; the materials also need to be able to be structurally capable of withstanding the dynamic forces of the pulsatile heart.

The structural proteins of the ECM are fibrous and some, such as collagen, are ribbon-like in shape.⁷⁴ The heart muscles are interwoven, and the extracellular matrix surrounding the heart reflects the interlaced patterns at different depths to provide strength in many directions.⁴⁹ Biomaterials made of similar fibrous morphologies to the ECM, could potentially provide the same mechanical properties as the native tissue. This thesis focuses on randomly oriented fibers. Figure 6a shows the microstructure of bovine pericardium (BVP) used for left ventricle restoration in the clinics.⁷⁵ The BVP is an acellular collagenous material of randomly oriented fibers (Figure 6a). The random orientation in the fibers in the pericardium anisotropically withstands deformation forces.⁷⁶ Self-assembly of collagen type I of both porcine and rat-tail tendon collagen yields randomly orientated fibers (Figure 6b,c). Other structures of synthetic polymers, such as random, cross-hatched (Figure 6e), and aligned (6f) were also fabricated in my work for both cardiac cells and the latter for nerve tissue engineering (Figure 6d-f). Neural tissue engineering benefits from directed growth of nerve axons that can be promoted by aligned fibrous scaffolds.⁷⁷ The PCL fibers offer a cylindrical shaped fiber that differs from the more ribbon like morphology of collagen fibers. The thiophene/quinoxaline, Figure 6g material discussed in **paper III**, was found to have a ribbon like morphology, and although they were 10 μm in diameter, they provide better cardiac cell adhesion than cylindrical PCL fibers (6d). Of course this is rudimentary statement, since the surface chemistries of TQ1 and PCL is quite different. Smaller

diameter, 1 μm , cylindrical fibers coated with conductive polymers (Figure 6h) were also shown to permit better nerve cell differentiation, but were only marginally better for embryonic cardiomyocyte proliferation (discussed in Chapter 4). The planar sheet of polycaprolactone, (Figure 6i), was the least suitable morphology for cell adhesion and proliferation even with serum protein pre-treatment, **Paper II**. However the flat morphologies made with collagen or collagen/cellulose materials, did elicit better cell proliferation than synthetic polymers of the similar structure.

Figure 6. Morphological differences of different materials: a) bovine pericardium used in the left ventricle restoration, b) self-assembled porcine collagen, c) self-assembled rat-tail tendon collagen, d) randomly oriented PCL, e) crosshatched PCL fibers, f) aligned PCL fibers, g) TQ1 ribbon fibers, h) randomly orientated PEDOT coated PCL fibers, i) spun coated sheet of PCL.

This thesis focuses on developing new biomaterials for cardiac regeneration, I found that, chemical and physical cues played an important part on deciding how to fabricate the materials. As mentioned before, the aim to produce a material with surface

energies similar to the tissue culture plate, but structurally similar to the extracellular matrix that surrounds cardiac cells was paramount. Although synthetic materials offer the ability to tune chemical and mechanical properties for an optimized cell adhesion, it is very difficult to mimic cellular recognition molecules found in the ECM. Within the *in vivo* environment there are a large number of different macromolecules, including growth factors that affect the ability of cells to proliferate and also provides cues for differentiation.⁷⁸ These interactions cannot be resolved by a reductionistic approach of merely measuring water contact angles on synthetic cell unrecognizable polymers, and then pronounce it biocompatible. It is important that we migrate from the idea that synthetics alone can do what natural materials have already evolved to do so well. Using constituents of the extracellular matrix ECM to form structurally and chemically apt materials is entirely possible to accomplish reproducibly. From collagen to hyaluronic acid and even using non-ECM biopolymers, such silk⁷⁹⁻⁸¹ we can create a biomaterial with varying morphologies and mechanical properties whilst retaining the cell recognizable motif.

“Adapt what is useful, reject what is useless and add what is specifically your own”

-Bruce Lee.

Chapter 3. Techniques

In tissue engineering, ECM mimics, known as scaffolds, are fabricated in varying ways to provide support for cells to grow into functioning tissue. As the name suggest, a scaffolds aims to provide the structural basis for cell support. The type of tissue regeneration strategy a material serves will dictate the types of fabrication techniques used to form scaffolds with the required morphology, topography and mechanical features. The choice of fabrication strategy also depends on the properties of the constituents used. For example, fabrication of structurally well-defined fibrous scaffolds using synthetic polymers, such as polycaprolactone, can be achieved using electrospinning. Biopolymers, such as collagen, can also be processed by electrospinning but the procedure will denature the protein structure potentially leading to a loss in biofunctionality. In the latter case self-assembly is a more rational choice for fabrication of scaffolds. Both electrospinning and self-assembly are cost effective and fairly simple techniques to create large quantities of reproducible materials.

This chapter focuses on electrospinning and plastic compression as means of creating fibrous scaffolds for structural extracellular mimetics. It also covers some aspects of synthesis of hydrogels based on collagen and collagen/cellulose derivatives as another way to make simpler forms of scaffolds for tissue regeneration.

3.1 Electrohydrodynamic Atomization: Electrospinning

Think of the spinning of cotton into yarn onto a spool. The starting material, a cotton bulb, in the solid state is fibrous, made of mostly cellulose. To get it into yarn, of which we then use to make textiles, the cotton bulb is carded and roved into the individual yarn fibers. Most synthetic polymers employed for biomedical applications, however, are not synthesized in an already fibrous form that can be roved; therefore other techniques are used to get a polymer solution into textile like fibers. Melt blowing is one such technique, where the polymer is heated and subjected to hot air as it is extruded.⁸² The use of electrically driven jets, however, produces a wide range of fiber diameters without heating. In this method a polymer solution is feed through a metal capillary that is subjected to a potential difference, the result is a filamentous jetting, at least $\frac{1}{3}$ of the diameter of the capillary in size, towards a grounded collector, Figure 7. The technique has been developed from a century of research into the surface tension of fluids, and electrical field effects on fluids, Figure 8.

Figure 7. A schematic of the electrospinning process and the resulting randomly oriented fibers.

The beginnings of understanding the electrical forces on fluids emerged in the early 20th century. Using a simple single plate apparatus, Figure 9, Zeleny described the changes observed when a drop of liquid was exposed to large static electrical fields.⁸³ The various instabilities that occurred at the surface of the droplet were further shown using

ethyl alcohol and glycerin (Figure 8a-h).⁸⁴ The surface tension of the liquids, ethyl alcohol, 25.3 dynes/cm and glycerin, 65.2 dynes/cm, are very different and result in different forms of the jet breakup.⁸⁵ In Figure 8e, the alcohol broke up into jets of smaller droplets, while in Figure 8g,h the glycerin produced a filamentous jet. Consequently surface tension plays a large role in developing droplets as in electrospraying, or fibers, as in electrospinning.

Figure 8. The different electrically driven droplets and jets from alcohol imaged in the early 1900s. At 5 kV a,b) the shielding effect of droplets, c) liquid breakup by forces from varying quickly in direction, d) higher magnification of 'c' showing the appearance of a Taylor cone, e) electrospraying, smaller diameter droplets forced out of the cone, f) increased voltage to have two areas of instability from one droplet, g,h) 7kV voltage to achieve $7\mu\text{m}$ thread ejected at a speed of 3 m/sec from glycerin. Adapted from ⁸⁴. Copyright © 1917. With permission from American Physical Society.

Surface tension is a measure of the cohesive force in which the molecules in the liquid will contract to form the smallest surface area. Water that drips from a tap experiences surface tension exerted on the liquid at the outlet of the tap. As the drop is then exposed to atmospheric pressure the droplet will decrease in size. Given a critical flow rate, a drop will be forced to move out of the capillary and breakup into smaller

droplets. If we then hold the flow of the water out of the capillary at a minimum rate, and then subject the water droplets to a voltage difference, where the field is the only force that acts on the water droplet to force it through the capillary, the breakup of the droplet will result in an axisymmetric breakup of a jet. The breakup, known as a varicose breakup, produces smaller volumes of liquid to be forced out of the tap.⁸⁶ The surface tension of water, 72 mN/m, is quite high for the electrospinning process. The lower the surface tension the less electrical force is needed to produce a cone in which a jet can form. Liquids with a surface tension greater than 50 mN/m were found to require an electrical field that exceeded the ionization potential of air.⁸⁷ This then meant that there would be electrical discharges (sparks), which is not safe, and actually will not result in droplets or fibers. It is thus important to keep the surface tension below 50 mN/m, which can be achieved by using more volatile solvents, like chloroform, or ethanol, or even the use of surfactants.

Volatile solvents also serve a second purpose, the more volatile the faster the rate of evaporation from the polymer solution as it jets out of the capillary and the more homogenous the fibers. The violent whipping of the jet after formation of the conical shaped droplet (Taylor cone), forms the fibers, by the evaporating solvent.⁸⁸ The polymer solution parameters: solvent, molecular structure, and weight of the polymer, will determine the latter step of elongation and formation of fibers as they solution whips towards the collector plate. Generally, the more viscous the solution, the more likely it will form fibers. However, homogenous fibers dictate that the polymer has a molecular weight > 50 kDa and a linear backbone structure. A linear polymeric backbone can be stretched and elongated from the droplet into a continuous filament.⁸⁹ A charged polymer tends to form ribbon like fibers, as seen with the collagen, and some thiophene-based polymers as seen in Table 3. The ribbon like morphology is a result of a polymer 'skin' that develops at the capillary tip.⁹⁰ As the solvent evaporates the hollow fibers collapse and form a thin elongated ribbon fiber.

The electrospinning setup has changed over the years, Figure 9 and 10. The setup used in this thesis was constructed with reproducibility and safety in mind. A closed insulating system increased the reproducibility in fiber production. The safety cabinet protected the user from the electric fields generated, especially with higher surface tension polymer solutions (i.e. requiring larger electric field strength). A safety switch was built in

since each high voltage system can supply 60 kV. When the cabinet was opened the voltage system was disabled.

Figure 9. The first known electrospinning equipment. The simple apparatus to illustrate the changes to charged droplets in a uniform electrical field, set up by Zeleny in 1914. It comprised, D, 6.3 cm brass disc 1.5 cm from capillary tip A, 1 mm diameter capillary where the current passed through, B rubber tubing connecting glass tubing, T, rubber tube, R, to connect to glass holding the fluid of which had a wire, W, immersed that supplied the voltage. The glass piece F could change the height at point, P, on the vertical scale, S. Inlet K, the crosshairs used to measure the meniscus. Adapted from ⁸³ Copyright © 1914. With permission from American Physical Society.

Figure 10. Image of electrospinning system used for this thesis, a) power supply for rotating mandrel, b) voltage supply, c) safety switch, d) voltage extension to capillary, e) infusion pump, f) rotating mandrel, g) collector. The system was designed by myself and built by Bo Thunér, 2010.

In my setup (Figure 10), there were means for both perpendicular and horizontal electrospinning. The latter was useful for more precious materials, e.g. collagen and the thiophene/quinoxaline semiconducting polymer TQ1 used in **paper III**. Other polymers like polycaprolactone (PCL) were spun perpendicularly. The rotating mandrel speed was linearly related to the input voltage from the power source. This particular system reached 2000 revolutions per minute. This produced varying fiber diameters of aligned fibrous polymers. The infusion pumps permitted dual electrospinning, or coaxial spinning, with up to four syringe holders. Examples of the different polymer structures made with this system during this thesis work are depicted in Table 3 with corresponding images in Figure 11.

In the initial phase of tissue engineering in the early 1990s, it was hypothesized that replicating the fibrous structure of the extracellular matrix with common polymer technologies was a feasible strategy to produce environments to regrow cells, and thus tissue. Three-dimensional materials with a large surface area caused by fibrous topographies offer more possibilities for cell attachment.⁹¹ Producing synthetic materials using electrospinning proved to be a fruitful research area, resulting in thousands of papers. Changing the polymer concentration, solvent, flow rate, electrical field and collector distance produced the changes in structure of different polymers for fabrication of well-defined scaffolds with large surface areas and fibrous ECM mimetic appearance.^{92,93}

In the first part of this thesis research, I focused on developing reproducible methods to fabricate electrospun fibers from polycaprolactone and a range of other polymers. The parameters and images of the fibers are shown in Table 3. Whilst the field is saturated with examples of electrospun scaffolds of synthetic polymers,^{94,95} the use of biopolymers is still relatively underdeveloped albeit, the electrospinning of silk, gelatin, cellulose, and other biomolecules has been described.⁹⁶⁻⁹⁹ Biomolecules can provide inherent integrin recognition motifs, which is beneficial for cell growth. Synthetic polymers, on the other hand, can be synthesized and electrospun to incorporate biomolecules that can cue cell attachment.^{100,101} The main advantage of synthetic polymers is the possibility for production of reproducible materials with the ability to vary the diameters of fibers during fabrication. Some biomolecules, however, will not

remain in their native folded state when exposed the solvent needed to produce uniform fibers. Collagen, for instance, is usually electrospun in fluororoalcohols and this has been shown to be a very costly way to produce the denatured collagen protein (i.e. gelatin) fibers.¹⁰²

Nonetheless, synthetic fibers, although poorer in ability to attract and adhere cells, can be electrospun to study the relationship between e.g. fiber diameter and cell attachment. In the case of polycaprolactone (PCL), a well-known biomaterial that is used consistently in the field,¹⁰³ it was important to determine how the changes in fiber diameter could affect certain cells. We have studied this phenomenon using human mesenchymal cells. Here we saw that the lower diameter fibers 1-0.5 μ m, although retaining the least amount of serum proteins, allowed for better cell attachment than large diameter fibers and planar substrates of PCL. This is discussed further in **Paper II**.

Composite electrospinning is useful for the incorporation of smaller but mechanically stronger materials into large polymer fibers, such as incorporating ceramics and carbon nanotubes (CNT) into polymer fibers.¹⁰⁴ In our work we used small concentrations of single walled CNTs to drastically increase the mechanical properties of polycaprolactone microfibers. The addition of thiophene conjugated single walled carbon nanotubes (T/CNT) in the PCL fibers increased the percentage of elongation of the fibrous materials, whilst retaining a similar ability to support cardiac progenitor cells growth (**Paper I**).

The work presented in this thesis has partly been based on the well-established methods of electrospinning to create fibrous meshes for tissue engineering. *In vitro* conceptualization of fibers and their effect on cell culture makes electrospinning a useful and very attractive technique, but the limits on the creation of biopolymer materials (e.g. collagen) by electrospinning also made me interested in the procedures to make materials by self-assembly, exploiting the natural process of fiber formation of collagen.

Table 3. Examples of materials and parameters used for electrospinning varying fibers.

Fibrous materials (Corresponding images in Figure 11)	EHD parameters					
	Weight %	Solvent	Needle Gauge	Flow rate (ml/hr)	Voltage (kV)	Distance from collector (cm)
PEDOT-S/PEG/fish collagen (1:2:3) No conductive coating during SEM		Water	21	0.5	21	10
Collagen	10	2,2,2, trifluoroethanol	21	0.5	6	15
Gold nanoparticles/collagen	7	2,2,2, trifluoroethanol	21	0.5	6	15
PCL random	10	Chloroform	21	0.5	5.4	15
PCL aligned	10	Chloroform	21	0.5	5.6	3.5 (1750 RPM)
PCL smaller diameters	10	Chloroform/Methanol 3:1	21	0.5-0.3	6	20-30
PCL/collagen 1:1	10/10	2,2,2, trifluoroethanol	21	0.5	6-7	15
Poly [2,3-bis-(3- octyloxyphenyl)quinoxaline- 5,8-diyl-alt-thiophene-2,5-diyl] (TQ1)	12	Chloroform	20	1	13.5kV	10

Figure 11. Corresponding scanning electron microscopy images of materials made in Table 3, a) PEDOTS/PEG/collagen, b) collagen, c) collagen AgNP, d) PCL random, e) PCL aligned, f) PCL smaller diameters, g) PCL/collagen, h) TQ1 fibers.

3.2 Collagen and Plastic Compression

The use of collagen as a biomaterial for tissue engineering has expanded over the years as isolation techniques have improved. Collagen biomaterials have been made into hydrogels, sponges, and nanofibrillar scaffolds for tendon, cartilage, and other connective tissue repair.¹⁰⁵ There has also been intense research on collagen mimetic peptides that can form robust synthetic (ECM) mimics.¹⁰⁶ One of which resulted in the development of peptide amphiphilic nanofibers that arrange with hydroxyapatite similar to the collagen in bone.¹⁰⁷ Similarly, isolated collagen reconstituted *in vitro* has emerged to form materials, which most closely mimic the healthy *in vivo* myocardial environment. With increasing fundamental knowledge of the large role collagen plays in regenerating tissues, how it is produced, and remodeled by cells in varying environments, *in vitro* adaptation has become easier.

The etymology of collagen traces back to the Greek words *kola-* for glue and *gen-* of a specific kind.¹⁰⁸ Collagen is a major structural protein of most connective tissue, and helps to provide the scaffolding that keeps multicellular organisms together.¹⁰⁹ The collagen that makes up most connective tissues aggregates to form fibers, and hence collagen is known as a fibrous protein. Evolution of fibrillar collagen has been shown to occur early on in the metazoan development, seen by the similarities between the vertebrate and invertebrate collagenous genes.^{110,111} Mammalian and fish collagen have been used for centuries by humans in products such as leather, yet the biomedical uses of collagen however, have only developed fairly recently. The growth of new techniques for studying collagen molecules have also filtered back into the leather industry, as X-ray diffraction studies have shown that thicker collagen fibrils result in stronger, more tear resistant leather.¹¹²

In the 1950s, biochemical assessment of the collagen molecule began with the X-ray diffraction studies of the collagen molecule by Ramachandran, where collagen from shark ray, kangaroo-tail and rat-tail tendon was examined in both the dry and wet state.¹¹³ Rat-tail tendon has since become the main model for collagen studies over the years. In 1955 Ramachandran and Crick simultaneously, but separately, published the structure of collagen in Nature.^{114,115} The amino acid sequence is a repetition of glycine in every third position. The glycine-X-Y repeated amino acid sequence is usually found to

have proline in the X position and hydroxyproline in the Y position. This allows for a polyproline II like alpha helix, which comes together to form the supercoil triple helix structure. The glycine residues are packed into the center axis of the coil, where the amide groups engage in interchain hydrogen bonding.^{114,115} Deletion of the glycine residue destabilizes the triple helix conformation along with monomer conformation.¹¹⁶ The hydroxylation of the proline residues increases the thermal stability of the collagen molecule through added hydrogen bonding.¹¹⁷

The distinctive triple helix super coil assembles into some 28 different types of collagen, ranging from fibrillar to basement membrane proteins.¹¹⁸ The triple helical collagen motif can also be found in at least 15 other molecules; including C1q, lung surfactant, mannose binding protein, acetylcholine esterase.¹¹⁹⁻¹²²

Collagen production *in vivo* is still a process under discussion, but generally collagen is produced by fibroblasts. The triple helical supercoil structure arises from three left handed alpha helix chains, brought together in the endoplasmic reticulum of the cell by disulfide bridges at the N and C terminal of the sequence.¹⁰⁹ The most abundant fibrillar collagen, type I, is heteromeric, in that it is formed from two alpha I and one alpha II chains. Collagen type III is homomeric with three alpha I chains. As the collagen molecule is passed into the extracellular spaces, procollagen proteinases cleave the disulfide rich N and C terminal, leaving an intact triple helical molecule to self-assemble.^{123,124} This is an oversimplification of the assembly as post-translation modifications occur in a crowded system where it is guided by integrins and several other molecules.¹²⁵

The mechanism of *in vitro* fibril formation is very different from the *in vivo* system, where cells are secreting the molecules and collagen fibrils are formed and enzymatically cross-linked.¹²⁵ Collagen that has already formed *in vivo* can be isolated and reconstituted in an acid soluble form. The mature collagen is considered insoluble and only during enzymatic degradation can this change. Fibril formation of the collagen molecule *in vitro* has been studied extensively and there are standard operating procedures that result in centrifuged pellets of collagen molecules to retrieve collagen fibers.¹²⁶⁻¹²⁹ The formation of fibrils from collagen dissolved in weak acidic solutions has been described since the early 1960s.¹³⁰ The more intact the triple helix is after isolation, the better the fibril formation, both with respect to time for monomer assembly, and the

number of hydrogen bonds between fibers. Proteinases, such as pepsin, are known to unravel the triple helical structure of the collagen during processing.¹³¹ However, collagens isolated by pepsin degradation can still form fibrils similar to those isolated without pepsin. The standard rat-tail tendon collagen and a pepsin treated porcine atelo collagen were used for proof of concepts studies in this thesis. Using the method described of gently raising the pH in a high salt environment to neutral, initiates nucleation of the collagen monomers,¹³² when the temperature is raised to 37°C, native collagen fibrils form within 10 minutes. Comparison studies of the rat-tail and porcine atelo (without telopeptides) collagen using circular dichroism, Figure 12a, showed little differences in the collagen supercoil spectra. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is utilized to separate proteins based on molecular weight and conformation. In the case for collagen it can show changes to the native state of the protein, whether there are more segmented pieces, or intact alpha chains. SDS-PAGE analysis of the atelo-porcine and rat tail collagen showed similar bands for the gamma (γ), beta (β) and alpha (α) 1 and 2 structures found in intact collagen molecules. The γ denotes the trimeric, and the β represents dimeric structure of the collagen. Further similarities are shown in the gel formation and fibrils in Figure 12c, d.

Figure 12. Comparison of rat-tail (RT) and porcine atelo (PA) collagen type I, a) CD spectra and b) SDS page of rat-tail tendon and porcine atelo collagen used in this thesis, c) collagen gels after 37°C incubation d) scanning electron microscopy of both collagens after compression of gels in 'c'.

3.2.1 Plastic compression

Compression molding is a technique that is used to shape plastics, in which a heated polymer is placed within a mold, and a plug with a fixed pressure is applied whilst maintaining the heat of the polymer.¹³³ This allows the polymer, usually thermoset polymers, to be shaped within the mold. Plastic compression (PC) of collagen uses the aforementioned means of collagen fibril formation that result in a viscoelastic gel. The compression expels most of the water from the collagen gel resulting in a sheet of collagen fibrils, mechanical stabilized by forced aggregation of the fibrils. The technique was first described by Brown et al. in 2005, and was developed to rapidly reproduce a

structural ECM mimic without cellular interactions.¹³⁴ Collagenous materials for mimicking the bladder and cornea have been described using this technique.^{135,136} In this thesis, plastic compression of collagen was adapted and optimized to enable the concentration of collagen to be increased to 5 -7 mg/ml. The stainless steel mold was modified to form different sized gels (Figure 13a). The relative ease of application allows for fabrication of homogenous collagen fibrous mats that are still hydrated to 90 % of their mass, Figure 12b. In the case of **paper IV**, this process was further optimized to allow collagen fibril formation in the presence of silver nanowires. The hydrogel-like material is not unlike the hydrated matrix surrounding tissues, but it is missing the crosslinking and incorporation of sugar moieties that help the hydrogel structure to withstand compression.¹³⁷

Figure 13. Plastic compression of collagen set-up, a) a representative stainless steel mold, b) schematic of the compression with compression load onto a pressure plate that pushes the liquid out of the collagen gel through metal and nylon meshes (both with 100 μm pore size).

3.3 Collagen Hydrogels

Hydrogels are dilute polymer networks that are able to retain a substantial amount of water to their weight, typically from 30 to almost 100%.¹³⁸ The use of organic hydrogels in industry preceded biomedical applications by a few decades as patents for titanium oxide hydrogels were filed already in the early 1900s.¹³⁹

A movement emerged to replace plastics used as 1st generation biomaterials with hydrogels, in an effort to ensure implant longevity. Proposed in a short letter in 1960, the materials were expected to satisfy three important criteria: high water content, biological inertness, and permeability to metabolites.¹⁴⁰ These criteria were presumably formulated based on observations of natural hydrogels, such as the cornea, where nanometer collagen fibrils are arranged with glycosaminoglycans to form a gel that is optically transparent and allows for easy exchange of certain metabolites.¹⁴¹

Hydrogels are made up of hydrophilic polymers, i.e. polymers that can form hydrogen bonds with water, and hence usually possess hydroxyl (–OH), carbonyl (–C=O), and carboxyl (–COOH) functional groups. The polymers are cross-linked, by either covalent (chemical) bonds or physical interactions to form a network. The extent and method for cross-linking depends on the polymer and affect both polymer degradation and hydrogel mechanical properties.

Polyhydroxyethylmethacrylate (poly-HEMA) hydrogels were one of the first used for biomedical applications, as intraocular lenses in the 1950s.¹⁴² Other synthetic polymers, such as polyethylene oxide, polyvinyl alcohol, and co-polymers of poly lactide glycolic acid/PCL are consistently used to make biocompatible hydrogels.¹⁴³ Temperature and pH sensitive polymers have also been used for fabrication of hydrogels for triggered release of drugs and to attract specific cells, for example, in wound healing.¹⁴⁴ Both self-healing hydrogels and hydrogels with tunable mechanical properties have been developed with alginate and polyacrylamide.¹⁴⁵ Fang et al. developed a nanofibrous hydrogel of poly (1-vinyl-2-pyrrolidinone) modified with chitosan and acrylamide that was able to withstand tensile forces of 0.1MPa, and provide up to 80% self healing capacity after cracks were induced in the material with promising biological results.¹⁴⁶

Increasing research on naturally occurring hydrogels, such as the cornea, have

inspired mimicry of the tissue *in vitro* using exogenous collagen,¹⁴⁷ e.g. the biosynthetic corneal implants.¹⁴⁸ Non-crosslinked collagen hydrogels have been used extensively to promote bone mesenchymal cell chondrogenesis, as dermal substitutes.^{149,150} Collagen has also been co-crosslinked with other proteins and macromolecules, such as fibroin, chondroitin sulfate and hyaluronic acid.^{151,152}

To fabricate hydrogels for tissue engineering in general, the cross-linking strategy used must not use or result in cytotoxic products. It is also important that the degree of cross-linking can be tuned to such an extent that the materials obtained possess tissue specific properties, which in turn depends on the intended applications. Whereas transparency is critical for collagen hydrogels for corneal applications, it is of little relevance for cardiac applications where mechanical properties are more crucial. Hence different cross-linking strategies must be considered. In this thesis a number of different strategies have been evaluated and exploited for fabrication of collagen hydrogels.

A suitable strategy for crosslinking of collagen is offered by the carboxyl reactive carbodiimide 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). EDC is often used in conjunction with N-hydroxysulfosuccinimide (NHS) and allows for crosslinking of primary amines and carboxylic acid functional groups.¹⁵³ As EDC is water-soluble it allows for cross-linking of the collagen hydrogel in a favorable hydrated environment. The EDC molecule forms a reactive *o*-acylisourea with carboxylic acid, and then reacts with the primary amine to form an amide bond with the water-soluble isourea byproduct. The soluble isourea and unreacted EDC are easily washed away with a phosphate based buffer and one is left with an intact hydrogel, Figure 14. The porcine collagen used in **Paper III** was crosslinked using this strategy. Other hydrogels made with this same system are cellulose hydrogels, and cellulose collagen hybrid hydrogels, Figure 13. For the cellulose hydrogels, amine groups were introduced using ethylenediamine. By crosslinking the cellulose the glass transition temperature was raised from 70°C to > 200°C.

Figure 14. Hydrogels prepared using the EDC/NHS crosslinking strategy, a) collagen b) cellulose, and c) collagen/cellulose hydrogel. Scale bar: 6 mm

The EDC/NHS is a safer alternative to the use of aldehydes since the by-product of the system, the isourea, is easily washed away in the processing. Also aldehyde crosslinking reactions are optimal at a pH of 9 and above¹⁵⁴ in which collagen molecules will aggregate into fibrils. *In situ* crosslinking of collagens or other polymers in the presence of cells, limits the use of most synthetic crosslinkers, as they tend to be cytotoxic.¹⁵⁵ Therefore the use of enzymatic crosslinking strategies was investigated as a means to improve the mechanical properties of the collagen.

3.3.1 Lysyl Oxidase-based Crosslinking of Collagen

Lysyl oxidase (LOX) is a copper amine oxidase enzyme, which activates the lysine residues in collagen and elastin to reactive aldehydes.¹⁵⁶ Following posttranslational modification of the lysine in the collagen molecule, the LOX enzyme initiate crosslinks within, and among, collagen molecules.¹⁵⁷ In healthy tissues, fibroblasts secrete the collagen, together with the enzymes that crosslink the collagen, into the extracellular spaces.¹⁵⁸ *In vitro*, lysyl oxidase has a high affinity for native collagen fibrils formed from weak acidic acid solutions.¹⁵⁹

In vitro crosslinking of collagen can be performed similarly to the *in vivo* processes either by addition of enzymes or, as examined here, using cellular secretion of LOX by genetic modification.

Human embryonic kidney cells (293T) and mouse atrial cardiomyocytes (HL-1) were used in this study to check the efficiency of transfection in different cell lines. 293T

cells are known to easily express recombinant proteins, as the lentiviral particles are produced in this cell type.¹⁶⁰ However, to extend the transfection to the cardiomyocyte line transfection, HL-1, pseudotyping plasmid containing the glycoprotein of the vesicular stomatitis virus (VSV-G) was employed. VSV-G enters the cell membrane via fusion with the phospholipids of the cell membrane.¹⁶¹ This technique extends to a larger host range than the HIV envelope, which increases the number of cell types that can be transduced.¹⁶²

In this study we used differential scanning calorimetry (DSC) to investigate the degree of crosslinking in the samples. Denaturing of collagen is considered a first order transition between collagen to gelatin.¹⁶³ This transition will show a change in the heat capacity of the material, due to the unraveling of the triple helix into the denatured gelatin state.

Figure 15. Western blot of lysyl oxidase expression in cell lysates of HL-1 and 293T cells. This confirms the expression of the lysyl oxidase by the cells.

Figure 16. Live Dead imaging of HL-1 cells within collagenous materials after 30 days of culture. Scale bars: 100 μ m

Western blot assays showed the 32 kDa band for the lysyl oxidase enzyme present in both the HL-1 and 293T cells lysate, and the active form (AcLOX) in the supernatant, Figure 15. Over-confluent but still viable HL-1 cells were kept 30 days in culture, as the largest effect in crosslinking took several weeks to appear. DSC results showed a significant increase in the ΔH of the collagen gels crosslinked by the LOX expressing cells. The gels showed a 4-6°C shift in the denaturing temperature and large changes in the enthalpy values, ranging from 11.8 J/g for the GFP expressing cells to 32.75 J/g for the AcLOX expressing cells, Figure 17.

Figure 17. DSC results for crosslinking collagen fibrous matrix with HL-1 cells.

The crosslinking of collagen fibers by the HL-1 LOX producing cells was optimally seen when the cells were cultured in a collagen gel after the fibrils had formed. However, after passage 69, the cells lost the expression of the LOX, although still remaining viable.

Optimization for the production of the LOX protein in conjunction with collagen fibril formation is still necessary, but the above set of results is a proof of concept study showing the possibility to use LOX expressing cells for crosslinking collagen fibers. The purified exogenous LOX protein has also been successfully used to crosslink articular cartilage implants in hypoxic conditions.¹⁶⁴ One modification of this study would be to change the characterization technique, as the changes to the surrounding collagen fibrils might not have been enough to be resolved with calorimetry. Other techniques such as micro rheology could easily detect small changes to the mechanical properties of the collagen fibrils¹⁶⁵, which should be the case if there is effective crosslinking.

3.4 Characterization Techniques

Materials fabrication only constitutes one part of the research activities, as describing and understanding how each change in fabrication alters the materials is just as important. For most biomaterials, an array of different techniques must be used in order to fully characterize and understand the properties of the materials. High resolution imaging techniques are employed to study the micro- and nanostructure of scaffolds and cell-materials interactions, whereas calorimetry, and mechanical testing are exploited to elucidate polymer phase transitions, degree of cross-linking, and overall mechanical properties. It is of course also vital to early on detect any potential toxicity to the cells and surrounding host environment when developing new biomaterials, which require both *in vitro* cell experiments and *in vivo* evaluations. The following techniques were consistently used in this thesis for all the biomaterials.

3.4.1 Scanning Electron Microscopy (SEM)

The resolution of microscopy techniques is determined by the diffraction limit, which is directly proportional to the wavelength (λ) of the radiation used (resolution = $\lambda/2NA$), where NA is the numerical aperture. Optical microscopy can generally not resolve features smaller than the about half the wavelength of visible light. For characterization of the fibers and materials developed in this thesis work it was hence necessary to use electron microscopy, since electrons have a significantly shorter wavelength. Scanning electron microscopy (SEM) uses a focused beam of electrons gathered from a tungsten filament to irradiate the sample. This technique is considered nondestructive, in the sense that the samples remain intact after the relatively high intensity of electron bombardment (>5kV) and can be reused for repeated imaging or elemental analysis. Bombardment of electrons into non-metallic materials, of high dielectric constants will result in charging, as these materials can withstand large electric fields. The charging results in blurred images, where it is impossible to resolve any features. To avoid charging effects, the samples are usually coated with thin metals, usually 10 nm thick. Most of the samples in this work were sputter coated with platinum. The electrons from the SEM gun interact with the material causing secondary electrons to form a few nanometers into the sample.

The secondary electrons are released and the detector then retrieves them, where only topographical differences can be resolved. By increasing the intensity of the electron beam, one can resolve further into the sample. Contrast in the sample is as a result of changes to the secondary electron ratio and the detection of backscattered electrons. Information about the elemental composition can be resolved from detecting X-ray from the samples. In this technique, energy-dispersive X-ray spectroscopy (EDS), a high-energy (in this case 20 kV) electron beam is used to scan the sample. The high-energy electrons used to irradiate the sample can excite or eject electrons, creating a hole. The hole is subsequently filled by an electron from an outer shell, resulting in an emission of an X-ray photon. The energy of the emitted X-rays provides information about the atomic number of the specimen, as they will have specific energies for different atoms. EDS was used in **Paper IV**, to resolve the differences between collagen and silver nanowires within the sample, as they both have similar size and shapes that could not always be distinguished in the SEM images.

A trade off with using the SEM is that the materials are imaged under high vacuum, and thus dry conditions. Drying biological samples is not a trivial process, as it can result in artifacts. To image materials and cells in SEM without causing artifacts due to the drying procedure it is important to reduce the surface tension of the solvent incrementally to preserve sensitive features. Traditionally the use of critical point drying was deemed to be the only way to dehydrate biological samples. In this technique, exchanging the liquids for carbon dioxide induces the preservation of the sample.¹⁶⁶ In the interests of simplicity, another technique was developed using increasing ethanol (surface tension = 22 mN/m) concentration washes followed by a bath in hexmethyldisilane (HMDS) (18 mN/m). In this way the water is incrementally replaced with more volatile solvents, thus retaining the morphology of the specimen.¹⁶⁷ HMDS is then used at the end to completely remove both ethanol and any remaining water. This is consistently used for biomaterial electron microscopy imaging^{70,168,169} and was employed throughout this thesis work.

3.4.2 Fluorescence Microscopy

Fluorescence imaging techniques are critical for characterization of *in vitro* cell experiments. Fluorophores are typically illuminated with specific wavelengths close to the absorption maximum of the dye. The absorbed photons excite electrons, which undergo a relaxation process to lower energies before emitting a photon with higher wavelength (lower energy). Imaging binding of fluorophore-labeled antibodies to proteins that are expressed differently based on cellular interactions with a biomaterial, such as alpha actin, connexin 40 and 43, are crucial for understanding biomaterial-cell interactions. The introduction of the green fluorescent protein (GFP), first isolated from jellyfish, allowed for researchers to tag and follow cells through many experiments without sacrificing the cells.¹⁷⁰ In one of the unpublished studies, we used atrial cells that co-expressed, GFP as a control for the transfection for the lysyl oxidase experiment. The low photo bleaching of GFP and low phototoxic effect on the cells during imaging,¹⁷¹ meant that we could follow cells for several weeks without losing the fluorescence signal. There are many variations to GFP, one of which is a ribonucleic acid mimic that could help researchers tract RNA activity over long culture times.¹⁷²

A commonly used fluorescence-imaging based method for initial investigations of the biocompatibility of materials is the LIVE/DEAD® assay. This assay provides a means to image and distinguish dead and living cells on the material surface, exploiting two different fluorophores. Ethidium homodimer is a fluorescent dye that cannot cross an intact cell membrane. The dye in solution is weakly fluorescent but has a strong affinity for nucleic acids and binding to DNA in dead cells with disrupted cell membranes leads to an intense fluorescence emission at 617 nm (i.e. it appears red) For detection of viable cells, fluorescent acetomethoxy derivate calcein (calcein AM) is used. The dye can enter the intact cell through the membrane where it will be enzymatically hydrolyzed intracellular by esterases, resulting in calcein that emits at 515 nm (i.e. it appears green). The combination of the ethidium homodimer and calcein AM for detection of cell viability was patented in 1994¹⁷³ and has since been used in every biomaterial lab.

To detect these interactions, fluorescence microscopes that can excite and detect the incoming fluorescence are used. Normal wide field fluorescence microscopy is suitable for imaging of two-dimensional structures, and is consistently used for 2D cell culturing on tissue culture plates. Most biomaterials, however, are three-dimensional and thus for imaging cells in several layers, confocal microscopy is primarily employed. The difference between wide field and confocal is that in the latter there is a pin-hole located in the optical path to the detector, removing unfocused light. Confocal microscopy has been so widely used in biology that it was already reviewed in 1989.¹⁷⁴ The differences between the two techniques with respect to imaging of fibrous scaffolds are illustrated in Figure 18. The inherently fluorescent fibers (TQ1, **paper III**) are imaged with (a) normal wide field fluorescent microscope, and (b) with a confocal microscope with images from many depths and rendered into one image.

Figure 18. Fluorescence imaging of TQ1 fibers using, a) regular wide field microscopy and b) confocal microscopy, rendered z-axis stacked image. Scale bar: 50 μ m.

3.4.3 Differential Scanning Calorimetry

The heat capacity of a material is the amount of heat needed to raise the temperature of a material by one degree. The heat capacity is directly related to the composition of the material. Differential Scanning Calorimetry (DSC) uses the changes in temperatures and heat flow through a sample to make a bulk assessment of the heat capacity of materials. Endothermic (heat flow into the sample), and exothermic processes (heat flow out of the

sample) can be qualitatively and quantitatively measured using DSC. In this technique, an aluminum pan is heated incrementally in a closed nitrogen purged chamber. A thermocouple electrode measures the specific changes in heat as a function of time and temperature ramping. A similar empty aluminum pan is used as the reference, such that only the changes in heat capacity of the sample material is recorded.

The heat flow (W/g) versus temperature ($^{\circ}\text{C}$) curves provides information about physical parameters of polymer materials, such as glass transitions, melting, cross-linking, and crystallization, Figure 19. Consequently, information about chemical modifications to materials can be assessed, such as the degree of crosslinking, which is important information for fabrication of hydrogels. Characterization of materials by DSC is often carried out on clinical implants according to ISO 11357-1, especially for elucidating effects of storing materials.

Figure 19. Thermal changes in seen in DSC spectra. In this instrument endothermic transitions are down and exothermic are up. Copyright © 2015 Els Verdonck, TA Instruments.

Calorimetry assessment of protein denaturing is useful especially when the proteins are concentrated solid materials. Heat capacity changes in protein unfolding are a result of hydrated groups being exposed to the outer solvent.¹⁷⁵ Although the curve of the DSC looks similar to a melting curve for polymer and other simple materials, the spectra of collagen is a lot more complicated. Earlier research on the changes seen in the spectra was thought to include melting of crystalline regions and suggested to be a phase

transition.¹⁷⁶ Heating collagen can result in a time dependent permanent conformational changes from the native triple helical to random coil states in collagen.¹⁷⁷ For all intents and purposes, this change is used to denote denaturing in the DSC spectra. As an example, in the case of the hydrogels made by EDC/NHS crosslinking, these still have a lower temperature of denaturing (42.9 °C) compared to the aggregated collagen fibrils without crosslinking (50.2°C), Figure 20.

Figure 20. Thermal changes of soluble collagen cross-linked into a clear hydrogel by EDC/NHS (black) and a fibrillated non-cross-linked hydrogel (red).

3.4.4 Mechanical testing

All materials have the ability to withstand forces to the point at which they fail. Material failure can be a crack, tear, or permanent deformation. The forces (F) exerted on a material per unit area (A), called stress ($\sigma = F/A$), and the forces that cause a change in length (l) of a material, strain ($\epsilon = \delta l/l$), are the determinants of the modulus of a material. The modulus is defined as the slope of the stress/strain curve. Generally the Young's modulus is a defined measurement of stiffness of a material. The ultimate tensile strength is the point at which the material breaks or undergoes permanent deformation, e.g. the force needed to stretch skin to the point it cannot return to its original length. The measurement is carried out by having the material clamped on opposing sides, with

one side pulling the material in one direction and measuring the changes in load as it extends. The thickness and length of the material between the clamps are recorded, and based on the load and extension data, the stress/strain curves can be resolved. To standardize this procedure materials are shaped as a dog bone, Figure 21a. This is to ensure that the forces on the larger areas are equal at both ends and that failure occurs in the middle of the sample rather than at the grips. A typical curve from the Instron Mechanical testing machine can have the load as a function of extension, which can show how much elongation (strain) the material can withstand before deforming, Figure 21b.

Figure 21. Mechanical testing using the Instron®, a) dog bone shape of materials as per standards to achieve reproducible stress/strain failure information at the center of the samples instead of at the edges, b) Load as a function of extension curves produced by the Instron®.

Arrangements of molecules, crosslinking, and morphology of materials can determine the mechanical characteristics of materials. *In vivo*, hydroxylapatite crystals added to collagen enhance the modulus, found in bone versus the skin, i.e. kPa to MPa. Similarly *in vitro*, carbon nanotubes added to polycaprolactone can increase the Young's modulus from 8.4 MPa to 11 MPa, **Paper I**. This change could be attributed to the increased stress transfer to the single walled carbon nanotubes in the materials, a process seen in many epoxy composites containing nanotubes.¹⁷⁸

3.4.5 Cell Viability

Cytotoxicity is described as imposed cell death. New biomaterials for clinical applications are always tested for their cell toxicity using standardized cells, according to International Organization of Standardization (ISO) criteria. However to answer tissue engineering research specific questions, usually the adapted tissue specific cells are used. There are

many ways to describe cell viability and lack thereof. As mentioned before the live dead assay is a technique that results in the green fluorescence for live cells and red for the dead cells. Other techniques are proliferation assays. With some materials, poor cell adhesion and thus lower rates of proliferation can be indicative of biomaterial cytotoxicity. The effect of materials on cellular proliferation is a complicated process, but cell attachment to the surface and communication between cells are important factors. Cell to cell communication is imperative to retain homeostasis.¹³⁷ Gap junction proteins are specialized cell to cell junctions that allow exchange of small intracellular signaling molecules.¹⁷⁹ Amongst cardiomyocytes gap junction protein, connexin 43, regulates the calcium ions¹⁸⁰ that allows for depolarization and contraction in the heart muscle. General viability of cardiomyocytes have been shown to be dependent on seeding density¹⁸¹, indicating that these cells need close range communications to survive.

The MTS (5-[3-(carboxymethoxy)phenyl]-3-(4,5-dimethyl-2-thiazolyl)-2-(4-sulfo-phenyl)-2H-tetrazolium inner salt), and WST-1 (sodium 5-(2,4-disulfophenyl)-2-(4-iodophenyl)-3-(4-nitrophenyl)-2H-tetrazolium inner salt) are used in simple colorimetric assays to determine the viability and proliferation of cells. In the MTS assay the yellow tetrazolium salt is converted to a purple formazan in the mitochondria of metabolically active cells.¹⁸² The WST-1 works in a similar manner producing a water-soluble form of the formazan, but has a different electron acceptor intermediate.¹⁸³ The combination of proliferation via mitochondria activity, and viability via intact cell walls helps to deliver a complete picture of biomaterial cytotoxicity. Most of these assays run for one week (7 days), as cell activity tends to have a delayed response to the materials, and usually at 7 days cells become confluent and would need to be split in normal conditions

3.4.6 Subcutaneous implantation

In vitro biocompatibility is key to determine cell interactions with materials. However, implantation of foreign materials always results in immune cell interactions before target cell interactions.¹⁸⁴ Although the cell viability assays give a depiction of specific cell interactions, during first implantations the injury to the area will create a foreign body response to the biomaterials, which will dictate the acceptance or rejection of the

material.¹⁸⁵ The biocompatibility of new materials can be investigated by subcutaneous implantation followed by careful evaluation of the inflammatory response, foreign body reaction and fibrous capsule formation at various time points. This will give an indication of local and systemic toxicity of the implant. According to the ISO standard 10993, there are various time points that necessitate various rat subcutaneous studies. The length of implantation is determined by the material's characteristics. Non-biodegradable implants must be followed for at least 12 weeks, where as biodegradable materials, should be followed for the duration of its existence. Clearance of the material should also be monitored until there is no more material left. The method of subcutaneous implantation for biological toxicity evaluation is depicted in Figure 22. The animals are sedated and then an incision is made in the dorsum, and the implant is introduced just under the muscle, then the site is closed and reopened at different time points. Controls are the same injury without implants, and no injury at all. A similar *in vivo* study was done in **Paper III** for 90 days, Figure 23. There were two controls, the rat tissue after injury, and collagen hydrogels. The samples were the thiophene/quinoxaline fibers (TQ1).

Figure 22. Rat subcutaneous implantation, a gold standard for biomaterial *in vivo* evaluation, a) measured lines drawn to accurately know depth and length of incision, b) anesthetized rat, c) antiseptic treatment of rat skin with povidone iodine, 'x' indicating the hip joints, d-f) at the center of the hip joints the skin was lifted, g-h) incisions with the marked scissors and pockets made, i-l) the U shaped flap was made, m-n) implant was placed on the subcutaneous muscle, o) a cell sheet was placed on the skin flap, p-r) the cell sheet was removed and the skin was sutured, s-u) animal was then revived and kept in clean environment. Scale bars: 10 mm. Adapted from¹⁸⁶ Copyright © 2011. With permission from Macmillan Publishers Ltd: Nature Methods.

Figure 23. Long-term biological evaluation by rat subcutaneous implantation for 90 days with TQ1 fibers. Hematoxylin and eosin stained slides of a-b) rat tissue control, c) collagen control, d) TQ1/collagen samples

Although this is a gold standard for biomaterial evaluation, campaigning from animal rights groups have made animal models and ethical concerns harder to defend for most biomaterial laboratories.¹⁸⁷ To combat this, co-culture systems, model inflammation and tissue culture are being developed by scientists to give *in vivo* quality answers with *in vitro* tests.¹⁸⁸⁻¹⁹⁰

“The end of surprise would be the end of science. To this extent, the
scientist must constantly see and hope for surprises”

-Robert Friedel

(Excerpt from Adapt by Tim Hartford)

Chapter 4. Multifunctional Biomaterials

In order to make materials that stimulate the body's own capacity for repair, a scaffold must in many cases provide additional functionalities than just structural support for cells to grow on. The natural extracellular environment is complex and dynamic and provides cells with numerous instructive signals that regulate cell growth, differentiation, et cetera, for the function of the tissues and organs. An inert and passive material mainly mimics the macroscopic properties of the tissue without any of the detailed chemical and structural complexity critical for cells to form functional tissue. Decellularized ECM from organs addresses some of these issues but still relies on donors. Development of new synthetic materials that mimic different features of the ECM is thus a flourishing field of research. Common strategies is to modify the scaffolds with short peptides or other biomolecules that stimulate cell adhesion, and recruit and bind growth factors, and enable scaffold remodeling. It is also possible to include elements that provide functionalities that are not naturally present in the ECM to improve the capacity of the materials to stimulate tissue regeneration, facilitate materials handling (e.g. suturing) and for tracking the implant. The focus of this thesis has been to develop and explore multifunctional materials that, in addition to good biocompatibility, were conductive, traceable and mechanically apt for cardiac tissue engineering and regeneration. This is an active research field and numerous beautiful examples have been described in the literature that has inspired this work.¹⁹¹⁻¹⁹³

4.1 Conductive Biomaterials

Electrical stimulation to promote regeneration of bone has been utilized since the 1850s, and subsequently for skin repair soon after.¹⁹⁴ Endogenous electrical fields generated after tissue injury are responsible for electrotaxis and other responses channeling tissue repair.¹⁹⁵ Research on tissue regeneration using electrical stimulation has, however, mostly centered on nerve tissue, owing to the fact that action potential propagation is vital to nerve and muscle tissue function. However, other tissues such as skin and cornea have all shown benefits from electrical stimulation in some form.^{196,197}

Most scaffold materials develop so far are, however, not inherently conductive and thus needs to be modified to improve their properties for regeneration of cardiac tissue and nerves. There are several considerations to take into account for fabrication of conductive biomaterials for tissue regeneration of any sort:

- 1) Preferential cell adhesion
- 2) Tissue/material mechanical matching
- 3) Charge inject/storage capacities
- 4) Stability of charges
- 5) Clearance of the degraded molecules
- 6) Remodeling of material to allow full tissue regeneration

Over the years, materials made of platinum, gold, iridium oxide, stainless steel (covered in silicone rubber mostly) have been consistently used as electrode materials, since their stability and charge injection capacities provide for long-term electrical stimulation.¹⁹⁸ These constitute the pacemakers and neuroelectrodes, deep brain stimulation electrodes, and even electrocardiogram electrodes (these are usually also made of silver).^{199,200} These materials are good conductors, however, they have really high ultimate tensile strengths, providing several orders of magnitude mismatch with soft tissue, e.g. the tensile strength of platinum alloy found in stents is 203 MPa²⁰¹, while the linear modulus of skeletal muscle is 447 kPa along the fiber.²⁰² Tissue/implant mechanical matching is important for longevity of the implant and regeneration of surrounding tissue. Using biopolymer or synthetic polymers fabricated into porous,

fibrous material, one can create biological electrodes and conductive scaffolds with a modulus tailored to the host environment. This was aptly shown with silk/polyimide electrodes implanted into feline brain tissue, which showed better recoding of the electrical signals because of the close tissue and electrode contact by the softer silk meshes.²⁰³ A number of different scaffolds have been developed: from gold nanowire/nanoparticles and conductive polymers incorporated into insulating synthetic and biological polymers,^{204,205} to implantable gelatin/carbon nanotube scaffolds that have helped to increase the functionality of an infarct rat myocardium.²⁰⁶ Interestingly, an increase in cardiac cell electrical coupling through the gap junction proteins can be seen on the materials without external electrical stimulation.¹⁹¹⁻¹⁹³

4.2 Conductive Polymers

Conductivity is measured with the SI unit of Siemens per meter (S/m). Resistance to a current flow (resistivity) is measured in ohm per meter (Ω/m), and is the reciprocal of the conductivity. For thick polymer films, a four-pointed probe can be employed to measure the bulk resistance of films.²⁰⁷ This provides an accurate measurement of polymers, as the conductivity of the conjugated polymer will change with the orientation of molecules and thickness of the material.²⁰⁸ Dopants are typically added to help carry the electrons along the polymer backbone, which increase charge mobility and thus conductivity. The dopants are either strong electron donor or acceptors within the polymer backbone.²⁰⁹

The development of conductive polymers attracted the Nobel Prize in 2000, since then the number of applications has been found to be endless, and perhaps surprisingly, especially as biomaterials. The first conductive polymer produced was polyacetylene in 1977.²¹⁰ A conductive polymer has a conjugated backbone, i.e. it has alternating single and double bonds. The pi-pi bonding where successive carbon atoms have sp^2 -hybridized orbitals allows for electron delocalization. This delocalization allows for the movement of the electrons and holes along the polymer backbone.²¹¹

Within biomedical engineering, conjugated polymers offer the ability to be fabricated as scaffolds whilst providing the electrical stimulation to cells and tissues for regeneration. Polypyrrole (PPy), polyaniline (PANI) and poly (3,4-ethylenedioxythiophene) (PEDOT) have been researched extensively for nerve, skeletal,

and more recently, cardiac regeneration.²¹²⁻²¹⁶ Out of the three, PEDOT has better thermal and conductive properties and is suitable for long-term implantation.²¹⁷ PEDOT is polymerized from the EDOT monomer, in the presence of the iron (III) tosylate (Fe (III)TOS. The Fe(III) TOS acts as both an oxidant for polymerization and a dopant.

Figure 24. Molecular structure of thiophene, TQ1 (thiophene/quinoxaline), and PEDOT: TOS. PEDOT:TOS. Adapted PEDOT:TOS from ²¹⁸. With permission of the Royal Chemical Society.

Thiophenes were described already in 1882 by Victor Meyer and published in 1888, *Die thiophengruppe*, and have been one of the most studied group of heterocyclic compounds.²¹⁹ Thiophenes (C₄H₄S), Figure 24, can be found in e.g. different dyes manufactured from petroleum.²¹⁹ The electrically conductive polythiophenes, e.g. PEDOT, are often used as organic semiconductors, and offer the possibility of producing cheaper, flexible solar cells and light emitting electrodes.^{220,221}

Throughout this thesis, thiophenes were used in several applications. In **paper I**, they were attached to the single wall carbon nanotubes to aid in miscibility of the carbon nanotubes. In **paper III**, a fully conjugated polymer with the thiophene moiety attached to quinoxaline was studied and found to be highly biocompatible, and increased blood vessel formation *in vivo*. In non-published work PEDOT, Figure 24, was intensively studied for its ability to differentiate neural cells and growth of primary cardiac cells.

Thiophenes in general have been used extensively for several applications in biomedical engineering, from probes for Alzheimer's disease to polymer solar cells.^{222,223} PEDOT in particular has found many interesting applications, including incorporation with clot binding proteins and to restoring electrical coupling in peripheral nerve gaps as

large as 20 mm.^{224,225} Degradable conjugated polymers of pyrrole/thiophenes tethered by ester bonds were also developed and showed a conductivity of 10^{-4} Scm^{-1} .²²⁶

Biocompatibility validation of PEDOT was seen in 2001 when used as electrodes for neural stimulation.²¹³ Further electrical stimulation of cells on the electrospun PEO/PEDOT mats was shown to drive differences in a neuroblastoma derived cell line (SH-SY5Y) adhesion and proliferation through possible changes in surface energy.²²⁷ It was also reported that neural cells could retain 80% viability when PEDOT was polymerized around them.¹⁶⁸

With this in mind, we investigated PEDOT coated polycaprolactone as a scaffold that potentially could have a positive effect on differentiating neural cells and then subsequently for cardiac cells. The PCL was electrospun as random and aligned fibrous meshes (see Table 3 for parameters), and then coated using both the spin coating and vapor phase coating techniques. In the former technique 150 μL of 10% Fe (III) TOS in butanol was added and then spin coated onto each mesh at 1200 RPM for 2 minutes. In the spin coated samples, 30 μL of 2,3-dihydrothieno(3,4- b)-1,4-dioxin(EDOT) was added to the Fe(III)TOS coated meshes, and also spin coated. For the vapor deposition, the EDOT monomer was placed on a glass dish, then the Fe(III)TOS coated meshes were further secured in an enclosed glass container and subjected to the vapors of the monomer, which was heated at 60 °C for 6 hours. The polymerization of PEDOT from the EDOT monomer occurs through oxidation of the monomer to the neutral polythiophene and then the oxidative doping of polymer by the Fe (III) tosylate.²¹⁸ There were significant differences in the coating between the methods, and the vapor deposition was found to result in the most homogenous polymerized coating of PEDOT on the PCL fiber, Figure 25.

Figure 25. Different coating strategies for PEDOT onto PCL fibers: a) spin coating, and b) vapor deposition. Scale bar 10 μm

Figure 26. Basic set up for electrical stimulation experiments with PEDOT coated PCL fibers.

Initial experiments used neural cells to study changes in proliferation or differentiation when subject to a current. Neuroblastoma derived cells, NDCs, are a sub-clone of the immortalized adult root ganglia and neuroblastoma cell lines, which are a model system for neuronal differentiation exhibiting properties of sensory neurons.²²⁸ These neural progenitor cells, previously optimized for differentiation in keratinocyte serum free basal media containing 0.1 μM dexamethasone, 50 $\mu\text{g/mL}$ ascorbic acid, 1.5 μM dimethyl sulfoxide, 20 μM cyclic adenosine monophosphate (cAMP), 0.5 $\mu\text{g/mL}$ nerve growth factor (NGF),²²⁹ were used to assess the ability of electrical stimulation to activate differentiation.

Figure 27. Differences in neural differentiation on the oxidized and reduced sides of the PEDOT coated PCL fibers (1 μm) after stimulation using 1.5V for 10 days. Scale bar: 10 μm

Following a similar set-up previously reported²²⁷, Figure 26, aligned and randomly coated PCL fibers with PEDOT:Fe(III)TOS showed differentiation on the reduced rather than on the oxidized side of the materials, Figure 27. Proliferation differences between the two sides had previously been reported using epithelial cell lines, as a result of lack of adsorbed fibronectin on the oxidized side of the materials.²¹⁵ Further tests showed that differentiation was only observed when supplementing the media with the growth factors (ascorbic acid, NGF and cAMP), as the NDCs did not differentiate after 10 days of electrical stimulation without differentiation media. The cells did not detach, but instead did not differentiate on the oxidized electrode. The differentiation seen on the reduced side of the sample could be due to the migration of charged media constituents to the negative electrode.

Repeating similar experiments with embryonic chicken cardiomyocytes (ECCM) were not as eventful. The cardiomyocytes did not proliferate on the PCL or PEDOT coated fibers. WST-1 proliferation assay showed a drastic decrease in cell numbers after day 1 of culture, Figure 28. Although PEDOT has been shown to be useful for neural regeneration, it appears to be of limited use for cardiac regeneration. Fundamental reasons behind the differences in cell survival on the material can be explained by the ECCM being primary cells, whilst the NDCs were immortalized. Also, it is imperative

for cardiac cells to be in close vicinity of each other for optimal proliferation, which is not as important for NDC proliferation.

Figure 28. WST-1 proliferation assay of ECCM cultured on 10 μ m PCL and PEDOT coated fibers.

Although the PEDOT was not able to sustain proliferation of the embryonic cardiomyocytes, a more encouraging result was obtained when investigating another, by now well-known conjugated polymer, developed for solar cell applications (TQ1). Although thoroughly characterized as a material for energy conversion, it has never before been shown to be biocompatible. In this thesis we have investigated the *in vivo* long-term biocompatibility and near infrared tracing properties of this polymer, as described in **Paper III**. TQ1 was also found to promote blood vessel infiltration and it further carries the notion that we can trace the polymer throughout its lifetime. This polymer could be of large interest in many regenerative medicine applications.

As mentioned before, to increase the conductivity of conjugated polymers, small molecules as dopants are added. The [6,6]-Phenyl C71 butyric acid methyl ester (PCBM) with TQ1 composite has been used as active layers in organic solar cells.²³⁰ Although the dopants had little effect on the viability of the cardiomyocytes, Figure 29, the amount of power needed to induce charge mobility in such thick fibers (10 μ m) resulted in temperatures that exceeded the limits for cell culture. The addition of the

dopant also made the polymer non-fluorescent and thus abolished the traceable functionality of the polymer, Figure 29b.

Figure 29. Myoblast cell line, C2C12, grown on a) TQ1 and b) TQ1/PCBM. There were no differences in the cell proliferation.

4.3 Conductivity in Collagen Scaffolds

To address cell adhesion and tissue mismatch head on, biopolymer scaffolds have always been better suited to this purpose as compared to synthetic polymers. However, biopolymers are not conductive and do not have suitable charge transfer abilities as electrode materials. One way to bypass this is to incorporate water-soluble conjugated polymers, or to add metal nanoparticles or nanowires into collagen meshes.

PEDOT-S (PEDOT with sulfonate group as dopant) is a water soluble conjugated polymer, of which a film gave a conductivity of 12 S cm^{-1} .²³¹ Coating of silk fibers 50-100 μm in diameter, has also been shown to be useful for fabrication of electrochemical transistors.²³² Addition of PEDOT-S during the growth step of collagen fibrils allowed for self-assembly of PEDOT-S coated collagen fibril, which could be imaged without conductive metal coatings in the electron microscope, Figure 30. However the resistivity was in the megaohm range. Nonetheless, other conductive nanomaterial were used to increase the conductivity of the material.

Figure 30. Scanning electron microscopy image of collagen fibrils grown in the presence of PEDOT-S. This image was taken without conductive coatings. Scale bar: 500 nm

Homogenous incorporation of gold and silver nanoparticle (AuNPs, AgNPs) in collagen fibrous scaffolds was accomplished by electrospinning. As mention previously, electrospinning of collagen, however, disrupts the native structure of the protein, making it a less attractive biomaterial. AuNPs and AgNPs were also photochemically synthesized in the presence of the collagen, and the NPs were effectively stabilized by the functional groups on the collagen.²³³ This however hindered collagen nucleation and growth into fibrils. Instead the use of *ex situ* synthesized silver nanowires (AgNWs), incorporated into the collagen gels, after nucleation, but just before fibril growth, allowed for a homogenous distribution of silver nanowires in the collagen mesh, Figure 30. These materials also showed mechanical properties suitable for embryonic cardiomyocyte growth and proliferation, whilst providing charge carrying capacities similar to the best conductive polymers. The added bonus of the AgNWs is that Ag is a known antimicrobial agent, used for many centuries, and found in many commercial products.²³⁴ In aiming for a conductive material, we created a mechanically apt,

antimicrobial, cell friendly, electrically active material. As such, the most multifunctional out of all the materials made in this thesis, and is described in **Paper IV**.

Figure 31. Electron micrographs, a) silver nanowires, scale bar: 10 μm , b) collagen/silver nanowires nanocomposites, * denotes the silver nanowires, scale bar: 1 μm .

“Science is a wonderful thing if one does not have to earn a living at it”
-Albert Einstein

Chapter 5. Summary of Papers

Four papers are included in this thesis; two published in peer reviewed journals, one under review, and one submitted. The first two papers address the efficacy of polycaprolactone materials as tissue engineering scaffolds, and the role of scaffold topography on protein adhesion and cell proliferation. The third paper focuses on developing a biocompatible material that could be traced using non-invasive optical techniques. The fourth paper is focused on developing collagen-based materials with high electrical charge carrier capacities, but with microbial resistance and the ability for primary cardiac cells to proliferate.

Paper I

Polycaprolactone-Thiophene Conjugated Carbon Nanotubes Meshes as Scaffolds for Cardiac Progenitor Cells

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J Biomed Mater Res B Appl Biomater, **2014**, 102, 1553-61

In an effort to make a scaffold that can buttress the scarring myocardium after infarct by providing mechanical stability, polycaprolactone (PCL) fibers were strengthened with single walled carbon nanotubes (CNTs). The fibrous meshes and thin films were made using electrospinning and spin coating, respectively. Thiophene conjugated single wall carbon nanotubes (T-CNT) were embedded into the electrospun fibers and sheets. The percentage of elongation to break increased by a factor of 2.5 with the addition of T-CNT. It was first found that isolated murine cardiac progenitor cardiac cells could not survive on the sheets but they grew better on the fibrous meshes. Immunofluorescent staining detecting stem cell markers showed greater expression of actin, ckit and Isl-1 on the PCL/T-CNT meshes than the PCL alone. However, expression of cardiomyocyte markers after differentiation showed no difference between the PCL and PCL/T-CNT fibers. Nonetheless, a Young's modulus of 11 MPa, and increased elasticity of the PCL/T-CNT fibers makes it a promising material for replacing the scarred myocardium.

Paper II

Influence of Polycaprolactone Scaffold Topography on Progenitor and Mesenchymal Cell Proliferation

Abeni Wickham, Sandeep Koppal, Staffan Dänmark, Daniel, Aili, Ebo de Muinck

2015 Submitted

In paper I we noticed that cardiac progenitor cells did not grow on sheets of polycaprolactone (PCL), only on the fibrous meshes. Therefore in this paper we tried to understand why varying topographies might lead to difference in cell adhesion. We investigated serum protein adsorption on varying topographies of PCL: a flat sheet, and PCL fibers with diameters of 20, 10, 1, and 0.5 μm . The sheets were fabricated by spin coating and the fibers by electrospinning. Both of these techniques tend to align polymer chains similarly. Surface energy calculations showed that PCL had a similar surface energy to Teflon, 20 mJ m^{-1} . The surface concentration of adsorbed proteins on sheets was significantly higher than on any of the fibers. However, this did not translate to cell adhesion and proliferation. Both the mesenchymal and cardiac progenitor cells were significantly less viable on the sheets and larger diameter fibers, than on the 0.5 and 1 μm diameter fibers. Cells cultivated on the scaffolds for up to 7 days showed no difference in growth when the materials were pretreated with serum. This study concludes that protein adsorption on PCL is not consistent with cell adhesion and proliferation on the materials.

Paper III

Near-Infrared Emitting and Pro-Angiogenic Electrospun Conjugated Polymer Scaffold for Optical Biomaterial Tracking

Abeni Wickham, Daniel Sjölander, Gunnar Bergström, Ergang Wang, Vijayalakshmi Rajendran, Camilla Hildesjö, Karin Skoglund, K. Peter R. Nilsson, Daniel Aili

Adv. Funct. Mater, **2015**, In press.

Regeneration by synthetic scaffolds is a fairly new feat in repairing diseased or depleted tissues. Scaffold topography and fiber morphology as well as surface energies seem to play a large role in protein adsorption and cell adhesion. For optical non-invasive tracing of the new materials through the regeneration process, a material should be excited and emit light in the optical window for tissue imaging, i.e. in the near infrared region of the spectrum. Fibrous meshes of poly [2,3-bis-(3-octyloxyphenyl)quinoxaline- 5,8-diyl-alt-thiophene-2,5-diyl] (TQ1), fit all the aforementioned requirements. Previously used as an active layer polymer for charge carrying, TQ1 was electrospun and fully characterized for cell adhesion, and subcutaneous murine evaluation of biological toxicity. *In vitro* studies showed that not only was it non cytotoxic but it attracted skeletal myoblasts and embryonic cardiomyocytes onto the fibers. After 90 days *in vivo*, the TQ1 fibers were shown to attract more blood vessels than a collagen hydrogel. The TQ1 emission peak is around 720 nm, and can be excited at 635 nm to reveal only the polymer, as tissue autofluorescence is not excited at this wavelength. Fluorescence lifetime imaging also showed relatively short lifetimes, 200-400 picoseconds, of which minimizes photobleaching and phototoxicity, enabling long-term tracking. Here we have shown that this new polymer can be used for tissue regeneration, where blood vessels, cell proliferation and non-invasive tracking are necessary.

Paper IV

Collagen-Silver Nanowire Composites as Electrically Active and Antibacterial Scaffolds for Embryonic Cardiac Cell Proliferation

Abeni Wickham, Mikhail Vagin, Hazem Khalaf, Sergio Bertazzo, Peter Hodder, Staffan Dnmark, Torbjrn Bengtsson, Jordi Altimiras, Daniel Aili

2015 Submitted

Another strategy to regenerate the myocardium is to provide electrical signals to couple the scarred myocardium to the synchronous contraction and relaxation of the healthy heart muscle. Conductive materials in electrodes, such as platinum and silver that are routinely used in implantable cardioverter defibrillators or pacemakers, mechanically mismatch the surrounding tissue. Therefore we developed a material that can provide a similar charge injection and storage capacity to that of metal and conjugated polymer electrodes, incorporated into extracellular matrix proteins that are mechanically apt to grow cardiomyocytes. Collagen meshes were fabricated using a tailored plastic compression technique that allowed for homogenous incorporation of silver nanowires (AgNW). Chronocoulometry characterization showed that the materials have charge storage and injection capacities of 2.3 mC cm^{-2} and 0.33 mC cm^{-2} . As a reference, platinum in pacemakers have a charge storage capacity of $208 \text{ } \mu\text{C cm}^{-2}$, and polypyrrole has a charge injection capacity of 1.17 mC cm^{-2} . Rheological fingerprints were established, and the collagen/AgNW nanocomposites retained a dynamic modulus in the 1 kPa range, which is apt for embryonic cardiomyocytes. Utilizing silver as a nanowire material we also added antimicrobial functionalities. The materials resisted colonization of *E.coli* and *S. epidermidis* bacteria. Here we have shown a material that could be remodeled whilst electrically coupled to the signals of the heart muscle.

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“Science is the quest to convince yourself and others of something you only guess to be true”

-Michael Brooks, Secret Anarchy of Science: Free Radicals.

Chapter 7. Prospective

Several different materials have been developed in this thesis and several conclusions can be drawn. The first of which, is that proliferation of primary cardiac cells is very low on polycaprolactone (PCL) compared to other materials developed in this thesis. PCL, however, can offer mechanical stability and be easily fabricated into scaffolds with various topographies and hence offers an interesting model for studies of the complex interplay between surface energies and fiber diameters on cell adhesion. Therefore at a fundamental level, PCL is still a useful polymer for research. Although not great for cardiomyocytes, it has still been shown to be useful in bone and other tissue engineering applications.

Secondly, if one recalls, in the early 20th century, the development of biomaterials was catalyzed by the industrial revolution. In this thesis the development of the near-infrared fluorescent polymer, TQ1, was driven by the need for renewable energy solutions through solar cell technology. Now, this polymer has not only been shown to be biocompatible, but its favorable interaction with freshly isolated embryonic cardiomyocytes, and its ability to attract blood vessels *in vivo*, offers another dimension to its capabilities.

Thirdly, telopeptide free collagen can also be formed into fibrous materials with the plastic compression techniques, and as such can be used to incorporate silver nanowires, or other nanoparticles, as a means to provide new functionalities. Considering that after infarct of the myocardium, proliferation of cardiomyocytes is one of the key challenges, this material offers a substrate for proliferating embryonic cardiomyocytes, as it is also mechanically similar to the healthy myocardial tissue. It is able to carry charges

throughout the scaffold and thus could facilitate to maintain the synchronous beating of the heart. Since the material is primarily made of native collagen, it can be remodeled to integrate into the host tissue while allowing for continuous contraction.

This is an exciting time for fundamental studies on biomaterials to be translated into clinical applications. Recently, it was shown that the salamander's ability to continuously regenerate is based on its macrophage population clearing senescent cells at the injured site.²³⁵ Also recently, cardiomyocytes with neonatal proliferative capacity have been located circulating in hypoxic conditions.²³⁶ How biomaterials strategies utilize the hypoxic environment to attract these cells to the injured myocardium, coupled with depleting senescent cells through regulated immune responses or local changes to the microenvironment will be a fruitful area of research. The changes from immune responses to regeneration, is a fundamental bottleneck that is steady widening. As biomaterial engineers, it is important that we utilize this continuous flow of new knowledge, that can certainly be translated into regenerative medicine therapies through cell-material interactions.

Publications

The articles associated with this thesis have been removed for copyright reasons. For more details about these see:

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