Electrospinning and electrospraying of unsaturated poly(macrolatone)s: alternative material for biomedical applications.

Fernando Cabral Sales de Oliveira
Royal College of Surgeons in Ireland, fernandooliveira@rcsi.ie

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Electrospinning and electrospraying of unsaturated poly(macrolatone)s: alternative material for biomedical applications.

A thesis for the degree of Doctor of Philosophy

Presented to

The Royal College of Surgeons in Ireland

By

Fernando Cabral Sales de Oliveira

School of Medicine
Department of Chemistry

Supervisor
Prof. Andreas Heise

Ph.D.
2018
Declaration

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree of Doctor of Philosophy (Ph.D.), is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

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Student number: 15144941

Date

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<tbody>
<tr>
<td>4MeCl</td>
<td>4-methyl caprolactone</td>
</tr>
<tr>
<td>AA</td>
<td>Adipic acid</td>
</tr>
<tr>
<td>ACY</td>
<td>Acyclovir</td>
</tr>
<tr>
<td>AFSCs</td>
<td>Amniotic Fluid Cells</td>
</tr>
<tr>
<td>AM</td>
<td>Ambrettolide</td>
</tr>
<tr>
<td>ASCs</td>
<td>Adult Stem Cells</td>
</tr>
<tr>
<td>BMP-2</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>Bp</td>
<td>Boiling Point</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CALB</td>
<td>Candida Antarctica Lipase B</td>
</tr>
<tr>
<td>DBSA</td>
<td>Dodecylbenzensulfonic acid</td>
</tr>
<tr>
<td>Dc</td>
<td>Dieletric constant</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
</tr>
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<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>DMEM+ GlutaMAX</td>
<td>Dulbecco’s modified eagle medium containing high glucose</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>DPP</td>
<td>Diphenyl phosphate</td>
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<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
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<td>DXO</td>
<td>1,5-dioxepan-2-one</td>
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<tr>
<td>EC-M</td>
<td>Enzyme-monomer complex</td>
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<td>ECM</td>
<td>Extra Cellular Matrix</td>
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<td>EDC</td>
<td>1-ethyl-3-(dimethylaminopropyl) carbodiimide</td>
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<td>EGMP</td>
<td>Ethylene glycol bis(3-mercaptopropionate)</td>
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<td>EtO</td>
<td>Ethylene oxide</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>Food and Drug Administration</td>
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<td>FSCs</td>
<td>Fetal Stem Cells</td>
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<td>Gl</td>
<td>Globalide</td>
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<td>-----------</td>
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<tr>
<td>GPC</td>
<td>Gel permeation chromatography</td>
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<td>HA</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric Acid</td>
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<tr>
<td>HDI</td>
<td>Hexadecalactone</td>
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<td>HDPE</td>
<td>High density polyethylene</td>
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<td>HFiP</td>
<td>1,1,1,3,3,3-Hexafluoro-2-propanol</td>
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<tr>
<td>IND</td>
<td>Indomethacin</td>
</tr>
<tr>
<td>iPS</td>
<td>Induced Stem induce pluripotent stem cell</td>
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<tr>
<td>ITS</td>
<td>Selenium premix</td>
</tr>
<tr>
<td>LE</td>
<td>Loading efficiency</td>
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<tr>
<td>MALDI-TOF</td>
<td>matrix-assisted laser desorption ionization time-of-flight mass spectrometry</td>
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<td>MES</td>
<td>2-N-morpholino-ethanesulfonic acid</td>
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<td>MH</td>
<td>Mercapto-1-hexanol</td>
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<td>Mw</td>
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<tr>
<td>n-ACA</td>
<td>N-acetylcysteamine</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium Hydroxide</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NPG</td>
<td>Neopentyglycol</td>
</tr>
<tr>
<td>P1</td>
<td>2,2 dimethoxy-2-phenyl acetophenone</td>
</tr>
<tr>
<td>P2</td>
<td>(2,4,6-trimethylbenzoyl) phenone oxide with 2-hydroxy-2-methylpropiophenone</td>
</tr>
<tr>
<td>PAm</td>
<td>Poly(ambrettolide)</td>
</tr>
<tr>
<td>PAN</td>
<td>Polyacrylonitrile</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PBT</td>
<td>Poly(butylene terephthalate)</td>
</tr>
<tr>
<td>PCl</td>
<td>Poly(caprolactone)</td>
</tr>
<tr>
<td>PCLA</td>
<td>Poly(L-lactide-co-e-caprolactone-diOH)</td>
</tr>
<tr>
<td>PCIEG</td>
<td>Poly(caprolactone-co-ethylene glycol)</td>
</tr>
</tbody>
</table>
PCIF  Poly(ε-caprolactone fumarate)

PDI  Pentadecalactone

PEO  Poly(ethylene oxide)

PEOT  Poly(ethylene oxide terephthalate)

PGA  Poly(glycolide acid)

PGI  Poly(globalide)

PGI-F  Fibres of poly(globalide)

PGI-XI  Crosslinked fibres of poly(globalide)

PGI-MS  Microspheres of poly(globalide)

PGI-XL-MS  Crosslinked microspheres of Poly(globalide)

PGI-CS  Core-shell microspheres of Poly(globalide)

PHDI  Poly(hexadecalactona)

PLA  Polylactide

PLCl  Poly(L-lactide-co-ε-caprolactone)

PLGA  Poly(DL-lactic-co-glycolic acid)
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PLLA</td>
<td>Poly(l-lactic acid)</td>
</tr>
<tr>
<td>PMMA</td>
<td>Poly(methyl methacrylate)</td>
</tr>
<tr>
<td>PPDI</td>
<td>Poly(pentadecalactone)</td>
</tr>
<tr>
<td>PSCs</td>
<td>Perinatal Stem Cells</td>
</tr>
<tr>
<td>PSf</td>
<td>Polysulfone</td>
</tr>
<tr>
<td>PTFALL$_{80}$</td>
<td>Poly($\varepsilon$-trifluoroacetyl-L-lysine)</td>
</tr>
<tr>
<td>PVA</td>
<td>Poly(vinyl alcohol)</td>
</tr>
<tr>
<td>PVP</td>
<td>Polyvinylpyrrolidone</td>
</tr>
<tr>
<td>RCM</td>
<td>Ring-closing metathesis</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine, Glycine and Aspartic acid</td>
</tr>
<tr>
<td>RI</td>
<td>Refractive Index</td>
</tr>
<tr>
<td>RM</td>
<td>Regenerative medicine</td>
</tr>
<tr>
<td>SCCO$_2$</td>
<td>Supercritical carbon dioxide</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electronic microscopy</td>
</tr>
<tr>
<td>sGAGs</td>
<td>Sulfated glycosaminoglycans</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>SPION’s</td>
<td>Supermagnetic iron oxide nanoparticles</td>
</tr>
<tr>
<td>TE</td>
<td>Tissue engineering</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TfOH</td>
<td>Trifluoromethanesulfonic acid</td>
</tr>
<tr>
<td>TGFβ3</td>
<td>Transforming growth factor β3</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>ValACY</td>
<td>Valacyclovir</td>
</tr>
<tr>
<td>XI-1</td>
<td>Bis(mercapto propionate)</td>
</tr>
<tr>
<td>XI-2</td>
<td>1,5- pentanediol</td>
</tr>
<tr>
<td>XPS</td>
<td>X-ray photoelectron spectroscopy</td>
</tr>
<tr>
<td>XRD</td>
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</tr>
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Objective

Polymers from macrolactones have structural similarities with commonly used biomedical polymers such as poly(caprolactone) but have not been fully utilised to date. In particular, unsaturated macrolactones offer possibilities of post-polymerisation modification and conjugation through double bond reactions. The objective of this project is to demonstrate the feasibility of processing unsaturated poly(globalide) into formats suitable for biomedical applications. The focus will be given to electrospinning and electrospraying techniques to obtain fibres and particles. A secondary objective is the demonstration of fibre/particles crosslinking and surface modifications utilising the polymer unsaturation and a preliminary validation of the advantages achievable by this process.
Abstracts

Chapter 1 – Introduction

Lactones are a promising class of monomers which enable production of a large number of polymers with different properties. This thesis aims to investigate materials derived from the unsaturated macrolactone globalide and their processing into variable structures by electrospinning and electrospraying. The first chapter provides an overview of the polymerisation techniques, spinning and spraying technology as well as the application of aliphatic polyesters in biomedical applications.

Chapter 2 – Optimization of electrospinning process using poly(globalide).

Poly(globalide) (PGl) has not be used in electrospinning to date. In this chapter the spinning conditions were optimised with the goal to obtain homogeneous fibres varying the polymer solution concentration, type of solvent, distance between the collector and the nozzle, voltage and flow rate. The results obtained showed that the process parameters played an important role in the process, which influence the morphology and the diameter of the obtained fibres. Moreover, cell viability tests confirmed that PGl is non-toxic, which qualifies it for the biomedical field.

Chapter 3 – Direct UV-triggered thiol–ene cross-linking of electrospun of poly(globalide)

Aiming to improve the mechanical properties of the PGl fibrous scaffolds, the possibilities of obtain a crosslinked fibres was evaluated. For this process, a thiol-ene click reaction during the spinning was applied. The samples obtained using the optimised process, showed that the crosslinking process improved the mechanical properties of the scaffolds. Another characteristic observed with the crosslinked fibres was the capacity of swelling with organic solvent without losing their fibre morphology, which enabled to perform a drug-loading by swelling. The cell viability tests confirms that the crosslinking process did not increase the toxicity of the samples and the drug releasing results confirms the possibilities of using this samples in drug delivery system.
Chapter 4 – Biofunctionalization of poly(globalide) fibrous scaffolds.

The surface hydrophobicity of the PGI scaffolds could limit cells attachment, promote rejection reactions and consequently interfere with the tissue regeneration efficiency. To overcome this, the surface biofunctionalization of PGI fibres was performed aiming to improve cells attachment and proliferation. For this process, initially amino functional groups were attached to the fibre surface and subsequently using EDC/NHS chemistry a sequence of amino-acid (RGD) was attached to the surface. XPS results confirm the increase of nitrogen concentration on the samples and the cell viability test demonstrated that the cells attachment after day 1 was 9.88 times higher than in the scaffolds without functionalization.

Chapter 5 – Electrospraying of poly(globalide) and the production of crosslinked and core-shell microspheres.

In this chapter, the possibility of producing PGI microspheres by optimisation of an electrospraying process was investigated. Subsequently, the production of crosslinked particles as well as core-shell structures using poly(ε-trifluoroacetyl-L-lysine) as hydrophilic shell and PGI as hydrophobic core was demonstrated. The results showed that it was possible to obtain particles with good morphology and homogenous diameters. Dye release test confirmed the possibilities of using PGI particles as a drug carrier. The core-shell samples were produced with good morphology; FT-IR and optical microscopy images confirmed the core-shell composition.
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Fernando Cabral Salles de Oliveira

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Chapter 1

Introduction
1. Introduction

1.1 Polyesters.

Since first described in 1930 polyesters have gained an important position among polymers produced in industry. Their versatility makes them adaptable for a broad range of applications from food packaging to biomedical devices. As for the latter, in particular aliphatic polyesters have been marketed as biodegradable polymers (e.g. poly(ε-caprolactone)) with variable mechanical and thermal properties.

Basically two techniques can be applied to synthesize polyesters, i.e. polycondensation, and ring-opening polymerization (ROP). Polycondensation is widely used to produce polyesters on industrial scale by reacting monomers with at least two functional groups such as hydroxy acids or diacids and diols groups, thereby promoting polymer chain formation under elimination of small molecules (e.g. water). Typically this technique is characterised by high temperatures, long reaction time, limited control over the molecular weight and polydispersity of the polymer, and also request the utilization of metallic complex catalysts (e.g. stannous octanoate (Sn(Oct)₂)). Alternatively, ROP of lactones offers possibilities to control the growth of the polymer chain as well as decreasing the polydispersity. It is, however, limited to aliphatic polyesters. The application of polyesters in the biomedical and pharmaceutical segment has increased in recent years due the possibilities of designing a wide range of different materials, such as, scaffolds for tissue engineering (TE) and particles for drug delivery system. As it is the technique applied in this work, a more detailed introduction to ROP to aliphatic polyesters and their bioaplications is provided below.

1.1.1 Ring opening polymerization.

ROP (Figure 1-1) have been extensively used to produce aliphatic polyesters and, when it occurs by chain-growth kinetics, this type of synthesis is considered a living polymerization, which is absent of breaking reactions. The polymers produced
under such conditions normally display a linear increase of molecular weight with conversion, and their molecular weight can be controlled by the monomer to initiator ratio (and conversion). In addition, this synthesis tends to result in a low dispersity and end-group fidelity of the polymer, which classify the ROP as a good route to produce and control the bulk material properties. A great number of lactones monomer, such as, glycolides, macrolactones, lactides, have been extensively used to produce polymers by ROP.

Figure 1-1. Schematic representation of ROP.

This type of polymerisation is typically initiated by a nucleophile and catalysed by different compounds (e.g. enzymes). One of the most prominent catalyst is Sn(Oct)$_2$ for which the mechanism is presented in Figure 1-2. In this example, the $\varepsilon$-caprolactone ROP initiates with the reaction with the impurities (e.g water) present on the system forming the stannous alkoxide species (1) and free 2-ethylhexanoic acid (3), posteriorly a stannous dialkoxide initiator (2) is formed and releases an equivalent of 2-ethylhexanoic acid (3). Furthermore, the presence of water in the system enable the catalyst deactivator formed with 1 and 2, decreasing the concentration of the catalyst activator and produce a stannous alcohol derivative (4). Finally, the reaction 2 with the monomer propagates the chain (5) initiating by the alcohol fragment and by stannous alkoxide. Moreover, during the polymerization, the equilibrium between activated and deactivated chain ends is promoted by the rapid exchange of protons among the stannous alkoxide and either hydroxyl groups initiators and hydroxyl chain end.
Figure 1-2. Adaptation Mechanism of initiation in Sn(Oct)$_2$ catalysed polymerization of $\varepsilon$-caprolactone, including (A, B) formation of stannous alkoxide initiator, (C) deactivation of catalyst with reaction by water, (D) coordination/insertion of monomer into the stannous alkoxide bond generating 1mer, and (E) chain transfer of active polymerizing center from 1mer to unreacted alcohol.\textsuperscript{8}

1.1.2 Enzymatic ring-opening polymerization.

Enzymes act as catalysts for metabolic reactions in the biosynthetic pathways in the living cells, producing natural polymers (e.g. polysaccharides, proteins, etc). In this context, enzymatic catalysts have been used to produce natural polymers in-vitro including polysaccharides like cellulose, amylose, xylan and chitin.\textsuperscript{9} Recently, enzymatic catalysts became an alternative for organometallic catalysts in the synthesis of synthetic polymers (Figure 1-3) in an effort to develop “green chemistry” approaches in polymer synthesis. Polymers without metallic contaminations can be produced decreasing the toxicity of the final product. Combined with the utilization monomers from renewable sources, enzyme catalysis is a promising strategy for sustainable materials and processes.\textsuperscript{10,11}
A great number of different enzymes have been tested in polymer synthesis but lipases are by far the most prominent class. For example, in a review paper published by Albertsson et al. around 30 different type of Lipases were described. Lipases are responsible to catalyse the hydrolysis of fatty acids esters and it is stability in aqueous as well as organic solvent environments makes the particularly useful to promote the reverse reaction, i.e. the formation of ester bonds. This type of enzyme usually comprises a lid or flap of amphiphilic $\alpha$-helix peptide sequence preventing access to the catalytic side. During the reaction, this lid opens and a hydrophobic supersubstrate is formed with the triade in the active site composed of a nucleophilic serine (Ser), which promotes a hydrogen bond in histidine (His) and aspartate (Asp)or glutamate (Ser105-His224-Asp187).

![Figure 1-3. Reactions scheme of eROP.](image)

Conventional ROP using organometallic catalysts has some limitation into polymerization of lactones of higher ring size (>6) produce polymers with low molecular weight. Duda et al. correlated this limitation to the thermodynamics of the system. While in larger lactones is driven by positive change in entropy ($\Delta S^\circ$), for smaller monomers the reaction is driven by negative change in enthalpy ($\Delta H^\circ$). The authors argue that the absence of ring-strain in macrolactones decreases the reaction enthalpy.
On the other hand, the polymerization of macrolactones is enabled by lipases, resulting in polymers with high molecular weight. This catalytic efficiency is correlated to the hydrophobicity of the macrolactones, which favours its recognition by the enzyme and consequently increases the ratio of the enzyme-monomer complex (EMC) formation and polymerization. Among the different Lipases, Candida Antarctica Lipase B (CALB) has been extensively used for the eROP of lactones. This reaction could be carried in many different solvents including toluene, fluorinated solvents, ionic liquids and supercritical fluids. Moreover, many different lactones (4 – 17 members) were polymerized using this catalyst, such as, e-caprolactone, δ-valerolactone, globalide, 12-dodecanolide, etc. In the eROP mechanism of lactones (Figure 3-1), the formation of an enzyme-activated monomer (EM) is considered the rate-determining step of the reaction. This EM is formed from the catalytic side of the enzyme (e.g. serine residue of lipases) and the monomer. The initiation of the reaction occurs through a nucleophilic attack of a nucleophile group (e.g. water or alcohol) onto the acyl carbon to form ω-hydroxy carboxylic acid that acts as a propagating species. Furthermore, the propagating species promotes new nucleophilic attack of terminal hydroxyl group onto the EMC and the polymerization proceeds by an “activated monomer mechanism”. The enzymatic process must be considered monomer activation and due the fact that the lipase does not distinguish between the ester bonds of the monomer (lactone) and the polymers the process is not controlled and polydispersity of the polymer produced tend to be higher.

1.2 Biomedical applications of aliphatic polyesters.

In the last years, aliphatic polyesters had been widely used in the biomedical and pharmaceutical segment due to their properties (e.g. biodegradability and good mechanical properties). Typical examples are poly-e-caprolactone (PCL), polylactide (PLA), poly(glicolide acid) (PGA) as well as theirs copolymers. Applications include sutures, bones screws, tissue engineering scaffolds as well as drug delivery systems. In this context, PLA, poly(DL-lactic-co-glycolic acid) (PLGA) has been extensively studied for drug delivery systems. The interest in PLGA is due to the fast and adjustable degradation ratio of this copolymer. However, when therapeutic release of proteins, peptides and DNA is desired, these polymers present some
disadvantages associated to their low interaction with the proteins and the acidification during the carrier degradation process, which negatively alters the efficiency of the drug delivery system.\textsuperscript{17} To overcome these drawbacks alternative techniques have been studied, for example, Samadi at al.\textsuperscript{18} compared the influence of uncapped and capped PLGA on the protein release profile. The authors treated the PLGA with NaOH (uncapped-PLGA) to degrades de polymer and consequently increase the presence of chain end group in the polymer, posteriorly they prepared particles encapsulating bovine serum albumin (BSA), using a double solvent evaporation technique. The results showed that the uncapped samples were more efficient for protein encapsulation as well as for the protein release. Another example of how to overcome the low interaction between the proteins and the this type of polymer was described by Choi et al.\textsuperscript{19} The authors prepared core-shell particles using PLGA and alginate for bone morphogenetic protein (BMP)-2 and dexamethasone dual release. The authors reported that this approach promoted the delivery of two different molecules with specific functions and also created possibilities of controlling the release profile by the positioning of the biomolecule in the core-shell domain.

Surface functionalization is another viable alternative to improve drug delivery systems, as it can provide amphiphilic materials and targeted carriers for a specific release. Gourdon et al. produced nanoparticles of poly(lactic acid)-co-poly(ethylene glycol)-NH\textsubscript{2} (PLA-PEG-NH\textsubscript{2}), and functionalized their surface with valine, glycylsarcosine, valine-glycine, and tyrosine-valine, aiming to enable PepT1 targeting and improving the absorption of the Acyclovir (ACY) or Valacyclovir (ValACY) drugs by the intestine, consequently increasing their bioavailability.\textsuperscript{20} The results showed that the particles functionalized with valine, increased permeability and the residence time of ACY in vivo.

Gene therapy is an important treatment for cancer, genetic disorder, and viral diseases. Nonetheless, the challenge of this treatments is to develop a system that enables a specific gene release pathway, for example, the RNA is quite instable in the bloodstream due to enzymatic degradation, limiting its delivery into the cell.\textsuperscript{21} Nelson et al. proposed copolyesters with pendant imidazolium groups using catalyst-free and solvent-free polycondensation of bromomethyl imidazolium-containing diol with neopentyglycol (NPG) and adipic acid (AA).\textsuperscript{22} The results showed that the DNA was
successfully bound to the imidazolium group on the polymer particles surface and good stability in aqueous environment was achieved. The in vitro experiments confirmed the efficiency of DNA transfect into the cell.

In addition, PCL has been widely used to produce scaffolds for tissue engineering. This polymer ideally matches the properties necessary for this kind of application, such as, good mechanical properties, biocompatibility, biodegradability as well as good processability.\textsuperscript{23} In addition, PCL is approved by Food and Drug Administration (FDA) as a safe material for biomedical applications. These characteristics made this polymer popular to produce samples by electrospinning, phase separation, solid freeform fabrication, microparticles, and also (due to its low melting point temperature) by blend production.\textsuperscript{24} However, PCL has some disadvantages, for example, its hydrophobicity that decrease the wettability and consequently alter the cell attachment. New technologies of surface modification have been shown to efficiently decrease the hydrophobicity of the PCL and make it more amenable for cell attachment. Luca et al. produced films of PCL and functionalized their surface attaching RGD (R: arginine; G: glycine; D: aspartic acid), using thiol (R-SH) chemistry.\textsuperscript{25} The results confirmed that the peptide immobilization on the film surface improve the cell adhesion and also could influence the cell morphology.

Other types of functionalized aliphatic polyesters have been studied for tissue regeneration application. Natarajan et al. synthesized 12 different samples basing them on poly(galactitol) crosslinked with glutaric acid, malic acid, maleic acid, succinic acid, citric acid, and tartaric acid.\textsuperscript{26} This introduced different functional groups to the sample and consequently altered their interaction with cells. 3D porous scaffolds were prepared using the salt leaching technique, and the results highlighted that all polymers produced were not toxic. However, the samples were crosslinked by citric acid and maleic acid promoted higher Young’s modulus and consequently decrease the release ratio suggesting their possible application for hard tissue regeneration.

1.3 Poly(macrolactones).

Macrolactones are defined as cyclic structures with 8 or more chain members and an ester function. These monomers were first described in 1927, with the isolation
of exaltolide 1 by Kerschbaum,\textsuperscript{27} since then these materials have been largely used by the industry in antibiotics and fragrances production. Some new efficient approaches for macrolactone synthesis have been developed, including lactonization of seco-acids, cross-coupling, ring-closing metathesis (RCM), and Nozaki-Hiyamakish reaction.\textsuperscript{27} The macrocyclization towards macrolactones starts with the activation of either the alcohol or the carboxylic acid terminal group, and depending on the structure different entropic and enthalpic factors are observed. For small lactones, the entropic factor is dominant due to the enthalpic characteristic from the strain energy in the ring being formed, and for larger lactones an opposite characteristic is observed, where the enthalpic factor decrease while the entropic factor increase due to the almost strain free formed structures.

The capability to substitute non-degradable thermoplastic products with polymers from macrolactones is an intensively discussed topic. Poly(pentadecalactone) (PPDI) is an example of this, its structure has a sequence of 14 methylene groups with a ester group in each repeat unit (Figure 1-4). It is comparable to high density polyethylene (HDPE) but having an ester group present. PPDI degradation occurs by hydrolysis of the esters groups in its backbone structure, and consequently promotes the chain breakage.\textsuperscript{28} eROP has been widely studied to produce a diverse range of functional polymers from macrolactones. Several different poly(macrolactones) have been synthesized by this techniques, for example, PPDI, (PHDi), poly(ambrettolide) (PAm), and poly(globalide) (PGl) (Figure 4-1).\textsuperscript{15,29} Poly(macrolactones) have also been extensively studied for biomedical application, for example, as materials for scaffolds in tissue engineering and particles for drug delivery system.\textsuperscript{15}
1.3.1 Polyglobalide.

PGl is an unsaturated, biocompatible and non-toxic aliphatic polyester derived from globalide (11/12-pentadecen-15-olide). This monomer is a 16-membered unsaturated macrolactone with two different isomers varying in the position of the alkene group between the 11 and 12 position. Globalide is used in the fragrance industry due to its musk odour, however, with the development of technologies for its polymerization PGl have become an interesting material for biomedical applications. In the literature, PGl has been synthesized using acid and enzymatic catalyst. Pascual et al. synthesized PGl using benzyl alcohol as initiator and three different acid catalysts including dodecylbenzensulfonic acid (DBSA), diphenyl phosphate (DPP) and trifluoromethanesulfonic acid (TfOH), varying their concentration between 0.5% and 10%. The results showed that for all investigated catalysts the molecular weight was influenced by the concentration and the reaction time, which increasing both parameters the molecular weight tended to be higher. However, with these conditions the polydispersity of the polymer reached values around 2.6. The authors correlated this with the higher viscosity of the medium, promoting transesterification reaction between esters groups present on the reaction. Several publications have reported enzymatic catalysis of globalide polymerization. van der Meulen et al. studied the eROP of different macrolactones, including pentadecalactone (PDI), hexadecalactone (HDI), ambrettolide (Am) and globalide (Gl). In addition, the thermal and mechanical properties of the samples were evaluated and their biocompatibility and
degradability tested. Comparing the thermal analysis results, PGl was less crystalline than the other samples, and the authors correlated this to the alkene groups on the backbone structure of the polymer, which decrease the possibility of ordered chain packing. Furthermore, the cytotoxic experiments confirmed that PGl is a non-toxic material in the cell cultures studied.

Supercritical fluids have been used to perform the eROP of globalide, as described by Guindani et al. who synthesized PGl, PCl and poly(globalide-co-e-caprolactone) (PGl-co-Cl) using supercritical carbon dioxide (SCCO₂). The copolymers were produced by varying the ratio between the monomers, using pure CO₂ and a mixture of CO₂ and dichloromethane (DCM) at the same pressure, temperature and reaction time. Subsequently, all samples were characterized by gel permeation chromatography (GPC), differential scanning calorimetry (DSC), nuclear magnetic resonance (NMR) and matrix-assisted laser desorption ionization time-offlight mass spectrometry (MALDI-TOF). Basing on the molecular weight of the samples, eROP performed with pure CO₂ was more efficient than the ones using a mixture of CO₂ and DCM. The authors attribute this to the higher mass transfer that the co-solvent system promotes during the reaction, which decreased the monomer concentration surrounding the enzyme pellets and consequently decrease the chain growth. In this context, Claudino et al. synthesized PGl-co-Cl by eROP and subsequently crosslinked the copolymers using photoinduced thiol-ene reaction. The results showed that the copolymerization of Gl and Cl increased the chains mobility and promotes a good distribution of the alkene groups along the polymer backbones structure, however, according with the authors, the formed crosslinked network was not uniform and a remaining crystallinity was observed.

Van der Meulen et al. aimed to increase the biodegradability of poly(macrolactones), and investigated the eROP of macrolactones (Am or Gl) with small biocompatible monomers (1,5-dioxepan-2-one (DXO) or 4-methyl caprolactone (4MeCl), varying the ratio between the monomers. Films of the copolymers were produced and thermally crosslinked using dicumyl peroxide. The results showed that the crosslinking process resulted in completely amorphous and insoluble networks and the incorporation of hydrophilic monomers in the backbone of the material increase their degradation ratio.
Ates et al. studied the possibilities of side-chain functionalization of PGl via thiol-ene click reaction. The authors used different compounds to functionalize the PGl, including mercapto-1-hexanol (MH) and N-acetylcysteamine (n-ACA), to introduce hydroxyl and amino groups respecting polymer structure. Moreover, Ates and Heise used ethylene glycol bis(3-mercapto propionate) (EGMP) to induce a photo-crosslinking on the PGl films, and subsequently functionalized these samples based on the procedure described above. Finally, Ates et al. studied the production of functional brush-decorated films using for bioconjugation. Initially, the authors prepared a crosslinked films according to the procedure previously described, posteriorly these samples were successfully grafted with poly(acrylic acid) and the fluorescent protein and chitobiase were immobilized on the surface of the films.

2. Electrospinning.

Electrospinning was first described in 1934, as “a process and apparatus for preparing artificial threads.” Initially this technique was not commercialized due to the great competition with the mechanical drawing process aiming to produce fibres. In the last decades, electrospinning has been improved and optimized, offering possibilities for its application in different fields. According to literature statistics, available on web of science database, in the last 10 years the number of publications containing the keyword “polymer electrospinning” has risen from 282 in 2006 to 1165 in 2016, (Figure 1-5) which represents an increase of 413%.
Figure 1-5. Number of published research articles containing the keywords “polymer” and “electrospinning”, from 2006 to 2016 (September, 2017, Web of Science Database).

This confirms that electrospinning is a promising technique with a wide applications range, such as filters,\textsuperscript{38} photonics and electronic devices,\textsuperscript{39} as well as scaffolds for regenerative medicine and drug delivery systems.\textsuperscript{40} Furthermore, with the development of new hardware, the variation of fibre shapes has led to further versatility, for example, coaxial tips producing core-shell fibres and nanotubes.\textsuperscript{41,42} The new technologies applied in electrospinning machines enabled to vary the fibre dimensions from micro and nanoscale, which significantly change the fibres properties. Additionally, further improvement of the electrospinning technique enabled the production of fibres an industrial scale.\textsuperscript{40}

Fibre formation in the electrospinning process uses a jet of a polymer solution and a high voltage field. Due to the potential difference between the tip and the collector the fibres are dried and collected. During the process the polymeric fluid is pumped through a metallic tip, where an electric charge is applied. When the surface tension is overcome, the Taylor cone is formed and a liquid jet is spun (Figure 1-6). However, process parameters play an important role in the fibre formation, such as, polymer solutions properties, voltage, and distance of the tip, which necessitates the
optimisation of different parameters to obtain well-defined fibres. The characteristics described above classify electrospinning as a versatile technique, that enables the processing of different polymers for the production of fibres with a particular morphologies and applications.

Figure 1-6. Schematic illustration of the electrospinning process.

2.1 Electrospinning parameters.

There are three different classifications that govern the electrospinning process such as parameters, the process parameters, solution parameters, and ambient parameters. The first one is correlated to the voltage, flow rate, distance of the tip, collector type, as well as shape and diameter of the nozzle used. The second group is linked to the polymer solution properties, such as viscosity, molecular weight and solvent properties. The last one is associate with the external factors such as temperature and humidity.
2.1.1 Voltage.

The voltage is crucial for the fibre formation during the electrospinning process. It is responsible for the Taylor cone formation that occurs when the surface tension of the polymer solution is overcome by the charge and subsequently the fibre is formed (Figure 1-7).

![Figure 1-7](image)

Figure 1-7. Adaptation of digital images (A-C) and illustrations (D-F) from the three stages of deformation of poly(vinylpyrrolidone) droplet under the influence of an increasing electric field forming the Taylor cone. 44

As illustrated in Figure 1-7, the high voltage applied to the polymer solution is held creating electrostatic repulsion in the drop is created. Due to the opposite charge on the collector (counter electrode) the liquid jet is stretched in the its direction, forming and drying the fibres. 45 In the literature many papers controversially describe how the voltage influences the fibre morphology. One of the initial papers describing electrospinning, published by Doshi and Reneker, 46 reported the production of poly(ethylene oxide) (PEO) fibres and the authors observed that voltage variation did not influence the fibres diameters. Others papers described that raising the voltage results in fibres with larger diameters. 47,48 Zhang et. al. studied the influence of the electrospinning parameters on poly(vinyl alcohol) (PVA) fibre morphology and the authors reported that by increasing the voltage the produced fibres presented larger diameters. 47 The same observation was described by Meechaise et al. who studied the
production of poly(desaminotyrosyltyrosine ethyl ester carbonate) (poly(DTE carbonate)) fibres by electrospinning. The authors described that raising the process voltage from 10 to 25 kV, fibre diameters varied from 2.5 µm to 5.4 µm, respectively. Additionally, the high voltage can contribute to bead formations and to increase in the fibres diameter distribution. According to Deitzel et al. this occurs because the stability of the liquid jet can be disturbed under high voltage resulting in the defect fibres. On the other hand, some papers describe that under higher voltage the fibres diameters tend to decrease. Mengzhu et al. studied the influence of the electrospinning parameter on poly(L-lactide-co-ε-caprolactone-diOH) (PCLA) fibre formation, and it was observed that the fibre diameters decrease with higher voltage. In another paper, Katti et al. studied the production of nanofibres for drug delivery systems, and the results obtained show that under higher voltage the PLGA nanofibre diameter decreased. These example demonstrate that the applied electric field controls the fibres formation during the electrospinning process, and its evaluation is crucial to reach an optimum process that results in homogenous fibre diameters without morphological defects.

2.1.2 Flow rate.

The flow rate also play an important role for the fibre diameter, shape and porosity. It is known that if the flow rate is not sufficient to maintain the Taylor cone shape on the tip during the process, the fibres formation is interrupted and the polymer solution tends to dry on the tip, blocking the liquid jet. The optimum flow rate is recognised by a balance between the rate of solution pumped through the needle and liquid drawn off it by the electrostatic field, resulting in well-shaped fibres with a homogenous diameter distribution. In the literature, many papers describe that at higher flow rates the fibre diameters tend to increase. They correlate these results to the lower conductivity of the polymer solution in the tip, which results in a thick liquid jet. Furthermore, bead formation can also occur by raising the flow rate. For example, Yuan et al. studied the morphology of polysulfone fibres and observed that beads were formed when the flow rate was increased from 0.4 to 0.6 ml/h. The authors correlated these results to low stretching force on the system. For polymer solutions using solvents of high volatility, its fast evaporation during the
electrospinning can result in the formation of pores in the fibres. Megelski et al. observed that in higher flow rates the pores formed presented larger diameters, and they defined that a thick liquid jet and the low stretching force during the electrospinning created conditions favouring their formation.\textsuperscript{56} Also ribbon-shape fibres can be formed by increasing the flow rate. This occurs due to a solid skin formed outside of the liquid jet during the electrospinning, which keeps the solvent inside the fibre and slow solvent evaporation results in skin shrinkage and consequently in a flat ribbon shape. This phenomenon was described by Chen et al. who studied the production of chitosan/PLLA membranes using electrospinning.\textsuperscript{60} In addition, at higher flow rate the liquid jet do not have enough time to fully dry, resulting in wet fibres. Chowdhury and Stylios\textsuperscript{61} studied the influence of the electrospinning parameters on the morphology of nylon 6 fibres, and they reported that the deposition of wet fibres on the collector created fused areas at the contact points.

2.1.3 Distance of the tip.

The distance between the tip and the collector defines the jet path and travelling time of the polymer solution, and it has a strong influence on the fibre drying process. To reach the optimum parameter it is necessary to evaluate the solvent dielectric constant as well as the solvent volatility, as their variation influence the electrostatic field strength.\textsuperscript{52,60,62} Bosworth and Downes\textsuperscript{63} studied the influence of the electrospinning parameters on poly(\(\varepsilon\)-caprolactone) fibres using solutions prepared in acetone. The results showed that the fibre diameters decreased and the fibres morphology had less imperfections when processed using large distances. The authors attributed these results to the faster solvent evaporation and the base pronounced fibre stretching. Similar results were described by Ayutsede et al. who studied the production of silk nanofibres by electrospinning.\textsuperscript{64} It is well known that the distance of the tip can also effect the formation of bead during the electrospinning.\textsuperscript{65,66} This characteristic is correlated to the liquid jet instability during the process, which interferes with the surface tension overcoming the forces responsible to the continuous Taylor’s cone elongation. Mazoochi et al. described beads formation on the polysulfone (PSf) nanofibre, performed at lower distance of the tip.\textsuperscript{67}
2.1.4 Type of collector.

With the technologies aiming to improve the process and opening new possibilities of application, different types of collectors have been developed. Initially, fixed flat collectors were used in the electrospinning machines. However, with the aim to produce fibres with diverse properties, morphologies as well as to increase the productivity, collectors with conveyor belts, rotating cylinders, petri dishes (Figure 1-8) were tested and also different conductive surface materials were developed.

![Figure 1-8. Sketches of the main possible types of electrospinning collectors.](image)

Conveyor belt collectors (Figure 1-8-A) the possibility to scale up the fibre production, improve their dispersion through the electrospun mats, and also resulted in fibres with homogenous diameters. Santos et al. used an electrospinning machine prototype that combined the printing needle with an conveyor collector. The authors described that fibres produced by the machine were more homogenous, aligned and scalable when compared with fixed flat plate collectors, hence increased the mechanical properties of the fibre mats. The rotation cylinder collector (Figure 1-8-B) is cited by several studies in the literature. This type of collector is well known.
to results in aligned fibres, which improve the mechanical properties of the electrospun mats as well as their suitability for cell attachment. Moreover, the cylinder diameter opens possibilities to produce scaffolds for vascular reconstruction as described by Tillman et al. The authors produced cylindrical scaffolds using PCl and collagen, these scaffolds are an alternative to traditional prosthetic vascular graft material. Petri dish plate collectors are usually applied to electrospinning process performed with polymers that require to be collected in some liquid to avoid the deformation of the fibres. This type of collector can be used to obtain crosslinked fibres as well, Thielke et al. studied the production of polybutadiene fibres crosslinked using thiol-ene click chemistry, and the authors used a plate collector with a solution of sodium chloride in methanol thereby letting the fibres float and increase the irradiation time of UV curing during the electrospinning process. Moreover, this collector type was used to produce particles when electrospraying is performed, for example, Duong et al. used a dish collector to produce micellar nanoparticles encapsulating the supermagnetic iron oxide nanoparticles (SPIONS’s).

2.1.5 Nozzle type.

The nozzle in the electrospinning machine is responsible for the charge of the polymer solution and consequently it has a great influence on the Taylor cone formation. Among the different types, the single, co-axial, tri-axial and multi-nozzle are the principal class of electrospinning nozzle (Figure 1-9).

![Figure 1-9. Sketches of the main possible types of electrospinning nozzle.](image)
Single nozzles of different diameters are commonly found in electrospinning machines. Heikkilä and Harlin,\textsuperscript{85} studied the influence the electrospinning parameters on the polyamide-6 fibres morphology, and they observed that nozzles with smaller diameters tend to decrease the fibres diameters. Multi-axial nozzle were developed to produce fibres or particles using different polymers combining their properties.\textsuperscript{36} Fibres and particles with core-shell structure are interesting materials for biomedical applications as these materials are bi-functional. Yanzhong et al. studied the production of core-shell nanofibres using Gelatin (core) and the PCl (shell).\textsuperscript{86} The authors used co-axial nozzles to perform the electrospinning and they demonstrated that the obtained samples could encapsulate a hydrophilic drug in the core, protecting it from degradation and ensuring its efficiency during release. Multi-nozzles were developed to scale up fibre production and optimize the electrospinning process. Varesano et al. studied the electrospinning with multi-nozzle using poly(ethylene oxide) solutions.\textsuperscript{84} The authors described that the nozzle arrangement can influence the continuous fibres formation because depending the distance between the nozzle, Coulomb repulsion can occur interfering the polymer solution spun.

2.1.6 Polymer solution properties.

The polymer solution properties are crucial to the fibre formation during the electrospinning, for example, viscosity play an important role in the Taylor cone formation.\textsuperscript{87} The polymer solution viscosity is dependent on the molecular weight and the polymer concentration. On this context, the concentration could be described by four different regimes; dilute, semidilute unentangled, semidilute entangled and concentrated. The transition between dilute and semidilute unentangled regimes is called the overlap concentration ($C^*$), and the transition between the semidiluted unentangled and semidiluted entangled is known as entanglement concentration ($C_e$). During the first transition, molecules start to overlap with one another and by increasing the concentration the entanglement of the chains starts to form by decreasing the hydrodynamic volume of the molecules, which is essential for the fibre formation during the electrospinning.\textsuperscript{88} In the literature\textsuperscript{88–90} it is well described that to reach an stable electrospinning process a concentration equal or higher than $C_e$ is necessary. This is rationalised because the polymer solution viscosity should be
sufficiently high to withstand the electrostatic repulsion forces during the fibre formation, avoiding bead formation by the continuous nozzle jet. Tiwari and Venkatraman \(^{88}\) studied the importance of the viscosity parameters on the coaxial electrospinning. They used the PVA as core and PLGA as shell, and the results showed that to obtain uniform fibres either core and shell solutions should have concentrations above \(C_e\).

As mentioned before, the \(C_e\) is responsible to maintain the polymer chain entanglement to form fibres instead the drops. The polymer molecular weight (Mw) has an important influence on this process due to its direct effect on the viscosity. In the literature \(^{91,92}\) some papers describe that independently of the other variables, increasing the Mw, the bead formation during the electrospinning decreased. However, decreasing the Mw of the polymer the fibre diameter and the porosity tended to decrease, as described by Pant et al. who studied the influence of the Mw of Nylon-6 on the fibres morphology. \(^{93}\)

Additionally the solvent conductivity can influence the fibre formation during electrospinning. Solvents with high dielectric constants require lower voltages to generate the critical density of free charges to initiate the required bending instability for fibre formation. In some cases the addition of salts is necessary to control the conductivity of the polymer solution to perform the electrospinning. \(^{40,94}\) Lee et al. studied the production of PCl nanofibers using solvents with different dielectric constant and the results showed that increasing the conductivity of the solvent, the fibres diameters and the beads formation decreased. \(^{95}\)

2.1.7 External factors.

External factors could interfere with electrospinning and their control is crucial during a process optimisation. Humidity directly influences the solvent evaporation creating condition for increased fusion of the fibres. Pelipenko et al. studied the effect of humidity on the electrospinning of PVA and PEO dissolved in DI water and acetic acid (3\% v/v), respectively. \(^{96}\) The results define that with the humidity increase the standard deviation of the fibres diameter distribution was 122\% for PVA and 57\% for PEO.
Temperature is an equally significant aspect in electrospinning as it can influence the polymer solution viscosity and the solvent evaporation rate, which consequently change the fibres morphology. For the combination of these factors, the fibre diameters decrease with the temperature increasing, as described by Yang et al. who performed electrospinning with polyacrylonitrile (PAN) and polyvinylpyrrolidone (PVP) solutions at different temperature. Moreover, the electrospinning performed at higher temperature could decrease the bead formation, as described on the results published by Desai and Kit, the authors attribute these results to the improvement on the stabilization on the liquid jet and consequently the spinnability of blend solution.

3. Electrospraying

The electrospraying or electrohydrodynamic process is a technique to produce particles and basically uses the same system as electrospinning. The particles produced could be from nano to micro scale, depending on the applied parameters. The process consists of breaking down a flowing polymer solution in an electric field (Figure 1-10).

![Figure 1-10. Schematic illustration of the electrospraying process.](image-url)
The morphology of the particles produced by electrospraying can be modulated by the processing parameters applied during the process. Essentially, the variable process parameters are the same as described for electrospinning, and their variation can define the size and morphology of the produced particles. Among the parameters that can influence the electrospraying results, the molecular weight has a significant effect on the particles morphology (Figure 1-11). For low Mw, the particles tend to shrink during the drying process due the mobility of the small chains. That could create new aggregation centres and form polymer debris. On the other hand, polymers with higher MW tend to form spherical particles as a consequence of the chains forming particles.

Figure 1-11. Schematic illustration of the molecular weight influence on the particles formations during the electrospraying process.

Increasing the flow rate in the electrospraying process tends to increase the diameter and the size distribution of the formed particles, as described by Enayati et al. for polymethylsilsequioxane. Songsurang et al. reported that the process voltage
decrease the particles size of Doxorubicin-Chitosan-Triphosphate particles by electrospraying due to the breakdown of the jet in smaller particles. Another effect observed by the authors was that at higher voltages the particles formed were oval instead of spherical, and they attribute this result to the jet stretching during the process. According to the literature using nozzle with smaller diameter result in a more stable electrospraying process. In addition, decreasing the nozzle diameter the formed particles presented better spherical morphology and a decreased size distribution.

It was also reported that at a shorter distance between the nozzle and the collector the electrospray process requires a higher voltage, and consequently particles with smaller diameters are formed. However, if the process distance is not large enough to dry the particles, wet material could start to agglomerate on the collector. The method used to collect the particles during the electrospray can also interfere with their morphology. There are many different setups to collect the particles from aluminium foil to dish plate varying the media to collecting them. The disadvantage of aluminium foil is the possibilities of particles agglomeration. On the other hand, using a collection media, commonly improvement of the morphology and the dispersion of the particles is observed. However, the surface tension and the interaction between the residual solvent and the collection solution might create interference. This was reported by Gao et al. who studied the influence of the collection media on the core-shell particles morphology. They observed that in cases of poor interaction between the polymer solvent and collector medium created conditions for the polymer chains to contract (due to the superficial tension of the collector media) resulting in hemi-spherical structures.

4. Electrospinning and Electrospraying for biomedical applications.

4.1 Regenerative medicine.

Regenerative medicine (RM) is considered a new field in medical research, although its principles have been known for a long time. Reports in the literature show
that the idea of RM started in 1938, with the publication of a book entitled “The cure of new organs” by Alexis Carrel and Charles A. Lindbergh.\textsuperscript{111} RM progress could have significant impact on people’s quality of life, by increasing life expectancy and creating new possibilities for disease treatment. The development of new technologies for RM is aiming to reduce treatment time and side effects, which could decrease the public expenses in the health sector.\textsuperscript{112} RM is a field that blends different sciences, such as tissue engineering (TE), cell transplantation, stem cell biology, biomechanics prosthesis, nanotechnology and biochemistry.\textsuperscript{113} Three principals should be well understood to ensure the success in RM procedures (Figure 1-12). The first is the manipulation, classification and cultivation of cells. The second is related to the substrate (called scaffolds) comprising either biological or synthetic materials to mimic the extra cellular matrix (ECM) and creating conditions for the cell growth. The last one is the combination of the scaffolds seeded with cells and implanted into the patient.\textsuperscript{113}

Figure 1-12. Adaptation of schematic representation of principles of regenerative medicine (RM).\textsuperscript{113}

The development of biomaterials is crucial to advance RM. Their initially concept was to provide treatments and replace any kind of tissue, organs, or functions of the body. However, this concept changed with new technologies available and it moved to a class of materials that interact with the body and influence the biological process for the tissue regeneration.\textsuperscript{114} These materials could be present on the ECM and their combination with cells results in the tissue formation. The ECM promotes the support to the cells proliferation, differentiation, migration, etc. Types of
biomaterials used in RM, fall into three different groups, ceramics, natural polymer and synthetic polymers.\(^{114,115}\) In the first group, ceramic materials are usually applied to produce scaffolds for hard tissues, for example, hydroxyapatite (HA) is used for bone regeneration.\(^ {116}\) The ceramic scaffolds normally present good mechanical properties, low elasticity, and hard brittle surfaces. Furthermore, for bone tissue regeneration HA shows good biocompatibility, and its interaction with osteogenic cells promote their differentiation and proliferation. However, in procedures that require shaping (e.g. dental implants) the brittle property of ceramics limits their application, and also the cells developed on these scaffolds have some limitation in remodelling due to the properties (e.g. high mechanical properties) of the material used to produce the scaffolds. In addition, some of these materials are primary constituents of bones (e.g. HA) and their degradation rate is difficult to control.

Natural polymers are extensively used in RM due their biocompatibility, good cell adhesion and biodegradability. This kind of materials can be integrate easily into the tissue during regeneration. They are obtained from living organisms and can be classified into polysaccharides, proteins, polynucleotides, polyisoprene and polyesters.\(^ {117}\) However, natural polymers suffer from some limitations, such as poor mechanical properties, large scale production, obtain homogeneous and reproducible scaffolds.\(^ {114,117}\)

Synthetic biodegradable polymers become a viable alternative for natural polymer because they could overcome some limitations found in natural polymers, such as production in large scale, good mechanical properties, and reproducibility of homogenous scaffolds. During recent years, the number of synthetic biopolymers applied in RM research increased with focus on polystyrene, poly-l-lactic acid (PLLA), PGA and PLGA.\(^ {55}\) Despite these advantages and versatility this class of material face some limitations correlated to the risk of rejection or incompatibility with the biological medium.\(^ {118}\) The ideal biomaterial for RM should allow full integration between the cells and molecular biology. Aiming at this integration, strategies combining synthetic biopolymer with molecular cues that mimic the characteristic of extracellular microenvironments were used.\(^ {119}\) One example of these strategies is the surface functionalization of synthetic biopolymer using RGD, that promotes the improvement of the cell adhesion, the migration and differentiation.\(^ {120}\)
4.1.1 Scaffolds for Tissue Engineering (TE).

Tissue engineering (TE) is a complex science that aims to regenerate, maintain or improve functions of tissues or a whole organ. Basically, this science combines concepts of engineering and biology creating conditions to build up and restore tissues lost to injuries or diseases. It utilizes essentially three tools: cells, biomaterials and biomolecules. The combination of these tools create condition to produce scaffolds mimicking the ECM for the reproduction of healthy cells in vitro and subsequently implant them.

In the last years, the development of new materials and process technologies increased the versatility of scaffolds applied to TE. If the optimum combination between the mechanical and biomimetic properties can be reached new application possibilities would arise. In addition, the materials used to produce scaffolds must present properties that support conditions for cell growth. Among these properties biocompatibility is one of the most important, because it translates to low toxicity and consequently minimize the inflamations and immune responses from de cells.

In the literature, many different process technologies as used to produce scaffolds, such as 3D printing, supercritical fluids, freezing drying, melt and solution base electrospinning, etc. All these techniques produce porous scaffolds with different shapes and morphology (Figure 1-13) attending specifics demand of the cells cultivated.
The morphology of the scaffolds influence the cell development, as it is responsible for the nutrient and oxygen distribution during cell proliferation. In addition, the scaffold morphology plays an important role in terms of the surface area, which directly influences the substrate available to the cell attachment. In this context, fibrous scaffolds have proven really versatile compared with the other types. This versatility is correlated to the possibilities of manipulating the fibres diameter from micro to nano scale (which influence the surface area) and also to produce scaffolds with different fibre orientation (aligned or random). Furthermore, controlling the fibres orientation provided possibilities of guidance to cultured cells that consequently influence their differentiation and elongation.

4.2 Electrospinning for biomedical application.

Electrospinning is a promising process to produce fibrous scaffolds for tissue engineering. This technique offers possibilities of processing both natural and synthetic biopolymers, and also combine their properties producing scaffolds with blends or core-shell fibres. Electrospinning presents advantages such as variation of
fibre diameters, porosity and porous size, fibres orientation, functionalization, crystallinity, mechanical properties and degradation.

4.2.1 Fibre diameter.

The variation of fibres diameters on the scaffolds has great influence on the cell proliferation, due to the variation of the surface area available to the cell attachment. With decreasing fibre diameter the surface area tends to increase and consequently the cell adhesion improves. The production of fibres from synthetic and natural biopolymer on micro and nano scale is extremely interesting for the TE. On this scale the fibres offer topologies to the cells that mimic the native ECM, which improve the recognition and the cells adhesion on the surface. Kwon et al. studied the production of nano and microfibers of copolyesters and the influence of their diameters on the scaffolds properties and the cell adhesion. The results showed that the high surface area of nanofibres enable the adhesion, spreading and proliferation of the anchored cell.

However, during the electrospinning process the production of the not homogeneous an beaded fibres could influence the cells adhesion. Moroni et al. evaluated the effect of poly(ethylene oxide terephthalate)-poly(butylene terephthalate) (PEOT/PBT) fibres morphology on the cells proliferation. The authors used fibre with diameters from 1 to 270 µm, and they observed the best results for cells proliferation occurred on samples with 10 µm, and in contrary to what was expected, the cells seeding efficiency was lower in scaffolds with smaller sizes fibres. The authors associated these results to the bead formation in fibres smaller than 10 µm, which suggest that the cells recognize not just the optimum fibres dimension but also the nano topography of the fibres surface. This demonstrates that for tissue engineering a process optimization aiming to obtain fibres with homogeneous diameters and without bead formation is crucial for the scaffolds efficiency.

4.2.2 Pore size and porosity.

The pore size play an important rule for the scaffold efficiency, as it influences the binding, migration, vascularization, morphology and phenotypic
expression of the cells. In the literature a common hypothesis defines that the ideal porous size should be large enough for the cell migrations into the structures, but small enough to provide a specific surface for a minimal ligand density between the cells.\textsuperscript{133}

In the literature, some techniques to vary the porosity of fibrous scaffolds were described. Firstly, Pham et al. produced PCl scaffolds with alternating layers of nano and microfibers, increasing the average of the porous size.\textsuperscript{134} Secondly, another technique used by Kim et al. applied salt leaching to increase the porosity on the fibrous scaffolds.\textsuperscript{135} Thirdly, Leong et al. processed the polymers using cryogenic electrospinning to promote a high porosity of the scaffolds.\textsuperscript{136} Lastly, some procedures were used to increase the porosity post electrospinning, including ultrasonication (which decrease the packing density of the fibres)\textsuperscript{137} and the production of scaffolds mixing sacrificial and stable fibres (which removing the sacrificial fibres increases the porous sizes).\textsuperscript{138}

Controlling the pore diameter is an important procedure in the scaffolds production because for some cell types the binding process could be improved with a specific porous size, for example, endothelial cells bind better to scaffolds with porous smaller than 80 mm, while fibroblasts tend to bind in scaffolds with porous bigger than 90 mm.\textsuperscript{133} Furthermore, some cells bind preferentially to scaffolds with a specific rage of pore sizes, for example vascular smooth muscle cells which prefer pore diameters between 63-150mm.\textsuperscript{139} In addition, a great number of cell types have preference to bind with scaffolds with pores larger than their own sizes. This preference is correlated to the bridging process of these cells, that request adjacent cells to act as a support structure, for example, the osteoblast adhesion.\textsuperscript{140} Based on all these characteristics, to design a scaffold for TE each type of cell requires specific pores size and it is crucial to consider them.

4.2.3 Fibre orientation.

The topography of a scaffolds plays an important role for the cellular activity. Its variation directly influences the attachment, morphology and proliferation. For fibre scaffolds, their orientation has a great influence on the cell adhesion, development, and differentiation. Moreover, the orientation of the fibres produced by electrospinning could be differentiate by using different collectors. For random
oriented fibres production, a stationary collector is used and for aligned oriented fibres a rotatory collector is applied. In this context, it is well known that for high oriented tissues aligned oriented fibres are required. In type of tissue the aligned fibres can stimulate the cells and consequently allow their vasoconstriction and vasodilation.55 Yin et al. studied the influence of the fibres orientation on the tendon stem cell differentiation, and the authors reported that with aligned fibres a microenvironment that promotes the cell differentiation was created, which also promoted the mechanotransduction of the cells.129

The possibilities of joining aligned and randomly oriented fibres in the same scaffold could provide a platform to treat injuries on tendon-to-bone insertion. Xie et al. studied this combination and produce “aligned-to-random” scaffolds, and the results shows that the cells were oriented according to the topography of the scaffolds, i.e. in aligned fibres the cells grew in longitudinal direction and the random fibres the cells were polygonal oriented.141 Furthermore, the scaffolds with random oriented fibres can increase the infiltration, provides the anchoring of the cells and also higher porosity, as described by Komvopoulus et al. 142

4.2.4 Surface modification.

Surface modification techniques increase the versatility of the scaffolds produced with synthetic biopolymers and enhance their feasibility in TE. This enhancement occurs because by surface functionalization the synthetic polymers properties can be combined with biological molecules that stimulates the cells adhesion and ECM production. Moreover, this modification can overcome the poor cell-adhesion due to the hydrophobicity on the fibres surface.

Many procedures could be applied to provide surface modification, including chemical treatment, plasma depositions, coating the fibres with proteins (e.g. collagen), and functionalization attaching bioactive molecules on the surface. Chemical modification requires treatment attacking the fibre surface using some chemical reagents, as described by Boland et al. who treated the surface of PGA fibres using concentrated Hydrochloric Acid (HCl).143 The authors reported that this procedure promoted a surface hydrolysis of PGA and increased the number of surface
carboxyl and hydroxyl groups, thereby improving the hydrophilicity of the scaffolds and consequently improved the cell adhesion. Secondly, plasma surface treatments promote a deposition of some hydrophilic groups to the fibres. Baker et al. studied the application of polystyrene on scaffolds for TE, the authors used plasma to treat the fibres surface and XPS results showed that after the treatment oxygen-containing groups where formed and the cell adhesion was improved.  

Thirdly, coating processes on the fibrous scaffolds aim to create a surface that improve the cell adhesion and their proliferation. One example was published by Zhang et al. that produced core-shell fibres performing coaxial electrospinning. The samples were prepared using PC1 (core) and collagen type I (shell). The authors described that the cells interactions with the scaffolds were improved with the coating.

Lastly, surface functionalization became a promising procedure to improve the efficiency of the scaffolds. Depending on the treated tissue, these techniques create possibilities of attaching a specific bioactive molecule, and consequently controlling the type of signal necessary for the cell development. Shin and Lim produced Poly(L-lactide-co-ε-caprolactone) (PCL) fibres and functionalized their surface attaching covalently RGD groups. The results showed that the presence of RGD on the surface of the scaffolds improved the cells adhesion, promoted the mature cytoskeletal structure of focal adhesion as well as differentiation. Cho et al. produced fibres of PCL/Poly(Cl-co-ethylene glycol) (PCIEG), and attached nerve grow factor (NGF) to their surface. The authors reported that this functionalization effectively influenced the cell differentiation of MSCs into neural-like cells, and also elongate their morphology.

4.2.5 Mechanical properties

Many factors influence the mechanical properties of the fibres produced by electrospinning, including the crystallinity, molecular weight, orientation and diameter of the fibres, etc. For TE, the scaffolds mechanical properties are responsible to provide shape stability to the cells. Moreover, these properties should match the requirements of the hosted tissue. These properties interfere directly to the mechanosensitivity of cells and consequently the morphology and adhesion of the
cells are altered. Many different types of mature cells demonstrated sensibility to the stiffness of the scaffolds, their cytoskeleton and ECM develops according to the signals promoted from the scaffolds. Furthermore, depending on the scaffolds stiffness the MSCs can be differentiate along the neuronal, muscle or bone lineages.

4.2.6 Degradability.

The scaffolds applied in TE provide physical support to the attachment and growth of cell, however this structure should be provisory and degrade during the tissue regeneration. Therefore, understanding and control of the degradation ratio of the scaffolds are important for TE. In this context, biopolymers applied in TE should present some hydrolytic or enzymatically sensitive bonds for cleavage, and some polymer properties play an important role in this process. One example is the polymer crystallinity, which restricts the chain mobility and consequently hydrolysis reactions are hampered. Crystal domains could act as a barrier for the diffusion of degradation products through the fibres. Henry et al. studied the application of polyesterurethane fibres in scaffold production, and the authors defined that the crystallinity of the samples could influence the degradation ratio and also the mechanical properties.

The surface area of the fibrous scaffolds, as well as their surface hydrophilicity can influence the degradation rate of the biopolymers. In the first case, higher surface areas tend to increase the degradation ratio. Bolgen et al. studied the degradation in vitro and in vivo of PCL nanofibres, and they observed that in fibres with smaller diameters (surface area) the degradation ratio was higher. The authors correlated these results to the smaller diffusion length to the degraded products during the process. In the second case, the hydrophilicity of the scaffolds can alter the hydrolysis reaction during the degradation process. This happens because of the higher wettability on this materials, that increase the water contact to the fibre surface and consequently facilitate the hydrolysis reaction during the degradation process. Kwangsok et al. studied the degradation rate of nanofibres produced using blends of PLA, PLGA and PEG. The results confirm that more hydrophilic sample the degradation ratio was higher.
5. Electrospraying for biomedical applications.

Electrospraying is a great alternative to produce polymeric micro and nanoparticles for biomedical application.\textsuperscript{154} By variation of the process parameters it is possible to vary the particles morphology and their diameter, and the possibilities of processing a wide number polymers, classify electrospraying as a promisor technique to produce particle for drug delivery system. During last decade, controlled drug release systems have received a lot of attention. Among the advantages are the possibilities of reducing the toxicity and side effects of the treatments as well also improve therapeutic efficiency. In this context, particles have been identify as a good alternative as drug carriers, as they can result in a less invasive administered route, increase the drug stability, control the releasing profile of the drug, elevate the bioavailability, and also the particles could be target for a selective tissues or cells type.\textsuperscript{155}

During recent years, biodegradable polymers have been extensively studied for application in drug delivery system. The utilization of micro and nanoparticles in drug delivery system is an interesting alternative, as they match all requisites necessary and also enable oral and intravenous administered.\textsuperscript{101} The drug release from the biopolymer particles is directly influenced by the polymer properties, such as, molecular weight, crystallinity, polymer blend, etc.\textsuperscript{156} In addition, the variation of these properties enable production of particles for an specific application. The drug release could occur by four different mechanisms: diffusion through water filled pores, diffusion through the polymers, osmotic pumping and erosion (Figure 1-14).

![Diagram](image)

Figure 1-14. Adaptation of a schematic representation of particles drug delivery mechanism: (A) diffusion through water-filled pores, (B) diffusion through the polymer, (C) osmotic pumping and (D) degradation.\textsuperscript{157}
Diffusion through water-filled pores, is normally used to describe the initial stage of the drug release, and occurs until the erosion release starts. It is directly dependent on the porous size.\textsuperscript{158} The diffusion through the polymer is used to describe the release of a small hydrophobic. Unlike the diffusion through the pores, this mechanism is not dependent the porous structures, however in particles with high porosity the surface area tends to be higher which could alter the drug release ratio. In addition, the drug diffusion ratio through the polymer would increase with higher temperature. The osmotic pumping mechanism, is correlated to the phenomenon caused by the water adsorption on the particles, causing an osmotic pressure and consequently drives and transport the drugs until they are released.\textsuperscript{159} In the degradation mechanism, the release occur without drug diffusion. It consist of the delivery the drug to the system during the polymer degradation, and the drug distribution homogeneity through the particles can alter the releasing profile.\textsuperscript{160}

The challenges for this systems are the difficulty of combining different properties from synthetic materials and natural compounds in the same dispositive, including processability, mechanical properties, biodegradation, bioconjugation, compatibility between the drug and drug carrier.

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Chapter 2

Optimization of Electrospinning Process Using Poly(globalide).
1. Introduction

Electrospinning is a versatile technique that has been used to produce fibres for different applications such as filtration, precursor for fabricating nanofibres composites, nanomaterials, drug delivery system, tissue engineering (TE), etc. Electrospinning became a prime technique to produce materials for biomedical applications due to the possibilities of processing different materials, produce fibres with variable diameters and properties, as well as the relatively low cost of its devices. Particularly, electrospinning of aliphatic polyesters has been applied to produce scaffolds for tissue engineering and drug delivery system. Among the different aliphatic polyesters used, poly(ε-caprolactone) (PCl) is the most widely applied polymer for these applications.

Generally, materials applied in regenerative medicine (RM), should create favourable grow conditions for cells. However, PCl presents some disadvantages in this respect, for example, its high hydrophobicity can prevent cell attachment and initiate inflammatory response. In this study, Poly(globalide) (PGl) is presented as an alternative to PCl. It has a similar chain composition but with an alkene group in the backbone structure that enables surface functionalization and also opens possibilities of crosslinking, which could result in a material with enhanced properties. However, there are no reports on the electrospinning of PGl to date. As highlighted in Chapter 1, processing parameters have a significant influence on the general success as well as the morphology of the fibres produced by electrospinning and must be optimised for every new material. In this chapter, PGl fibre production by electrospinning varying the process parameters, including spinning solvents, polymer solution concentration, voltage, distance of the nozzle, and flow rate is presented. The morphology of the fibres was determined by scanning electronic microscopy (SEM) and the thermal and mechanical properties as well as the degradation and cell viability of the PGl fibres were determined.
2. Experimental procedures.

2.1 Materials.

Globalide (Gl) was purchased from Symrise, Novozyme435 (Candida Antarctica Lipase B immobilized on acrylic resin) was purchased from Novozymes A/S. Dulbecco’s modified eagle medium containing high glucose (DMEM+GlutaMAX), Fetal bovine serum (FBS), penicillin/streptomycin and insulin, transferrin, selenium premix (ITS) were purchased from Biosciences. Transforming growth factor β3 (TGFβ3) was purchased from Prospec Biosciences. All other reagents and solvents used were purchased from Sigma-Aldrich and used without further purification.

2.2 Methods

2.2.1 eROP of Globalide.

For the eROP of Gl, 2.16 g (0.009 mol) of the monomer was added to a Schlenk flask containing 80 mg of enzymatic catalyst Novozyme 435. Toluene (1.6 g) was added to the mixture, and the reaction flask was purged with nitrogen, placed in an oil bath at 60°C, and stirred during 4 hours. Posteriorly, DCM was added to Schleck flask, to inhibit the enzymes and separate them by filtration. In the last step of the synthesis, the filtered solution was dropped in cold methanol and the precipitated PGl was filtered, furthermore the polymer was vacuum-dried at room temperature over a 24-hours period. The dried PGl was weighted and the yield of each reaction was calculated by the following equation.

\[
\text{Yield (\%)} = \left( \frac{\text{Polymer mass}}{\text{Monomer mass}} \right) \cdot 100
\]
2.2.2 Gel Permeation Chromatography.

The GPC measurements were performed using an Agilent 1200 series instrument equipped with GPC control software. All measurements were carried out using a Polymer Laboratories Gel 5 µm Mixed-C 300 x 7.5 mm column, at 40 °C with DAD and RID detection. Tetrahydrofuran (THF) was used as the eluent at a flow rate of 1 mL/min. The molecular weights of all polymers were calculated based on polystyrene standards.

2.2.3 Electrospinning of PGI.

The electrospinning was initially performed using a Mecc nanon (Figure 2-1 a) and posteriorly Spraybase (Figure 2-1 b) electrospinning machine, both with stationary collector.

![Mecc nanon](image1.png)  ![Spraybase](image2.png)

Figure 2-1. Mecc nanon (a) Spraybase (b) electrospinning machine.

The electrospun solutions were prepared solubilizing PGI in DCM and THF with different concentrations (10-65%wt.). In addition, the process parameters including voltage, distance of the nozzle and flow rate were variated used on the Mecc nanon and Spraybase electrospinning machine were described on the Table 2-1 and Table 2-2, respectively.
Table 2-1. Parameters used to optimization process of PGl electrospinning performed by Mecc nanon.

<table>
<thead>
<tr>
<th>PGl Solution</th>
<th>Voltage (kV)</th>
<th>Distance of the nozzle (cm)</th>
<th>Flow rate (mL/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15% wt. THF</td>
<td>11 – 25 – 30</td>
<td>10 – 12.5 – 15 – 17.5 – 20</td>
<td>0.5 – 1 – 2 – 5</td>
</tr>
<tr>
<td>25% wt. THF</td>
<td>11 – 25 – 30</td>
<td>10 – 12.5 – 15 – 17.5 – 20</td>
<td>0.5 – 1 – 2 – 5</td>
</tr>
<tr>
<td>35% wt. THF</td>
<td>11 – 25 – 30</td>
<td>10 – 12.5 – 15 – 17.5 – 20</td>
<td>0.5 – 1 – 2 – 5</td>
</tr>
<tr>
<td>45% wt. THF</td>
<td>11 – 25 – 30</td>
<td>10 – 12.5 – 15 – 17.5 – 20</td>
<td>0.5 – 1 – 2 – 5</td>
</tr>
<tr>
<td>55% wt. THF</td>
<td>11 – 25 – 30</td>
<td>10 – 12.5 – 15 – 17.5 – 20</td>
<td>0.5 – 1 – 2 – 5</td>
</tr>
<tr>
<td>60% wt. THF</td>
<td>11 – 25 – 30</td>
<td>10 – 12.5 – 15 – 17.5 – 20</td>
<td>0.5 – 1 – 2 – 5</td>
</tr>
<tr>
<td>65% wt. THF</td>
<td>11 – 25 – 30</td>
<td>10 – 12.5 – 15 – 17.5 – 20</td>
<td>0.5 – 1 – 2 – 5</td>
</tr>
<tr>
<td>10% wt. DCM</td>
<td>11 – 25 – 30</td>
<td>10 – 12.5 – 15 – 17.5 – 20</td>
<td>0.5 – 1 – 2 – 5</td>
</tr>
<tr>
<td>15% wt. DCM</td>
<td>11 – 25 – 30</td>
<td>10 – 12.5 – 15 – 17.5 – 20</td>
<td>0.5 – 1 – 2 – 5</td>
</tr>
<tr>
<td>20% wt. DCM</td>
<td>11 – 25 – 30</td>
<td>10 – 12.5 – 15 – 17.5 – 20</td>
<td>0.5 – 1 – 2 – 5</td>
</tr>
<tr>
<td>30% wt. DCM</td>
<td>11 – 25 – 30</td>
<td>10 – 12.5 – 15 – 17.5 – 20</td>
<td>0.5 – 1 – 2 – 5</td>
</tr>
<tr>
<td>40% wt. DCM</td>
<td>11 – 25 – 30</td>
<td>10 – 12.5 – 15 – 17.5 – 20</td>
<td>0.5 – 1 – 2 – 5</td>
</tr>
<tr>
<td>50% wt. DCM</td>
<td>11 – 25 – 30</td>
<td>10 – 12.5 – 15 – 17.5 – 20</td>
<td>0.5 – 1 – 2 – 5</td>
</tr>
<tr>
<td>60% wt. DCM</td>
<td>11 – 25 – 30</td>
<td>10 – 12.5 – 15 – 17.5 – 20</td>
<td>0.5 – 1 – 2 – 5</td>
</tr>
</tbody>
</table>

Table 2-2. Parameters used to the optimization of PGl electrospinning performed by Spraybase.

<table>
<thead>
<tr>
<th>PGl Solution</th>
<th>Voltage (kV)</th>
<th>Distance of the nozzle (cm)</th>
<th>Flow rate (µL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% wt. DCM</td>
<td>8 – 9 – 10</td>
<td>15 – 20</td>
<td>25 – 50 – 100 – 200</td>
</tr>
<tr>
<td>50% wt. DCM</td>
<td>8 – 9 – 10</td>
<td>15 – 20</td>
<td>25 – 50 – 100 – 200</td>
</tr>
<tr>
<td>60% wt. DCM</td>
<td>8 – 9 – 10</td>
<td>15 – 20</td>
<td>25 – 50 – 100 – 200</td>
</tr>
</tbody>
</table>
2.2.4 Optical Microscopy.

In the first step of the PGI electrospinning optimization, the samples were collected on glasses slides and dried overnight under vacuum. The results of the tested parameters were analysed using an optical microscope Leica model DMIL.

2.2.5 Scanning Electronic Microscopy.

The images were obtained by a Hitachi variable pressure SEM, model S-3400N, ranging acceleration voltage between 10-20 kV, and before starting the experiments the samples were sputter-coated with gold. Posteriorly, the obtained images were treated and the average and the frequency fibre diameters were measured using the microscopy software.

2.2.6 X-Ray Diffraction (XRD).

The XRD analysis was carried out on a Bruker AXS D8 Advance with a 3kW tube with a copper anode using the K-alpha line. The sample was mounted on a six-axis goniometer and a locked coupled scan used. The samples were then scanned through an angle theta and the detector was maintained at twice that angle theta.

2.2.7 Differential Scanning Calorimetry.

The thermal properties of the samples were characterized by DSC, and their melting point ($T_m$) and the melting enthalpy ($\Delta H_m$) were measured on TA instruments Q200 DSC. The analysis were performed using aluminium pan with temperature range from -60 °C to 120 °C, and with a heating rate of 5 °C per minute, under nitrogen atmosphere. The results of the first scan was used to evaluate the influence of the electrospinning process on the crystallinity of PGI.

2.2.8 Mechanical Properties.

The mechanical properties of PGI fibres were measured using a tensile testing machine Zwick/Roell model Z2 with loading of a cell of 50N at 5 mm/min, using samples cut in dog-bone shape. These analyses were performed in triplicate, and with
the average of the results the tensile strength and the Young’s Modulus of the samples were assessed.

2.2.9 Degradation.

To evaluate the degradation of the fibres, samples with dimensions of 1x1 cm were weigh and sterilized using UV-light for two minutes on each side. The samples were incubated in a PBS solution (0.01M, pH 7.4) for 90 days at 37°C; three samples were used for each experimental point. After the hydrolytic degradation, the samples were rinsed with 50 mL of distilled water and dried under vacuum over 48h. Degradation was measured by the mass difference of the samples and converted to percentage of the initial weight.

2.2.10 Cell Seeding and Culture Conditions.

Bone marrow derived porcine stem cells (MSCs) were isolated as previously described and expanded in growth media consisting of high-glucose Dulbecco’s modified Eagle’s medium DMEM+ GlutaMAX supplemented with 10 % FBS, penicillin (100 U/mL)-streptomycin (100 µg/mL) and 0.25 µg/mL amphotericin B (all Gibco, Biosciences) at 20% pO₂. Electrospun scaffolds (Ø 8 mm) were punched out from the electrospun mesh and sterilized by ethylene oxide (EtO). To prepare for cell seeding, scaffolds were hydrated by progressive washes in ethanol and several washes with ultra-pure water. Scaffolds were incubated in expansion media overnight before cell seeding. At the end of passage 2, cells were trypsinized and seeded onto one side of the electrospun scaffolds at a density of 100,000 cells per scaffold. Cell-seeded scaffolds were maintained expansion medium at 20 % pO₂ for 7 days, with medium changed twice. MSC-seeded scaffolds were cultured on custom-made wells suspended above the underlying tissue culture surface.

2.2.11 Metabolic activity assays.

Metabolic activity of the cells was quantified via reduction of Alamar Blue after 24, 48 and 72h in culture; cells plated on cell culture plates were used as control (n = 5). The absorbance was read at 570 nm using 600 nm as a reference wavelength.
2.2.12 DNA quantification.

On day 7, scaffolds were digested overnight at 60°C using 3.88 U/ml papain ($n = 4$). DNA content was quantified via the fluorometric Hoescht 33258 assay (Quant-iT ssDNA Assay Kit, Biosciences) and DNA standards according to manufacturer’s protocol. Fluorescence was measured on a plate reader (BioTek).

2.2.13 Statistics.

Statistics. Statistical difference was established at $P <0.01$ by two-way ANOVA followed by a Bonferroni’s post hoc test for pairwise comparisons of Alamar Blue reduction in control, PGI and PGI-XI. All data are reported as means ± standard deviation (GraphPad Prism 5, GraphPad Software).

3. Results and Discussion.

3.1 Synthesis of PGL

In this work, enzymatic polymerization of globalide (Figure 2-2) was applied following a standard procedure using Novozym435 (*Candida Antarctica* immobilised on resin beads) as a catalyst in toluene. After precipitation of PGI in methanol GPC analysis (Figure 2-3) revealed a polymer molecular weight of Mn 25,000 g/mol and polydispersity (PDI) of 2.1. As can be observed in the Figure 3-2, the obtained chromatographic peak was relatively broad as typical for this type of synthesis. The PDI of the synthesized samples of around 2 is a consequence of possible trans-esterification and side reaction promoted by the enzyme catalyst.
3.2 Electrospinning optimization.

As already stated, PGl had never been tested in electrospinning. The first tests were carried out on Mecc Nanon machine (Figure 2-1 a; Trinity College Dublin) and it was found that fibres could be produced at about 30% wt. in THF. While these were of low quality, promising first test prompted us to carry out an extensive and systematic investigation varying all the process parameters to identify optimum condition. We defined those conditions as those which produce homogenous fibres mats. Electrospinning operating parameters such as flow rate, intensity of the electric...
potential and distance between the electrode and the collector can influence the obtained results. Moreover, solution properties such as viscosity, conductivity, dielectric constant, solvent boiling point and surface tension, are important parameters that affect the experimental outcome.\textsuperscript{7,8}

The solvent influence on the electrospinning process using PGL solutions of different concentrations in THF (Figure 2-4) and DCM (Figure 2-5) was first evaluated. These solvents were selected because they efficiently solubilise the polymer. In both cases fibre formation was observed. However, the electrospinning performed with DCM resulted in fibres with more homogeneous diameters, good morphology and less fusion points, while the ones performed in THF resulted in inhomogeneous fibres with uneven surfaces and fused fibres regions, possibly due to the ineffective solvent evaporation during the fibre forming process.

Figure 2-4. Selected optical microscopy images of PGL materials obtained by electrospinning in THF.
These results could be correlated to the solvents properties. Boiling point (Bp), the dielectric constant (ε) and vapour pressure are influencing the solvent evaporation process and the conductivity of the solutions, respectively. DCM has higher Dc and lower Bp than THF (Table 2-3). In addition, DCM facilitated fibres at 20% wt. (Figure 5-2 condition 4) while in THF this was just observed at solution concentrations of 35% wt. (Figure 2-4 condition 4). This is in agreement with literature reports, i.e. solvents with low volatility tend to form fused or welded fibres on the collector, while solvents with medium volatility can provide conditions to stretch and dry the fibre before deposition.9,10 Moreover, the higher dielectric constant of DCM might be beneficial for the electrospinning process due to the increased conductivity of the polymer solution.10 Lee at al. studied the influence of the solvent on the PCl nanofibres formation during the electrospinning.11 The results showed that by increasing the Dc of the solvents the fibre diameters tended to decrease. Furthermore, the fibre diameter distributions tended to be narrow using solvents with low BP, which
corroborates the results obtained in this project. All following experiments were carried out with DCM as a solvent.

Table 2-3. Properties of solvents used in the electrospinning of PGI

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Boiling point (°C)</th>
<th>Dielectric constant at 25°C (ε)</th>
</tr>
</thead>
<tbody>
<tr>
<td>THF</td>
<td>66.0</td>
<td>7.5</td>
</tr>
<tr>
<td>DCM</td>
<td>39.6</td>
<td>8.9</td>
</tr>
</tbody>
</table>

In this work, PGI with an average $M_n$ 25,000 g/mol was used. It should be noted that the preferred molecular weight of, for example, PCl in electrospinning is much higher ($\geq 80,000$ g/mol) to meet the required solution viscosity.\textsuperscript{12,13} However, the intrinsically higher solution viscosity of PGI compared to PCl for a comparable $M_n$ allowed to formulate solutions with a sufficient viscosity at a concentration of $\geq 30$ wt%. Below this concentration the electrospinning process was unsuccessful irrespectively of other conditions and resulted in the formation of beads similar to electrospraying. As pointed out by Tiwari and Venkatraman,\textsuperscript{14} in order to electrospin fibres the solution should be above the overlap concentration. At this concentration the topological constraints induced by the larger occupied fraction of the available hydrodynamic volume in the solution develop into chain entanglements creating conditions for fibre formation.

Due to the availability of a new electrospinning machine in our own laboratory, all following test were carried out on the Spraybase system (Figure 2-1 b). Using a Spraybase electrospinner with a stationary collector, and samples were collected on aluminium foil and sample morphologies were analysed by SEM (Figure 2-6). In the SEM images, it can be observed that the polymer concentration directly influenced the fibre morphology. Comparing the conditions 3, 14, 23, 39 in Figure 2-6, it is possible to define that at lower concentrations bead formation occurred and the heterogeneity in the fibres diameters tends to increase. This could be correlated to the viscoelastic and electrostatic force on the surface tension, that results in the minimization of the surface area which tends to convert the liquid jet into spherical drops.\textsuperscript{10} At higher polymer concentration the morphology of the fibres was more
homogeneous (condition 31), the solvent evaporation was efficient thus avoiding conglutinated networks in the scaffolds. Bosworth and Downes studied the influence of electrospinning process parameters on the PCl fibres morphology.\textsuperscript{15} PCl was solubilized in acetone and the electrospinning was performed varying the concentration of the solutions, voltage, distance of the tip and flow rate. The results confirmed bead formation for solutions at lower concentrations which agrees with the results obtained in this project.

Figure 2-6. SEM images of fibres obtained in DCM collected in aluminium foil:
(3) 30% wt., (14) 40% wt., (23) 50% wt. and (39) 60 % wt.

Comparing the conditions of PGI 50% wt. in DCM, voltage 8 kV, distance of the nozzle 15 cm and varying the flow rate, it is possible to observe the influence of the flow rate on the fibre diameters (Figure 2-7). The average fibre diameter decreased from $10.4 \pm 1.1 \, \mu\text{m}$ to $7.5 \pm 1.5 \, \mu\text{m}$, using 25 $\mu\text{l/min}$ and 100 $\mu\text{l/min}$, respectively. In addition, increasing the flow rate imperfections and the diameters on the fibres tended to increase (Appendix 2-1, conditions 6, 7 and 8). These results could be correlated to the influence of the stability of the Taylor cone formation during the process. If the liquid jet is not sufficient to maintain the cone shape through the capillary, the fibre jet is altered resulting in inhomogeneous fibres. By increasing the flow rate, the
formed fibres tend not dry completely before reaching the collector, which explains the results obtained in the experiments. \(^{16}\)

Next, the influence of the voltage on the PGI fibres formation was examined comparing the conditions where the flow rate was kept at 100 µL/min, distance of the nozzle 15 cm and varying the voltage in 8 kV, 9 kV and 10 kV (Appendix 2-1, conditions 18, 21 and 24). The average fibre diameter (Figure 7-2) decreased around 20% (from 10.4 ± 1.1 µm to 8.3 ± 1.9 µm, using 8 kV and 10 kV, respectively). This result could be correlated to the electrostatic stress on the liquid jet, which results in a liquid jet stretching decreasing the average of fibres diameter. \(^{17}\)

![Figure 2-7. Average fibre diameters obtained from PGI solutions of 50 % wt. in DCM using flow rates of 100 µL/min, 50 µL/min and 25 µL/min, distance of the tip 15 cm and voltages of 8, 9 and 10 kV.](image)

Analysing the influence of the distance of the nozzle to the collector on the fibres morphology, increasing the distance the fibres diameter tends to decrease (Figure 2-8). The electrospinning performed using parameters of 9kV, flow rate 100 µL/min, PGI solution 60 % wt. in DCM, and different distances 15 cm and 20 cm (Appendix 2-1, conditions 39 and 35) resulted in fibres with average of 11.3 ± 2.3 µm to 9.6 ± 2.7 µm respectively, which represents a reduction of 15.4 %. This results could be associated to the longer stretching time of the solution into fibres promoted
by higher distances.\textsuperscript{18} Liu et al. studied the optimization of the electrospinning process of poly(L-lactide-co-e-caprolactone-diOH) and the results show that increasing the distance, the fibre diameters and bead formations decreased, which the authors correlated to fibres stretching. However, in the same investigation, for distances higher than 22 cm the fibre diameters started to increase and bead formation occurred again. The authors defined that these results occurred due to the reduction of the field strength.

![Graph showing fibre diameter distribution](image)

\begin{align*}
\text{AVG} &= 11.3 \mu m \\
\text{STD} &= 2.3 \\
\text{AVG} &= 9.6 \mu m \\
\text{STD} &= 2.7
\end{align*}

Figure 2-8. Average fibre diameters obtained from solutions of PGl 60\% wt. in DCM using distance from the nozzle 15 and 20 cm, flow rate of 100 µl/min. and voltage 9kV.

The electrospinning optimization for this type of PGl, confirms that the process parameters have a great influence on the fibres formation and consequently on their morphology. From these experiments, the optimum fibres (Figure 2-9) were obtained using a voltage of 8 kV, distance from the nozzle of 15 cm, rate flow of 50 µl/min (Appendix 2-1- condition 23), and PGl solution with a 50\%wt. in DCM. Moreover, these parameters were used to prepare samples for the thermal and mechanical analysis, as well as for the cell viability testes.
3.3 Properties of PGI fibres.

The crystallinity of a polymeric material is an important characteristic to be quantified and well understood because it may influence many properties of the material, for example, thermal and mechanical properties, degradation mechanism, surface morphology, etc.\textsuperscript{19} To evaluate the effect of the electrospinning process on the PGI crystallinity analysis by XRD and DSC were performed using samples of PGI fibres and bulk. The influence of the electrospinning on the crystallinity and crystal orientation of the semicrystalline PGI was first investigated by XRD. Both the spectra (Figure 2-10) of bulk PGI (PGI-B) and PGI fibres (PGI-F) display reflection peaks at 2\(\theta\) values at 21.4\(^\circ\) and 23.7\(^\circ\), which indicates \(d\)-spacings of 0.415 and 0.375 nm, respectively.\textsuperscript{20} It suggests that the electrospinning process tends to induce more homogeneous crystal packing and facilitates better growth of the crystals.\textsuperscript{21} From the spectra the size of the crystallites in the PGI-B and PGI-F were calculated to be \(~10.8\) nm and \(~13.9\) nm, respectively. Furthermore, the crystallinity of the PGI-B and PGI-F were 76% and 87%, respectively.
Figure 2-10. XRD diffraction spectra of PLG bulk and fibres.

The thermograms of the samples (Figure 2-11) corroborate the XRD results (Figure 10-2). The melting enthalpies from the DSC results are in agreement with the higher crystallinity of PLG on the fibres ($\Delta H_m = 179.1$ J/g; melting point: 40.2°C) compared to the PLG-B ($\Delta H_m = 128.6$ J/g; melting point 40.3°C); These results illustrate that the electrospinning conditions enhance the growth of the spherulite as well as their orientation. This has also been observed with PLA:PCI.22 Once deposited, the remaining solvent in the fibres upon continued evaporating, could create conditions to increase the size of the crystallites.19,23 A similar result was reported for PPDL in melt extrusion.24

Figure 2-11. DSC thermograms first heating cycle of samples PLG-B (black) and PLG-F (blue).
The mechanical properties of a polymer fibrous scaffolds are influenced by different characteristics, such as, molecular weight, crystallinity, fibres diameters and orientation, etc.\textsuperscript{1} The measurement of this property is crucial to define the material profile for TE, as it is responsible for the signals received by the cell and consequently altering their differentiation process and morphology.\textsuperscript{25} However, analysis of mechanical properties of the fibres meshes is not trivial due to the difficulties in producing representative homogenous samples. For this project the PGI-F mechanical properties were determined by tensile test using samples in dog bone shape and they were performed in triplicate. The results should only be considered preliminary.

![Stress-strain curve of PGI-F](image)

Figure 2-12. Stress-strain curve of PGI-F.

Based on the average of the stress-strain behaviour of PGI-F (Figure 2-12), the tensile strength average was 0.19 ± 0.01 MPa and the Young’s modulus 4.75 ± 1.1 MPa. These relatively low values could be correlated to the relatively low molecular weight of the PGI compared to PCl used to produce fibrous scaffolds described in the literature (80,000 g/mol). For example, Nunes et al. investigated the influence of the molecular weight on the mechanical properties of the polymer meshes.\textsuperscript{26} The mechanical property is related to the capability of a material to absorb energy when force is applied. The results obtained on Nunes study suggested that the mechanical
properties of the samples were proportional to their molecular weight. The authors attributed this to the greater elongation prior to fracture of the samples with higher molecular weight. In addition, the higher elongation enables a higher orientation on the polymer chains before failure.

The hydrolytic degradation rate is an important criterion to determine the application profile of a material. For being classified as biodegradable polymer, the material should present some sensitive bonds for hydrolytic or enzymatically cleavage.²⁷ To evaluate and define the hydrolytic degradation profile of PGI-F, PBS solutions was used and the samples were incubate over 90 days. For PGI-F a small mass loss of 10 % of a 1 x 1 cm mesh sample in buffer solution was found within the first 30 days (Figure 2-13). Over another 30 days a similar mass loss rate was determined after which the degradation slowed to a mass loss of 25 % after 90 days. The PGI-F has to be classified as slowly degrading, probably due to its hydrophobicity, the degradation profile is typical for semicrystalline polymers, where degradation starts in the amorphous region by ester hydrolysis. In contrast, chain hydrolysis ratio in the crystalline region tends to decrease due to the low chain mobility. ²⁸

![Figure 2-13. Relative mass loss of PGI fibres in buffer solution in correlation with the average polymer molecular weight of samples from remaining fibres.](image)

The feasibility of PG nanofiber meshes as medium for cell growth was studied in cytotoxicity and cell proliferation experiments to validate this polymer for tissue engineering applications. The experiments were performed with PGI-F and PCl
fibres (PCI-F) as a comparison. Cytotoxicity was first explored and the viability of cells seeded on PGI electrospun meshes was compared with PCI. The SEM images (Figure 2-14) show cell proliferation in both materials, although with higher uniformity in the PGI scaffolds. While the SEM images suggested a better cell development on PGI, the metabolic activity that define the cell proliferation on the scaffolds during the firsts days, confirmed a similar cell growth in both materials (Figure 2-15). The higher cell density on the PGI surface could be due to the smaller diameters of the fibres. In the PGI scaffolds the average diameter is $7.89 \pm 0.97 \mu m (n = 20)$, while it is $13.9\pm2.7 \mu m$ for the PCI. As described in the literature, for materials with smaller fiber diameters cell growth tends to occur on the surface of the scaffolds and have better distribution, due their higher surface area and limited porosity. In contrast, materials with larger fibre diameter have higher porosity creating conditions for the cell infiltration into the scaffolds.\textsuperscript{29,30} A quantitative proliferation study by means of a proliferation assay revealed that MSCs underwent a decay in cell proliferation during the first 48 h after which their proliferation rate increased within 72 h (Figure 2-15). The initial apparent decrease in cell metabolic activity on the electrospun fibre mats of either PGI-F or PCI-F compared to plated cells is due to the porous structure of the electrospun fibre mats that is unable to retain all of the cells during seeding. However, after 72 h, cells seeded on electrospun fibre mats proliferate to the extent of plated cells proliferation. Under these experimental conditions, the metabolic activity assay showed that PGI-F allow for cell proliferation without any deleterious response in MSCs viability, and thus it is considered non-cytotoxic.

![SEM images of PGI-F (A) and PCI-F (B) electrospun scaffolds seeded with mesenchymal stem cells (MSCs) after 21 days.](image-url)

Figure 2-14. SEM images of PCI-F (A) and PGI-F (B) electrospun scaffolds seeded with mesenchymal stem cells (MSCs) after 21 days.
Figure 2-15. Cell metabolic activity mesenchymal stem cells (MSCs) seeded on PGI-F and PCl-F compared to plated cells (control) after 72h.

In order to evaluate if PGI would allow for extracellular matrix deposition, seeded scaffolds were cultured in differentiation media. The biochemical composition (Figure 2-16) of the deposited matrix after 21 days of culture was evaluated for sulfated glycosaminoglycans (sGAGs) and collagen synthesis, extracellular components in musculoskeletal tissues such as muscle, cartilage, and bone, and in other tissues such as nerves and skin. MSCs seeded on PGI electrospun fibre mats resulted in a lower proliferation compared to the one displayed by MSCs seeded on PCl electrospun fibre mats. However, when the amounts of GAG and collagen deposited were normalized to DNA at day 21, PGI-F was not significantly different from PCl-F.

Figure 2-16. Biochemical analysis: DNA, sGAG and collagen synthesis
4. Conclusion.

The fabrication of PGI fibres was possible using the electrospinning technique. The results showed that the process parameters influence the morphology and the diameter of the fibres. It was observed that the voltage plays an important role to the fibres diameters and the solution concentration alters the viscosity and consequently the fibres spinning. The biological tests showed that the PGI is a non-toxic material, which open a new range of possibilities in biological applications. The cell development confirms that it is viable to be used as scaffolds in tissue engineering. Complementary studies are necessaries to explore the possibilities of PGI functionalization, which can improve its properties resulting a feasible material to bioengineering.

5. References.


Chapter 3

Direct UV-Triggered Thiol–ene Cross-Linking of Electrospun of Poly(globalide)
1. Introduction.

Currently there is a demand for developing biopolymers for electrospinning that offer additional functionality for immobilizing bioactive cues or cross-linking to enable post spinning manipulation such as drug loading. Fibre functionalization via click-chemistry has been explored and reviewed \(^1\)–\(^3\) but only a few examples of in situ cross-linked electrospun fibres have been disclosed to date.\(^4\),\(^5\) This is surprising since polymer crosslinking can enhance or modify the characteristic of the polymers, such as crystallinity, mechanical properties, degradability, etc.\(^5\),\(^6\) Of the examples reported in literature, the crosslinking process was performed after electrospinning, which normally results in a heterogeneous crosslinking throughout the fibres.\(^7\),\(^8\) In one example of in situ crosslinking reported by Shanmuganathan et al. the production of thermoset fibres was investigate, with high elongation and elastic recover. The samples were prepared using multifunctional vinyl ether monomers with pentafunctional acrylate and tetrafunctional thiol, and electrospun under simultaneous UV-cross-linking. The authors observed that the curing kinetics of vinyl ethers were faster than that of (meth)acrylates, which results in a more homogeneous network and the tensile test showed higher elongation.\(^4\) In another example, Kalaoglu-Altan et al. studied the production of poly(2-oxazoline) fibres cross-linked by a UV-triggered thiol-ene reaction. In addition, surface functionalization was achieved using the remaining thiol and ene groups of the material by attaching two different fluorescent dyes via radical thiol-ene and Michael-type nucleophilic conjugation. The results confirmed that the crosslinking promoted the modification on the nanofibre structures and also enable their surface functionalization.\(^9\) Thielke et al. used a similar process to produce crosslinked polybutadiene fibres. The fibres were deposited in a liquid collector containing a solution of sodium chloride 1% wt. in methanol.\(^5\)

To the best of our knowledge, there is no example of cross-linked electrospun polyester fibres matching the versatility of poly(ε-caprolactone) (PCL) or poly(lactic-co-glycolic acid) (PLGA). One barrier is the quite challenging chemistry to introduce functional groups along the polyester chain via ring opening polymerization (ROP), which typically requires rigorous multistep procedures to derivate lactide or lactone monomers. With the intention to enhance the mechanical properties of the PGI-F fibres
it was evaluated the possibilities of crosslinking the polymer chain. For this study, an in-situ crosslinking based on thiol-ene chemistry was used. To reach the optimum parameter, different cross-linkers, photo-initiators, and also the collector type were tested, posteriorly the crosslinked samples (PG1-Xl) were characterized and compared to PG1-F. Moreover, the drug loading and releasing experiments were performed on the PG1-Xl fibres.

2. Experimental procedures.

2.1 Materials.

Gl was purchased from Symrise, Novozyme 435 (Candida Antarctica Lipase B immobilized on cross-linked polyacrylate beads) was purchased from Novozymes A/S. Methanol, dried toluene and dichloromethane (DCM) were purchased from Sigma and used as received unless otherwise noted. Dry DCM for the electrospinning process was obtained from Sigma. To the degradation tests, phosphate buffer tablets (PBS) (pH 7.4) was obtained from Sigma. Dulbecco’s modified eagle medium containing high glucose (DMEM+ GlutaMAX), Fetal bovine serum (FBS), penicillin/streptomycin and insulin, transferrin, selenium premix (ITS) were purchased from Biosciences. Amphotericin B, sodium pyruvate, bovine serum albumin (BSA), dexamethasone, L-ascorbic acid 2-phosphate, linoleic acid, L-proline, and phosphate-buffered saline were purchased from Sigma-Aldrich. Transforming growth factor β3 (TGFβ3) was purchased from Prospec Biosciences. All other reagents and solvents used were purchased from Sigma-Aldrich and used without further purification.

2.2 Methods

GPC measurements were performed using an Agilent 1200 series instrument equipped with GPC control software. All measurements were carried out using a Polymer Laboratories Gel 5 µm Mixed-C 300 x 7.5 mm column, at 40 °C with DAD and RID detection. Tetrahydrofuran (THF) was used as the eluent at a flow rate of 1
mL/min. The molecular weights of all polymers were calculated based on polystyrene standards. Scanning Electronic Microscopy (SEM) images were obtained by a Hitachi variable pressure SEM, model S-3400N, ranging acceleration voltage between 10-20 kV, and before starting the experiments the samples were sputter-coated with gold. Posteriorly, the obtained images were treated and the average and the frequency fibre diameters were measured using the microscopy software. The FT-Raman measurements were performed on PerkinElmer – Raman station 400F, with a laser source of 785 nm, and each spectra were based in 16 scans. The XRD analysis were carried out on a Bruker AXS D8 Advance with a 3kW tube with a copper anode using the K-alpha line. The sample was mounted on a six-axis goniometer and a locked coupled scan used. The samples were then scanned through an angle theta and the detector was maintained at twice that angle theta. The thermal properties of the samples were characterized by Differential Scanning Calorimetry (DSC) and their melting point (T_m) and the melting enthalpy (ΔH_m) were measured on TA instruments Q200 DSC. The analysis was performed using aluminium pan with temperature range from -60 °C to 120 °C, and with a heating rate of 5 °C per minute, under nitrogen atmosphere. The mechanical properties of PGl fibres were measured using a tensile machine Zwick/Roell model Z2 with loading of a cell of 50N at 5 mm/min, using samples cut in dog-bone shape. These analyses were performed in triplicate, and with the average of the results the tensile strength and the Young’s Modulus of the samples were defined. Water contact angles were measured on a FTA125 (First ten Angstroms, USA), using 4 drops of distilled water in each sample. The results were analysed using the software of the machine.

2.2.1 eROP of Globalide.

For the eROP of Gl, 2.16 g (0.009 mol) of the monomer was added to a Schlenk flask containing 80 mg of enzyme catalyst Novozyme 435. Toluene (1.6 g) was added to the mixture, and the reaction flask was purged with nitrogen, placed in an oil bath at 60°C, and stirred during 4 hours. Posteriorly, DCM was added to Schleck flask, to inhibit the enzymes and separate them by filtration. In the last step of the synthesis, the filtered solution was dropped in cold methanol and the precipitated PGl was filtered, furthermore the polymer was vacuum-dried at room temperature over a
24-hours period. The dried PGl was weight and the yield of each reaction was calculated by the follow equation.

\[
\text{Yield (\%) = } \frac{\text{Polymer mass}}{\text{Monomer mass}} \times 100
\]

2.2.2 Electrospinning of PGl-XI.

Experiments were carried out according to those described in Chapter 2 using a Spryabase system equipped with a UV lamp. In this setup the polymer solution jet is exposed to the UV-light during the electrospinning process, which enable the initiation of photochemistry reactions on the samples. Process parameters listed in Table 3-1 were used.

Table 3-1 Parameters used to the optimization of PGl electrospinning performed by Spraybase electrospinning.

<table>
<thead>
<tr>
<th>PGl Solution</th>
<th>PGl / (Cross-linker and Photoinitiator) (20% wt./wt.)</th>
<th>Voltage (kV)</th>
<th>Distance of the nozzle (cm)</th>
<th>Flow rate (μL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60% wt. DCM</td>
<td>XI-1/P-1</td>
<td>8–10–12–14–16–18</td>
<td>15–17.5–20</td>
<td>50–100–200</td>
</tr>
<tr>
<td>70% wt. DCM</td>
<td>XI-1/P-1</td>
<td>12–13–14–15–16</td>
<td>15–17.5–20</td>
<td>50–100–200</td>
</tr>
<tr>
<td>60% wt. DCM</td>
<td>XI-1/P-2</td>
<td>12–13–14–15–16</td>
<td>15–20</td>
<td>50–100–200</td>
</tr>
<tr>
<td>60% wt. DCM</td>
<td>XI-2/P-2</td>
<td>14–12</td>
<td>18–20</td>
<td>150–200</td>
</tr>
</tbody>
</table>

2.2.3 Degradation.

To evaluate the degradation of the fibres, samples with dimensions of 1x1cm were weigh and sterilized using UV-light for two minutes on each side. The samples were incubated in a PBS solution (0.01M, pH 7.4) for 90 days at 37°C; three samples were used for each experimental point. After the hydrolytic degradation, the samples were rinsed with 50 mL of distilled water and dried under vacuum over 48h.
Degradation was measured by the mass difference of the samples and converted to percentage of the initial weight.

2.2.4 Cell Seeding and Culture Conditions.

Bone marrow derived porcine stem cells (MSCs) were isolated as previously described \(^{10}\) and expanded in growth media consisting of high-glucose Dulbecco’s modified Eagle’s medium (DMEM) GlutaMAX supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL)-streptomycin (100 µg/mL) and 0.25 µg/mL amphotericin B (all Gibco, Biosciences) at 20% pO\(_2\). Electrospun scaffolds (Ø 8 mm) were punched out from the electrospun mesh and sterilized by ethylene oxide (EtO). To prepare for cell seeding, scaffolds were hydrated by progressive washes in ethanol and several washes with ultra-pure water. Scaffolds were incubated in expansion media overnight before cell seeding. At the end of passage 2, cells were trypsinized and seeded onto one side of the electrospun scaffolds at a density of 100,000 cells per scaffold. Cell-seeded scaffolds were maintained expansion medium at 20% pO\(_2\) for 7 days, with medium changed twice. MSC-seeded scaffolds were cultured on custom-made wells suspended above the underlying tissue culture surface.

2.2.5 Cell viability and metabolic activity assays.

Cell viability was assessed by staining cell-seeded scaffolds with 4 mM calcein-AM (green: ‘live’), and 2 mM ethidium homodimer-1 (red: ‘dead’) for 1 h at 37 °C. Samples were examined under a scanning confocal microscope (Olympus FV1000). Metabolic activity of the cells was quantified via reduction of Alamar Blue after 24, 48 and 72h in culture; cells plated on cell culture plates were used as control (n = 5). The absorbance was read at 570 nm using 600 nm as a reference wavelength.

2.2.6 DNA quantification.

On day 7, scaffolds were digested overnight at 60°C using 3.88 U/ml papain (n = 4). DNA content was quantified via the fluorometric Hoescht 33258 assay (Quant-iT ssDNA Assay Kit, Biosciences) and DNA standards according to manufacturer’s protocol. Fluorescence was measured on a plate reader (BioTek).
2.2.7 Statistics.

Statistics. Statistical difference was established at $P < 0.01$ by two-way ANOVA followed by a Bonferroni’s post hoc test for pairwise comparisons of Alamar Blue reduction in control, PGl and PGl-Xl. All data are reported as means ± standard deviation (GraphPad Prism 5, GraphPad Software).

2.2.8 Degree of Swelling.

The dried samples of PGl-Xl were cut into pieces (1x1 cm), weight and immersed in 20 mL of DCM. The wet samples were blot dried with a filter paper and weight again. The swelling degree of the samples was defined using the following equation

$$SD = \left( \frac{M_2 - M_1}{M_1} \right) \times 100$$

where $M_2$ is the mass of swollen sample and $M_1$ is the mass of the dried sample. 11

2.2.9 Dye Encapsulation.

A sample of PGl-Xl with dimensions of 1x1 cm were immersed, during 10 min, in THF Rhodamine-B 0.1% wt. solution and dried under vacuum overnight. The dried sample was washed five times with 10 mL of water:ethanol 1:1 solution until colorless solution was obtained.

2.2.10 Indomethacin Encapsulation.

Drug loading capacity of the PGl-Xl were studied using Indomethacin (IND) as a hydrophobic model drug. The samples were prepared by cutting discs of 8 mm in diameter. Essentially, the first loading method was by swelling and the second one, for comparative purposes, 10% wt. of the drug was incorporated in the polymer solution before the PGl fibres were electrospun. In the first case, discs of PGl-Xl (n=3) were immersed during 6 hours in IND solutions in DCM with concentrations of 10 and 20%wt. separately. The different concentrations was used to compare their
influence on the drug encapsulations by swelling. Additionally, to verify the drug loading capacity by swelling of the PGI-XI samples, aliquots of the drug solution before and after of swelling were collected and analysed by high-performance liquid chromatography (HPLC, Agilent 1120 Compact LC with a Phenomenex Gemini 5u C18 110, LC 250x4.6mm Column). Ten microliters of the samples were injected and a solution of acetonitrile: PBS pH 8 (50:50, v/v) was used as a mobile phase, the detector wavelength used was 254 nm, flow rate of 1 mL/min and run time of 10 min. The standard curve was prepared using solutions of IND in release medium ranging from 0.098 to 196 mg/mL (Appendix 3-1).

2.2.11 Drug Release studies.

The drug release performance of PGI-XI and PGI samples were analysed by HPLC described before. The samples (n=5) were placed in Eppendorf tubes containing 2 mL of medium release solution (PBS and methanol 80:20, v/v), afterwards incubated at 37°C in a water bath shaker. The release medium were completely removed at predetermined time point and replaced with fresh solution. The loading efficiency (LE) of the PGI-XI and PGI fibres were calculated as:

$$\text{LE(\%)} = \frac{(\text{LC} - \text{BR}) \times 100}{\text{LC}}$$

where, LC is the loading capacity and BR is burst release.

3. Results and Discussion.

3.1 Electrospinning of PGI-XI.

For the optimization of PGI-XI electrospinning the PGI was synthesized according to chapter 2 with an average molecular weight 25,000 g/moL and polydispersity of 2.1. To facilitate in-situ UV-crosslinking a UV-lamp was installed inside the Spraybase electrospinning cabinet (Figure 3-1). This adaptation enables UV-light exposure directly to the polymer liquid jet during the electrospinning,
thereby promoting the initiation of the thiol-ene photochemical reaction. Another necessary adaptation on this machine was to cover the tube that feed the nozzle during the process, which avoided the tube blocking due to presence of crosslinked polymer in it. In addition, the influence of different type of PGI-XI collectors was evaluated, using a flat collector or a dish plate collector, in cold methanol or aluminium foil, respectively.

![UV lamp adapted inside of electrospinning SprayBase spinner](image)

Figure 3-1. UV lamp adapted inside of electrospinning SprayBase spinner: (1) High voltage supplier connection, (2) Feeder connection, (3) Nozzle, (4) UV-lamp, (5) Camera and (6) collector.

The optimized electrospinning parameters for PGl were used as a reference to electrospin PGI-XI. For this step PGI at concentrations raging from 60 - 70 wt% (DCM), a voltage range of 8-15 kV, flow rate 50-200 µl/min, and distance of the tip 15-25 cm were used (Figure 3-2). Two different reagents were applied as cross-linkers, ethylene glycol bis(mercaptop propionate) (XI-1) and 1,5- pentanedithiol (XI-2), both were added separately to different polymer solutions at the proportion of 20% wt/wt. As photo-initiators, two different solutions were tested, i.e. 2,2 dimethoxy-2-phenyl acetophenone (P1) and (2,4,6-trimethylbenzoyl) phenone oxide with 2-hydroxy-2-methylpropiophenone (P2) in a proportion of 50:50. The PGI samples were collected on aluminium foil while PGI-XI samples were collected in methanol. All the samples were then vaccum-dried for 48 hours and stored in a desiccator until further use.
The alkene group in the PGl structure opens possibilities of production modification on the polymers structures. Ates et al. evaluated this by producing crosslinked PGl films using thiol-ene chemistry under UV irradiation. The experiments were performed using different ratios of Xl-1 as a cross-linker and P-2 as an UV initiator. Based on this, the initial tests to produce the PGl-Xl were performed incorporating Xl-1 and P-1 into a PGl solution in DCM. Initially, the material was spun using the optimum conditions identical to chapter 2 and the samples were collected on glasses slides for optical microscope analyses and on aluminium foil for the SEM analysis (Figure 3-3).
The obtained samples in the initial tests presented some inhomogeneous fibres formation and they were not completely dried during the process. The collection of wet fibres created fusion points between the fibres, which largely eliminate porosity and the characteristic of fibrous scaffolds. These results are probably caused by unreacted XI-1 that remained on the fibres due to its high boiling point (255 °C) preventing complete evaporation and creating condition for fusion points formation. In addition, the polymer solution was not stable and started to cross-link inside the syringe prior to electrospinning.

In the second step of the electrospinning optimization a new photoinitiator (P-2) was tested, and following the same procedure used previously the samples were evaluated using optical microscope. The parameters that showed most promising fibre formation were repeated and the fibres were collected on aluminium foil for SEM imaging (Figure 3-4)
Figure 3-4. SEM images of PGl-XI produced using XI-1 and P-2.

The main purpose of the optimization using P-2 was to evaluate the possibilities of improvement on the process and to avoid the cross-linking reaction inside syringe. The SEM images (conditions X52 and X53) confirm the presence of porosity in the samples, however, the fusion points still occurring and the fibres morphology was not homogenous. As it was hypothesized that the fusion points occurred due incomplete drying of fibres before they touch on the collector, the last step of the PGl-XI optimization process, the combination of XI-2 and P-2 was tested. The SEM images (Figure 3-5) confirmed the efficiency of the electrospinning, the samples produced with this combination presented a better fibre morphology with less conglutinated networks. However, again issues were observed with fused fibres (condition X62).
To overcome this, methanol, a non-solvent for PGI was used to collect the fibres directly after they passed through the UV light. This technique was first described by Thielke et al. to prevent fibres fusing on the collector for cross-linked electrospun polybutadiene fibers.\textsuperscript{5} Under these conditions a homogeneous fibre morphology was obtained similar to the non-cross-linked fibres. The optimum fibres (Figure 3-6) were obtained using PGI solution 60% wt. in DCM, combination of XI-2 and P-2, voltage of 14 kV, distance of the nozzle 20 cm, flow rate 200 µL/min and collecting the sample in methanol. The fibres presented average diameter of 9.2 ± 1.6 µm (n = 20), and this optimum parameter was used to prepare the samples for the fibre property characterization.

Figure 3-6. PGI-XI fibres obtained by optimum electrospinning process.
First evidence of a successful cross-linking reaction was obtained from the fact that the fibres were not soluble in THF anymore. Spectroscopically, cross-linking was confirmed from the characteristic Raman double bond bands (Figure 3-7) around 1660 cm\(^{-1}\), which were significantly reduced for the cross linked fibres PGI-XI compared to the non-crosslinked fibres (PGI-F). Moreover, PGI-XI samples display a signal at 1000 cm\(^{-1}\) characteristic of C-S bonds, which is not present in the spectrum of the PGI-F (Figure 7-3).  

![FT-Raman spectra of PGI-F (black) and PGI-XI (red).](image)

**Figure 3-7.** FT-Raman spectra of PGI-F (black) and PGI-XI (red).

### 3.2 Properties of PGI-XI

The influence of the crosslinking process on the fibres crystallinity was investigated by XRD. As mentioned in chapter 2, PGI-F display reflection peaks at 20 values at 21.4\(^\circ\) and 23.7\(^\circ\), which indicates a \(d\)-spacing of 0.415 and 0.375 nm. These peaks were not observed in the spectrum of the PGI-XI (Figure 3-8). Moreover, as a consequence of the cross-linking, the degree of crystallinity of electrospun fibres was significantly reduced, the PGI-XI samples presented broad peaks suggesting that the crosslinking process resulted in mostly amorphous fibres. These results can be explained by limitation of the chain mobility in crosslinked polymers, which decrease the possibilities of chains reorganization in crystallographic planes and consequently decrease the degree of crystallinity of the polymer.
The DSC thermograms of the samples (Figure 3-9) corroborate the XRD results. The melting enthalpies from DSC are in agreement with the higher crystallinity of PGI-F ($\Delta H_m = 179.1$ J/g; melting point: 40.2°C), while in the PGI-Xl did not present an endothermic peak, confirming the absence of crystalline domain in its structure. In agreement with previous studies on cross-linked PGI bars, cross-linking significantly reduces the crystallinity by distribution of the crystalline domains, so that nearly fully amorphous fibres were obtained.$^{15,16}$ Moreover, it was possible define that the cross-linked network influences the crystallites formation, as described by Nojima et al. who studied the crystallization process of cross-linked copolymer poly($\varepsilon$-caprolactone)-block-polybutadiene (PCI-b-PB). Their results showed that the cross-linked samples promoted non-homogeneous crystallite formations on the polymer structure, which was attributed to the limitation on the chain mobility during the crystallization process.$^{17}$

Figure 3-9. DSC thermograms first heating cycle of samples PGI-F (black) and PGI-Xl (red).
The crosslinking process could considerably change the mechanical properties of the polymer, for example, Wang et al. studied the properties of photo cross-linked poly(ε-caprolactone fumarate) (PClF). The tensile results showed that with increasing cross-linking density the tensile modulus tends to be higher. However, the samples with higher degree of crosslinking became brittle and the elongation at break was low in these samples. As previously mentioned, the cross-linking process was performed on the PGI fibres aiming to improve the mechanical properties of the scaffolds, as the initial results of PGI-F confirm the low Young’s modulus and tensile strength. As for PGI-F samples tensile tests were performed in triplicate using samples cut in dog bone shape. The average of tensile test results of PGI-F and PGI-Xl (Figure 3-10 A) were used to define the tensile strength (Figure 3-10 B) and Young’s modulus (Figure 3-10 C) of both samples. Comparing the results, the crosslinking process resulted in an increase in the mechanical properties, the tensile strength and Young’s modulus increased 194% and 40%, respectively, which agrees with a successful crosslinking reaction.

![Figure 3-10](image)

**Figure 3-10.** Stress-strain curve of PGI-F (black) PGI-Xl (red) (a), tensile strength (b) and Young’s modulus (c) of PGI-F and PGI-Xl.

The hydrolytic degradation rate is an important criterion to determine the application profile of a resorbable material. For PGI-F a small mass loss of 10 % of a 1 x 1 cm mesh sample in buffer solution was found within the first 30 days. Over another 30 days a similar mass loss rate was determined after which the degradation slowed to a mass loss of 25 % after 90 days (Figure 3-11). The PGI-Xl presented faster mass loss in the first 30 days reducing 14% of its initial mass; for the next time points analysed the mass loss rate continued in 10 % steps and after 90 days the samples lost around 34% of their mass. These results can be correlated with the difference in the
surface hydrophobicity between the samples. The average water contact angle was 102.3° to PGI-XI and 110.5° for PGI-F which classify the crosslinked fibres as less hydrophobic. This higher wettability of PGI-XI could influence the degradation, as it results in a faster penetration of the water into the fibres increasing the hydrolysis reaction rate and the surface erosion.\(^\text{18}\) While PGI has to be classified as slowly degrading, probably due to its hydrophobicity, the degradation profile is typical for semicrystalline polymers, where degradation starts in the amorphous region by ester hydrolysis. Chain hydrolysis in the crystalline region is retarded due to the low chain mobility.\(^\text{19}\) Confirmed by XRD results, PGI-XI does not present a strong crystalline domain and this could influence the water diffusion into the polymer structure, which consequently resulted in a faster mass loss.

![Figure 3-11. Relative mass loss of PGI-F and PGI-XI in buffer solution after 30, 60 and 90 days, and water contact angle of each sample. Mass loss experiments were carried out in triplicate, error bars represent standard deviation.](image)

3.3 Cell viability.

Ultimately, the feasibility of PGI for biomedical applications lies in its biocompatibility and nontoxicity. Adult mesenchymal stem cells (MSCs) have been used in preclinical models for tissue engineering of bone, cartilage, muscle, and other mesenchymal tissues with significant promise.\(^\text{20}\) MSCs were seeded on PGI-F and PGI-XI fiber meshes to assess their viability, metabolic activity and proliferation to validate these polymers for tissue engineering applications. Live/dead imaging was
performed to qualitatively evaluate cell viability (Figure 3-12 A, B). MSCs remained viable on PGI-F and PGI-XI electrospun fiber scaffolds after 24 h in culture. There was no significant difference between PGI-F and PGI-XI in terms of cell viability according to their cell metabolic activity (Figure 3-12 C). Long term viability was quantifying by measuring the DNA content of both PGI-F and PGI-XI. DNA content was higher after 14 days of culture compared to day 0 (Figure 3-12 D).

![Figure 3-12](image)

Figure 3-12. Viability and proliferation of MSCs on PGI and PGI-XI. Representative live/dead images of cells at day 1 in (A) PGI and (B) PGI-XI (live: green, dead: red indicated with arrows). Alamar blue reduction readings in the scaffolds after 1 day in culture are shown in (C), blank and cells in tissue culture plates (TCP) are included for comparison. DNA amount at day 1 and day 14 are plotted in (D). * P < 0.05 (n = 5).

Porcine bone marrow mesenchymal stem cells were used to evaluate the cytotoxicity of these polymer formulations. Cytotoxicity was evaluated qualitatively by using live/dead viability assay, and quantitatively with alamar blue cell metabolic activity assay. Cell proliferation was measured using pico green assay for DNA
quantification. Results showed that PGI-F and PGI-XI supported cell metabolic activity.

3.4 Drug Encapsulation and Releasing.

These results highlight that PGI electrospun fibers are a suitable alternative scaffold material to PCL or PGLA in tissue engineering applications. However, the possibility of crosslinking PGI opens opportunities not achievable with conventional polyesters. While PGI-F dissolve in THF, PGI-XI swell up to 14% and retains its structural integrity. SEM micrographs in Figure 3-13 demonstrate that; while some deformation of the fibers is apparent, the fibrillar structure and the fiber dimensions remain unchanged even after evaporation of the THF. This can potentially be exploited to load the hydrophobic fibers with hydrophobic active ingredients such as drugs to facilitate or support tissue regeneration. We demonstrated the feasibility of this process by the loading of PGI-XI with Rhodamine B as a model molecule. In this process, a PGI-X fiber mesh was placed in a THF solution of Rhodamine B. After removal from the solution the mesh shows the typical red color of the dye (Figure 3-13C). The fiber mesh was then extensively washed with water:ethanol 1:1 to remove all surface adsorbed Rhodamine B until the washing solution was optically free of color (Figure 3-13G). The fact that the extensively washed fiber mesh still contains Rhodamine B suggests the incorporation of the dye into the fiber bulk by the soaking process.
To further demonstrate the drug loading capacity and efficiency of PGl-XI fibers, loading of the hydrophobic anti-inflammatory drug IND was studied. In one approach, loading was achieved in a conventional process by adding IND to the spinning solution. A limitation of this approach is that the maximum loading capacity of the drug in the fibres is determined by its solubility in the spinning solution, in this case was 10% (wt/wt) of IND in total mass of PGl used. Increasing the loading capacity is not easily possible as increasing the amount of solvent would compromise the spinning outcome. In the second approach, PGl-XI fibres were soaked in a DCM solution of IND at concentrations of 10 (PGl-XI-10) and 20% wt/v (PGl-XI-20). The actual loading was determined by monitoring the IND concentration in the solvent before and after loading by HPLC. Figure 3-14 shows the amount of IND loaded in PGl-F-Ind., PGl-XI-10 and PGl-XI-20 samples.
Figure 3-14. Drug loading capacity by pre-spinning (PGF-F) and by swelling using IND. solutions in DCM at concentrations of 10 (PGl-XI-10) and 20 (PGl-XI-20) %wt/v.

The results demonstrate that 21 ± 2 mg IND per 100 mg PGl could be loaded in the PGl-XI-10 sample, while 35 ± 4 mg per 100 mg PGl was loaded in the PGl-XI-20, which is significantly higher than that obtained by the direct spinning approach (10 mg IND/100 mg PGl). The drug release performances of PGl-F-Ind, PGl-XI-10 and PGl-XI-20 was investigated by placing them in a buffer/methanol solution. The IND in the release medium was determined by HPLC at time intervals. An initial burst release regime comprising release of physisorbsorbed drug and diffusion of drug near the surface is seen for the first 180 min for all samples (Figure 3-15).
Figure 3-15. In vitro release profiles of indomethacin encapsulate pre-spun (PGl-F-Ind.) and by swelling using IND. solutions in DCM at concentrations of 10 (PGl-XI-10) and 20% wt/v (PGl-XI-20).

An initial amounts of IND incorporated in the bulk of the fibres can be calculated which will only be released upon fibre erosion (not further studied here),\(^{21,22}\) i.e., 90 µg/mg PGl for PGl-F, 145 µg/mg PGl for PGl-X-10, and 205 µg/mg PGl for PGl-X-20. These experiments highlight not only that a higher drug loading is achievable by the swelling approach using cross-linked fibers, but also that the total amount of drug can be controlled by the drug concentration in the swelling solution.

4. Conclusion.

The results highlight that PGl-XI eletrospun fibers are a potential alternative scaffold material to PCI or PGLA in tissue engineering applications. Comparing the PGl-XI and PGl-F it was possible to conclude that the crosslinking process improves the mechanical properties of the fibres as well as alter the crystallinity of the material. Moreover, the benefit of cross-linking was highlighted by the possibility of solvent swelling under retention of the fibre morphology. This opens possibilities to load fibers with active ingredients post spinning, thereby omitting the need to optimize spinning protocols for each drug/polymer formulation. Moreover, higher loading
capacity as well as the opportunity to vary loading amounts by their concentration in the swelling medium mark an advantage over conventional polyester fibres

5. References.


731–738.


Chapter 4

Biofunctionalization of Poly(globalide) fibrous scaffolds
1. Introduction.

Due to the versatility of properties synthetic polymers has been extensively studied to produce biomedical devices.\(^1\) Their applications vary from prostheses and implants to drug carriers and scaffolds for tissue engineering.\(^2\) Polymers for tissue engineering scaffolds have to meet specific properties, including good mechanical properties, biodegradability and non-toxicity in order to be qualify for this application. Of particular importance is that the material interface facilitates good cell adhesion and proliferation. Poor interaction between these materials and the cells could cause immunogen response and limit the treatment efficiency.\(^3\) In this context, polymer surface functionalization have been largely studied to decrease the rejection reactions and also to enhance the cells attachment and potentially increase their growth. Applied procedures enable the interfacial interaction of synthetic materials with biological entities for example cells. Ideally, the scaffold material should also avoid inflammations, infections, aseptic loosening, local tissue waste and implant encapsulation as well as thrombosis and embolization.\(^4\)

There are many different procedures for surface functionalization, for example surface graft polymerization.\(^5\) Another promising approach is the surface attachment of biocompatible groups varying from amino acids to proteins. Among those, molecules presented in the extracellular matrix (ECM) including elastin, fibronectin, and laminin present a particular advantage due to their capability of binding with specific proteins (integrin) on the cell.\(^6\) However, these large biomolecules present some challenges on their manipulation, purification and bioconjugate on the polymer surface, which could promote their fast degradation. Small molecules composed of amino acids are a great alternative for surface functionalization, as they are more stable than proteins and could promote a specific stimulation on the cells. Of interest are for example amino acid sequence including RGD, YIGSR, or IKVAV.\(^7\) Notably, the concentration of these functional groups on the surface has to be controlled as it could alter the cell migration. For example, at higher concentrations a strong cell adhesion would occur thereby decreasing the cell migration, which is a disadvantage if full proliferation throughout the scaffold is desired.\(^8\)
The next generation polymeric fibrous scaffolds should be a combination of a substrate promoting cell reproduction and at the same time act as a drug reservoir, slowly releasing suitable drugs for example to avoid tissue inflammation during the cell growth. There are clear advantages of using electrospun fibres for this purpose due to the high surface-to-volume ratio, which would facilitate efficient drug release by diffusion, degradation or a combination of both. As demonstrated in Chapter 3, loading of PGI-XI by soaking is highly efficient. In this chapter the functionalization of PGI-XI fibres by covalent surface attachment the RGD was evaluated. Subsequently, the effect of this functionalization on cells viability was studied.

2. Methods

2.2.1 eROP of Globalide.

For the eROP of Gl, 2.16 g (0.009 mol) of the monomer was added to a Schlenk flask containing 80 mg of enzymatic catalyst Novozyme 435. Toluene (1.6 g) was added to the mixture, and the reaction flask was purged with nitrogen, placed in an oil bath at 60°C, and stirred during 4 hours. Posteriorly, DCM was added to Schleck flask, to inhibit the enzymes and separate them by filtration. In the last step of the synthesis, the filtered solution was dropped in cold methanol and the precipitated PGI was filtered, furthermore the polymer was vacuum-dried at room temperature over a 24-hours period. The dried PGI was weight and the yield of each reaction was calculated by the follow equation.

\[
\text{Yield (\%)} = \left( \frac{\text{Polymer mass}}{\text{Monomer mass}} \right) \times 100
\]
2.2.2 Gel Permeation Chromatography (GPC).

The GPC measurements were performed using an Agilent 1200 series instrument equipped with GPC control software. All measurements were carried out using a Polymer Laboratories Gel 5 μm Mixed-C 300 x 7.5 mm column, at 40 °C with DAD and RID detection. Tetrahydrofuran (THF) was used as the eluent at a flow rate of 1 mL/min. The molecular weights of all polymers were calculated based on polystyrene standards.

2.2.3 Electrospinning of PGI-XI.

The electrospinning was performed using Spraybase electrospinning machine with an UV lamp adapted inside of the machine. With this adaptation is was possible to start the cure process of the PGI-XI during the electrospinning. The electrospinning was performed using the parameters of the optimum process, obtained previously including PGI solution of 60% wt. in DCM, combination of 1,5-pentanediithiol (XI-2) and (2,4,6 – trimethylbenzoyl) phenone oxide with 2-hydroxy-2-methylpropioifenone (P2) in a 1:1 wt/wt, voltage of 14kV, distance of the nozzle 20cm, flow rate 200 μL/min and collecting the sample in methanol.

2.2.4 Amine Functional Fibres.

To prepare the fibres with amino-functional group on the surface (PGI-NH₂) thiol-ene reaction as used on the surface with 2-(Boc-amino) ethanethiol. Equal moles of photo initiator 2,2 dimethoxy -2-phenyl acetophenone and 2-(Boc-amino) ethanethiol (0.35 mmol) were mixed with 0.5 mL THF and then transferred onto fibres surface followed by UV. The deprotection of the Boc group was conducted by immersing samples into trifluoroacetic acid (TFA) for one hour followed by neutralizing with excess of sodium hydroxide (NaOH) 0.1mol/L solution and washing with DI water. ¹⁰

2.2.5 RGD Functional Fibres.

The immobilization of RGD on the fibres (PGI-RGD) was performed using the PGI-NH₂ samples was immersed on a 1-ethyl-3-
(dimethylaminopropyl)carbodiimide (EDC) solution 0.1M dissolved in 2-N-morpholino-ethanesulfonic acid (MES) buffer 0.1 M (pH 5.5) to increase reactivity. Posteriorly, the samples were removed from this solution and replaced in petri-dishes and a new solution with RGD (Arg-Gly-Asp), EDC and N-hydroxysuccinimide (NHS) were prepared in MES solution 0.1M added on the surface of the scaffolds and maintained during 3 h at room temperature. The RGD and the EDC solution were combined in a 1:1 molar ratio and NHS was added to that solution in a molar ratio of EDC:NHS of 4:1, and the proportion of RGD was de 0.2mg per mm² of the scaffolds surface area. In addition, the samples were washed and sterilized for the cells viability and XPS studies.

2.2.6 X-ray Photoelectron Spectroscopy.

X-ray Photoelectron Spectroscopy was performed under ultra-high vacuum conditions (< 5×10⁻¹⁰ mbar) on a VG Scientific ECSAlab Mk II system using Al Kα X-rays (1486.6 eV). The analyzer pass energy was set to 100 eV for survey spectra and 30 eV for high resolution core scans recorded. An electron flood gun was used for charge compensation and the binding energy scale was referenced to the adventitious carbon 1s core-level at 284.8 eV.

2.2.7 Mechanical Properties.

The mechanical properties of the samples were measured using a tensile machine Zwick/Roell model Z2 with loading of a cell of 50N at 5 mm/min, using samples cut in dog-bone shape. These analyses were performed in triplicate, and with the average of the results the tensile strength and the Young’s Modulus of the samples were defined.

2.2.8 Water Contact Angle.

To define if the crosslinking reactions interfered in the hydrophilicity of the scaffolds, the water contact angle was investigated. The tests were performed by contact angle analyser FTA125 (First ten Angstroms, USA), using 4 drops of distilled water in each sample. The results were analysed using the software of the machine.
2.2.9 Cell Seeding and Culture Conditions.

Human mesenchymal stem cells (MSCs) were isolated from the iliac crest of normal human donors 20-30 years old (Lonza Biologics PLC) according to a previously established protocol (Barreto, et al., 2017) and expanded in growth media consisting of low-glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and penicillin (100 U/mL)-streptomycin (100 µg/mL) at 37°C under humidified atmosphere (5% CO₂ and 95% relative humidity). Medium was replaced every 3 days and upon reaching 80–90% confluency, cells were passaged using trypsin–EDTA solution. For the following experimental analysis cells were used up to passage 6. Electrospun scaffolds (Ø 8 mm) were punched out from the electrospun mesh. To prepare for cell seeding, scaffolds were hydrated by progressive washes in ethanol and several washes in sterilized PBS. Finally, cells were seeded onto one side of the electrospun scaffolds at a density of 100,000 cells per scaffold. Cell-seeded scaffolds were maintained expansion medium at 20 % pO₂ for 8 days, with medium changed twice.

2.2.10 DNA quantification.

After 1, 4 and 8 days of cell seeding, DNA was quantified in scaffolds as a indicative of cell proliferation. First, 1mL of lysis buffer (0.2M Sodium Carbonate (Na₂CO₃) + 1% V/V Triton X in dH₂O) was added per scaffold, followed by three freeze-thaw cycles in liquid nitrogen, in order to rupture cells membrane and exposure DNA content. Finally, DNA was quantified with Quant-iT™ PicoGreen ® dsDNA (Invitrogen), according to manufacture instructions. To calculate the doubling time, i.e., the time needed for the DNA content to double between day 1 and 2, the following formula was used: doubling time=duration of time x log(2)/log(Final DNA content)−log(Initial DNA content). To calculate percent growth rate, first the percent change was calculated as Percent Change = 100 × (Final DNA content – Initial DNA content) / Initial DNA content. Finally, the percent growth rate was calculated as Percent Growth Rate = Percent Change / duration of time.
2.2.11 Statistics.

Statistics. Statistical difference was established at P <0.01 by two-way ANOVA followed by a Bonferroni’s post hoc test for pairwise comparisons of DNA Content in control, PGl-F, PGl-Xl, PGl-NH$_2$ and PGl-RGD. All data are reported as means ± standard deviation (GraphPad Prism 5, GraphPad Software).

3. Results and Discussion

3.2.1 Synthesis of RGD functional PGl

The average PGl molecular weight used for the experiments was around 25,000 g/moL and the polydispersity 2.1. Cross-linked PGl (PGl-Xl) was produced using the optimum condition according to Chapter 3 using PGl solution in DCM (60% wt.), combination of XI-2 and P-2, voltage of 14 kV, distance of the nozzle 20 cm, flow rate 200 µL/min and collecting the sample in methanol. The fibres used for these experiments presented average diameter of 9.2 ± 1.6µm. The functionalisation strategy comprises two steps. In the first step, surface attachment of an amine compound was sought assuming sufficient remaining double bonds after PGl cross-linking. In the second step, the amino groups were coupled with RDG (Arg-Gly-Asp) sequences.

The PGl-NH$_2$ fibres were produced by thiol-ene reaction of Boc-amino-ethanethiol with surface alkene group present on the PGl-Xl fibres (Figure 4-1). A solution of photo initiator 2,2 dimethoxy -2-phenyl acetophenone and Boc-amino-ethothoni in THF was add on the PGl-Xl. The sample was then irradiated with UV light. The deprotection of the Boc group was conducted by immersing the samples into TFA followed by extensive washing with DI water.
Figure 4-1. Reaction schemes of thiol-ene reactions between 2(Boc-amino) ethanethiol and alkene group on the PGI-XI fibres, and posteriorly the deprotection.

Initial qualitative confirm for the presence of primary amino groups on the PGI-NH$_2$ samples was obtained by the ninhydrin test. This test consist in a chromogenic reaction between the ninhydrin and an amino group at pH 5.5, promoting the formation of soluble chromophores. In addition, when ninhydrin reacted to primary amino groups a purple dye called Ruhemann’s purple (RP) is formed. Basing on this, the ninhydrin test was used for an initial evaluation of amino groups formation on the PGI-NH$_2$ surface, posteriorly the nitrogen quantification on the samples was performed by XPS. For this procedure, discs with diameters of 8 mm were cut from the fibre mesh and immersed in a solution of ninhydrin (0.1% wt.) in a mixture of acetic acid and DI water (10:90 v/v) for 20 min. The results of this initial test confirms the presence of primary amino groups on the PGI-NH$_2$ surface (Figure 4-2) as evident from the typical purple colour upon the reaction between the amino group and ninhydrin. Notably, the test was negative for a sample that was not deprotected.
RGD is an amino acid sequence present on the extra-cellular matrix (ECM) proteins, such as fibronectin and collagen. RGD is crucial for modulating cell adhesion, migration, and differentiation of various cells. It was envisaged that RGD functionalisation of the PGL-NH$_2$ fibres would facilitate polymer/cell interaction. The RGD immobilization on the PGL-NH$_2$ fibres surface was performed using classical EDC/NHS chemistry (Figure 4-3) by immersion a PGL-NH$_2$ mesh into an EDC/NHS/RGD solution. Thorough washing with DI water after the reaction ensured the removal of all unbound material.

Figure 4-2. Ninhydrin test with PGI-NH$_2$ before (A) and after (B) the deprotection procedure.

![Figure 4-2. Ninhydrin test with PGI-NH$_2$ before (A) and after (B) the deprotection procedure.](image)

Initial confirmation that the reaction occurred was obtained from water contact angle measurements. Figure 4-4 shows a drop of water placed on PGI-F, PGI-
XI, PGI-NH$_2$ and PGI-RGD. There is a slight reduction in contact angle from 110 to 102$^\circ$ due to the cross-linking process. A significant drop of water contact angle by approximately 25% was detected after amine functionalisation, which is in agreement with the expectation of higher hydrophilicity of this sample. Notably, the surface hydrophilicity of a scaffold plays an important rule for the cells attachment and migration, as well as the rejection reaction.\textsuperscript{12}

![Contact Angle Images](image)

Figure 4-4. Contact angle images for PGI-F, PGI-XI, PGI-NH$_2$ and PGI-RGD.

X-ray photoelectron spectroscopy (XPS) is a sensitive technique for surface characterization, presenting a probe depth of typically <5 nm for organic materials. This technique can provide the chemical composition of the surfaces and therefore allow monitoring the success of the surface reaction.\textsuperscript{13} XPS analysis was performed to specifically evaluate the efficiency of the surface functionalization process by detecting and quantifying nitrogen (N) on the PGI-F, PGI-XI, PGI-NH$_2$ and PGI-RGD samples. Figure 5-4 shows the survey scan of the PGI-F and the expected oxygen (O1s; binding energy 532 eV) and carbon (C1s; 285 eV) signals are present. The situation is similar for the cross-linked sample PGI-XI. No sulphur signals can be assigned in this spectrum probably due to the low penetration depth of the technique. Analysing XPS spectrum of PGI-NH$_2$, beside the C and O signals a small nitrogen 1s peaks is present around 399 eV, in agreement with the amine functionalisation of the fibre surfaces.
After RGD attachment the relative intensity on the amine 1s signal is increased due to the introduction of additional nitrogen atoms in the amide bonds. The interpretation of the XPS spectra is in agreement with work published by Pourcelle et al. The authors studied the production of PCl-PEG (Poly(ethylene glycol) nanoparticles grafted with GRGDS (Gly-Arg-Gly-Asp-Ser), and the XPS results obtained presented the amine and amide bound peaks. From the spectra the relative elemental concentration of the samples can be calculated. The summarised results in Table 4-1 show that the nitrogen concentration for PGI-NH$_2$ and PGI-RGD were 1 and 7%, respectively. This difference confirms the success of the functionalization process, which with the covalent attachment of RGD on the fibres surface the ratio of N increase proportionally. Furthermore, the analysing the O/N and C/O ratios between PGI-NH2 and PGI-RGD it was possible to observe that it dropped from 13 to 2.3 and 6.4 to 4.7, respectively, these results could be correlated to the higher amount of oxygen on RGD structure.

Table 4-1. Relative elemental concentration of the PGI-F, PGI-XI, PGI-NH$_2$ and PGI-RGD.

<table>
<thead>
<tr>
<th>Sample</th>
<th>C$_{1s}$ (%)</th>
<th>O$_{1s}$ (%)</th>
<th>N$_{1s}$ (%)</th>
<th>C/O</th>
<th>O/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGI-F</td>
<td>83.4</td>
<td>16.6</td>
<td>-</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>PGI-XI</td>
<td>85</td>
<td>12</td>
<td>-</td>
<td>7.1</td>
<td>-</td>
</tr>
<tr>
<td>PGI-NH$_2$</td>
<td>83</td>
<td>13</td>
<td>1</td>
<td>6.4</td>
<td>13</td>
</tr>
<tr>
<td>PGI-RGD</td>
<td>76</td>
<td>16</td>
<td>7</td>
<td>4.7</td>
<td>2.3</td>
</tr>
</tbody>
</table>
To evaluate the effect of the functionalization process on the mechanical properties of the scaffolds, tensile tests were performed with PGI-F, PGI-XI and PGI-RGD. For fibrous scaffolds, the morphology of the fibres directly influences the tensile
strength as well as the Young’s modulus. The amorphous phase of the fibres is responsible to their elastic properties and the crystalline phase play an important role to the dimensional stability.\(^{14}\) In this context, the tensile test results (Figure 4-6) demonstrate that the functionalization process altered the Young’s modulus of the scaffold, which comparing to the PGl-F and PGl-Xl it is around 75% and 90%, respectively. These results could be correlated to the fibres swelling during the amino functional step, that promote their reorganization in the scaffolds. This reorganization, probably increase the space between the fibres eliminating the conformation obtained during the electrospinning. Moreover, this characteristic probably contributed to the easy energy distribution of the samples during the tensile test, which resulted in the Young’s Modulus decrease. On the other hand, comparing the tensile strength results the functionalization process did not significantly alter this property. As pointed out before, mechanical test of fibre meshes are not straightforward and without a more detailed study should not be over interpreted.

![Stress-strain curve of PGI-F(black), PGI-Xl (red) and PGI-RGD (blue) (A), Young’s modulus (B) and tensile strength (C) of PGI-F, PGI-Xl and PGI-RGD.](image)

3.3 Cell Viability.

In the previous cell viability tests performed for Chapter 3 Porcine bone marrow mesenchymal stem cells (MSCs) were seeded on PGl-F and PGl-Xl fibre meshes to assess their viability, metabolic activity and proliferation to validate these polymers for tissue engineering applications. Moreover,\(\textit{in vitro}\) tests results defined that the PGl-F and PGl-Xl supported cell metabolic activity. MSCs have been used in
preclinical models for tissue engineering of bone, cartilage, muscle, and other mesenchymal tissues with significant promise.\textsuperscript{15} Based on this, the cell viability analysis was performed using similar parameters as in the previous PGl fibres studies, aiming to evaluate the RGD effect on the cell proliferation. At this moment, human MSCs were used, which can further increase the translational potential of this current technology to the clinics.

The DNA quantification was used to define the cell proliferation on the PGl-F, PGl-XI, PGl-NH\textsubscript{2} and PGl-RGD scaffolds. The results (Figure 4-7) demonstrate that DNA content of PGl-RGD was 9.88 times higher than PGl-F samples on day 1, indicating that the RGD surface functionalization enhanced cell attachment to the scaffolds. However, comparing the growth rate and the doubling time between day 1 and 8, it is noticeable that PGl-F growth rate was higher than PGl-XI, PGl-RGD and PGl-NH\textsubscript{2}, in that order (Table 4-2). This result could be explained with the strong cell adhesion on the PGl-RGD and PGl-NH\textsubscript{2} samples, which could be limiting cell migration and proliferation.\textsuperscript{8,16} Nevertheless, it is important to highlight that even with a smaller growth rate, PGl-RGD samples presented after 8 days of culture a DNA content 3.57 times higher than PGl-F samples.

\begin{table}
\centering
\caption{Doubling time and percent growth rate of samples based on DNA content between days 1 and 8 of culture.}
\begin{tabular}{|c|c|c|c|}
\hline
 & PGl-F & PGl-XI & PGl-NH\textsubscript{2} & PGl-RGD \\
\hline
Doubling time (days) & 3.28 & 4.34 & 8.36 & 8.28 \\
\hline
Percent growth rate (%) & 55.05 & 32.31 & 11.76 & 11.92 \\
\hline
\end{tabular}
\end{table}
4. Conclusions

The results presented on this chapter, confirms the versatility of the PGl for biomedical application. An efficient surface functionalization process was devised proving the RGD bioconjugation on the PGl fibres surface, which enhanced the cell attachment. However, the strong interaction between the cell and RGD probably limited the cell migration compared to the non-modified PGl fibres. Indeed, biocompatible scaffolds in which cells easily adhere to their surface and allow cell proliferation are a goal in tissue engineering and regenerative medicine, suggesting future clinical applications of the PGl-RGD scaffolds. Future cell differentiation and extracellular matrix deposition studies both in vitro and in vivo are necessary to reinforce this suggestion. Moreover, a future goal is to combine the surface functionalisation with drug loaded scaffolds.

5. References.

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Chapter 4


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Chapter 5

Electrospraying of poly(globalide): the production of crosslinked and core-shell microspheres.
1. Introduction.

Initial drug delivery systems made use of drug carriers from lipoproteins which protected the drug from the enzymatic degradation as well as provided some targeting to the site of action. However, these materials present some limitations with respect to their low encapsulation efficiency and the poor storage stability.\(^1\) Therefore, the encapsulation of a drug in biodegradable polymers in the form of micro or nano particles was introduced as an alternative. Benefits of polymeric particles are for example, avoiding the degradation of the non-released drugs, controlling the drug release and the amount released, release of the drug in a specific location and increasing the bioactivity of the drugs.\(^2\) For a polymeric material to be applicable in drug delivery it must possess some specifics characteristics, such as, chemical and physical stability in a biological environment and the polymer as well as its degradation products have to be non-toxic.\(^3\) Depending on the application, the polymer particles itself should also fulfil some specific characteristic. For example, the particles size plays an important role for the interaction with the cell membrane and defines the level of penetration across the physiological barriers. Another aspect is the blood circulation time, which can be increased by modifying the particle surface by functionalization or coating with hydrophilic molecules.\(^4\)

In the literature, many different techniques were applied to produce biodegradable polymer particles including oil in water (O/W) or water in oil (W/O) emulsions, coacervation and spray drying. However, these techniques can be challenging in terms of achieving uniform particles sizes and the homogeneous drug distribution throughout the particles. Since these particle characteristics directly influence the cell penetration and drug release profiles, they have to be tightly controlled. In this context, the development of systems that produce monodisperse particles with well-defined morphology became a key issues in particle supported drug delivery.\(^5\)

The electrospraying process is a great alternative to produce biodegradable polymer particles for drug delivery systems. This technique allows the processing of many different types of polymers and the samples produced by electrospraying tend to present small size distribution and also promote homogenous drug encapsulation. Due
to these characteristics, the possibilities of PGl microspheres (PGl-MS) production by electrospraying was evaluated. Initially an optimization was performed to evaluate the influence of the process parameters on the particles morphology. Subsequently, the production of crosslinked particles (PGl-MS-XI) and finally coaxial electrospinning was performed to produce core-shell particles with different hydrophilicity with a hydrophobic PGl core a poly(ε-trifluoroacetyl-L-lysine) shell (PGl-CS).

2. Experimental procedures.

2.1 Materials.

Globalide (Gl) was purchased from Symrise was used for the PGl eROP, novozyme 435 (Candida Antarctica Lipase B immobilized on cross-linked polyacrylate beads) was purchased from Novozymes A/S. Methanol, dried toluene and Dichloromethane (DCM) were purchased from Sigma. For the PTFALL₈₀ synthesis, ε-trifluoroacetyl-L-lysine, allylamine, dimethylformamide (DMF) and tetrahydrofuran (THF) purchased from Sigma were used. To produce the PGl-MS-XI it was used 1,5-pentanediol, (2,4,6 – trimethylbenzoyl) phenone oxide with 2-hydroxy-2-methylpropiophenone in a 1:1 wt/wt were purchased from Sigma.

To PGl electrospinning process, it was used dried THF and DCM both from Sigma. To the degradation tests, Phosphate buffering tablets (PBS) (pH 7.4) was obtained from Sigma. In addition, to prepare the reference samples for cell viability tests, the PCI (Mₘ 70 – 90 kDa) used was purchased from Sigma.

2.2 Methods

2.2.1 eROP of Globalide.

For the eROP of Gl, 2.16 g (0.009 mol) of the monomer was added to a Schlenk flask containing 80 mg of enzymatic catalyst Novozyme 435. Toluene (1.6 g) was added to the mixture, and the reaction flask was purged with nitrogen, placed in an oil bath at
60°C, and stirred during 4 hours. Posteriorly, DCM was added to Schleck flask, to inhibit the enzymes and separate them by filtration. In the last step of the synthesis, the filtered solution was dropped in cold methanol and the precipitated PGl was filtered, furthermore the polymer was vacuum-dried at room temperature over a 24-hours period. The dried PGl was weight and the yield of each reaction was calculated by the follow equation.

\[
\text{Yield (\%)} = \left( \frac{\text{Polymer mass}}{\text{Monomer mass}} \right) \times 100
\]

2.2.2 Synthesis of poly(ε-trifluoroacetyl-L-lysine).

The NCA of ε-trifluoroacetyl-L-lysine (4.10 g, 15.30 mmol) was added to a Schlenk flask and dissolved in a 40 mL mixture of anhydrous CHCl₃/DMF (4:1). Allylamine (10.92 mg, 1.91×10⁻¹ mmol) was prepared in 5 mL of dry CHCl₃, and quickly added to the Schlenk flask via syringe. The solution was allowed to stir for 3 hours at 25 °C. The sample was taken directly via syringe to measure the molecular mass using GPC. The samples precipitated in diethyl ether and dried under vacuum overnight and characterized by FTIR.

2.2.3 Gel Permeation Chromatography.

The GPC measurements were performed using an Agilent 1200 series instrument equipped with GPC control software. All measurements were carried out using a Polymer Laboratories Gel 5 µm Mixed-C 300 x 7.5 mm column, at 40 °C with DAD and RID detection. Tetrahydrofuran (THF) was used as the eluent at a flow rate of 1 mL/min. The molecular weights of all polymers were calculated based on polystyrene standards.

To determine the dispersity and molecular weight of PTFALL₈₀, GPC was conducted in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) using an PSS SECurity GPC system equipped with a PFG 7 µm 8 × 50 mm pre-column, a PSS 100 Å, 7µm 8 × 300
mm and a PSS 1000 Å, 7µm 8 × 300 mm column in series and a differential refractive index (RI) detector at a flow rate of 1.0 mL min⁻¹. The systems were calibrated against Agilent Easi-Vial linear poly(methyl methacrylate) (PMMA) standards and analysed by the software package PSS winGPC UniChrom.

2.2.4 Fourier-Transform Infrared Spectroscopy (FTIR).

The FTIR measurements were performed using a Nicolet IS10-FTIR (Thermo Scientific) by the accumulation of 32 scans at 4 cm⁻¹ resolution in the wave number from 4000-400 cm⁻¹. Specimens were placed on the diamond ATR crystal using a top-plate and pressure-arm accessories (smart iTX accessory). The data obtained were analysed using the software Ominc 8 (Thermos Scientific).

2.2.5 Electrospraying of PGI.

The electrospraying was performed using Spraybase electrospraying machine for the PGI-MS, variating the process parameters including flow rate, voltage and the polymer solution concentration and keeping the distance of the nozzle in 15cm (Table 5-1). The samples were collected in cold methanol.

Table 5-1. Parameters used to optimize electrospraying of PGI-MS.

<table>
<thead>
<tr>
<th>PGI Solution</th>
<th>Voltage (kV)</th>
<th>Flow rate (µL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% wt. DCM</td>
<td>10 – 15 – 20</td>
<td>50 – 100 – 200</td>
</tr>
<tr>
<td>10% wt. DCM</td>
<td>11 – 25 – 30</td>
<td>50 – 100 – 200</td>
</tr>
<tr>
<td>15% wt. DCM</td>
<td>11 – 25 – 30</td>
<td>50 – 100 – 200</td>
</tr>
<tr>
<td>20% wt. DCM</td>
<td>11 – 25 – 30</td>
<td>50 – 100 – 200</td>
</tr>
</tbody>
</table>

As in the PGI-XI electrospinning process, for PGI-MS-XI process an UV lamp was adapted inside of the machine to start the cure process of the samples during the electrospraying and both samples were collected on cold methanol. The PGI solution was prepared using the same cross linker and photo initiator used to produce PGI-XI samples (described on chapter 3). To the process optimization it was varied the voltage, flow rate and distance of the tip (Table 5-2).
Table 5-2. Parameters used to the optimization of PGI-MS-XI electrospraying.

<table>
<thead>
<tr>
<th>PGI Solution (with photo initiator and cross linker)</th>
<th>Voltage (kV)</th>
<th>Distance of the nozzle (cm)</th>
<th>Flow rate (µL/min)</th>
</tr>
</thead>
</table>

For the PGI-CS samples a coaxial nozzle with shell and core of 28/22 Gauss respectively, was used. The PGI inner solution was prepared using 10% wt. in DCM, the PTFALL\textsubscript{80} outer solution was prepared in 10% wt. in a mixture of methanol and THF (50:50% v/v). The samples were collected in a mixture of Phosphate buffer (PBS) (pH 7.4) and methanol with 40:60% v/v, and the process parameters including voltage and flow rate were varied (Table 5-3). For all produced samples, the same flow rate was used to the outer and the inner solution.

Table 5-3. Parameters used to the optimization of PGI-CS electrospraying.

<table>
<thead>
<tr>
<th>PGI Solution (core) + PTFALL\textsubscript{80} Solution (shell)</th>
<th>Voltage (kV)</th>
<th>Distance of the nozzle (cm)</th>
<th>Flow rate (µL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% wt. DCM (core) + 10% wt. THF+Methanol 50% v/v.</td>
<td>9 – 10 – 11 – 12 – 13</td>
<td>15</td>
<td>50 – 100 – 150</td>
</tr>
</tbody>
</table>

2.2.6 Scanning Electronic Microscopy (SEM).

The images of PGI-MS were obtained by a Hitachi variable pressure SEM, model S-3400N, ranging acceleration voltage between 10-20 kV, and before starting the experiments the samples were sputter-coated with gold. Posteriorly, the obtained images were treated and the average of the particles diameters were measured using the Image J.

The images of PGI-MS-XI were obtained by SEM Carl Zeiss Ultra, ranging acceleration voltage between 5-10 kV, and before starting the experiments the samples
were sputter-coated with platinum. Posteriorly, the obtained images were treated and the average of the particles diameters were measured using Image J.

2.2.7 Optical Microscopy.

To evaluate the morphology and verify the core-shell structure on the particles, after defining the best parameter by SEM, the samples were suspended in a mixture of ethanol and DI water, and a drop of this suspension was added on the slide glasses and the images were obtained. The results of the tested parameters were analysed using an optical microscope Leica model DMIL.

2.2.8 X-Ray Diffraction.

The XRD analysis were carried out on a Bruker AXS D8 Advance with a 3kW tube with a copper anode using the K-alpha line. The sample was mounted on a six-axis goniometer and a locked coupled scan used. The samples were then scanned through an angle theta and the detector was maintained at twice that angle theta.

2.2.9 Differential Scanning Calorimetry.

The thermal properties of the samples were characterized by DSC, and their melting point (T_m) and the melting enthalpy (ΔH_m) were measured on TA instruments Q200 DSC. The analysis were performed using aluminium pan with temperature range from -60 °C to 120 °C, and with a heating rate of 5 °C per minute, under nitrogen atmosphere.

2.2.10 Releasing profile.

To evaluate the releasing profile of PGI-MS, it was used as a model drug rhodamine B, that was loaded 10% wt./wt. in proportion to the polymer mass used to prepare the solution before starting the process. The electrospraying was performed using the optimum parameters defined during the optimization of PGI-MS, and the samples were collected in methanol and dried under vacuum overnight. Posteriorly, 50 mg of samples (n=5) were placed in Eppendorf tubes containing 2 mL of medium release
solution (PBS and methanol 80:20, v/v) and then incubate at 37°C in a water bath shaker. The release medium was completely removed at predetermined time point and replaced with fresh solution. The measurement of the amount of released rhodamine B was performed by UV-spectroscopy using a calibration curve (Appendix 5-1).

3. Results and Discussion.

3.1 PGI and PTFALL80 synthesis.

PGI was synthesized by enzymatic ROP of globalide with an average molecular weight of 25,000 g/mol and polydispersity of 2.1. PTFALL80 was provided by a group member and obtained by the ROP of ε-trifluoroacetyl-L-lysine N-carboxyanhydride (NCA according to Figure 1. The average molecular weight of PTFALL80 was 19,400 g/mol and the polydispersity 1.07 as measured by GPC. (Figure 5-1)

![Reaction scheme for the synthesis of PTFALL80 and GPC chromatogram the polymer product.](image)

The FTIR spectrum of the polypeptide (Figure 5-2) present a characteristic amide-I band at 1649 cm⁻¹ for the peptide backbone C=O and amide II band at 1545 cm⁻¹ for peptide backbone N-H bending. The band at 1709 cm⁻¹ is due to the C=O stretching of the side chain amide protecting group of the PLys(TFA). These bands are
characteristic of \(-\text{helical secondary structure}. The polypeptide was subsequently deprotected to produce poly(lysine).

![Figure 5-2. FT-IR spectra for PTFALL\(_{80}\).](image)

3.1 Electrospraying of PGI.

The optimization of PGI-MS electrospraying was performed by varying the process parameters (Figure 3-5). The optimum process was reached using solutions with 20% wt. of PGI in DCM, voltage of 15 kV, flow rate of 200 \(\mu\)L/min and nozzle distance of 15cm. The particles obtained on these conditions (Figure 5-5) presented smooth surface, spherical shape and narrow size distribution (diameter=15.2 \(\mu\)m, SD=1.7 and \(n=20\)).
<table>
<thead>
<tr>
<th>Sample</th>
<th>Voltage</th>
<th>Flow Rate</th>
<th>DCM %</th>
<th>Distance</th>
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<td>7.5kV</td>
<td>100µL/min</td>
<td>5 %</td>
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</table>

Figure 5-3. SEM images of PGI-MS samples obtained during the optimizing process.
Notably, it was found that the particles size and their distribution could conveniently controlled by the polymer solution concentration. The results depicted in Figure 5-4 demonstrate that by increasing the polymer solution concentration the particle diameter became bigger and more homogeneous. This characteristic is in agreement with the results obtained by Xu et al.⁸, who studied the possibilities of water-soluble protein encapsulation in polylactide (PLA). The authors correlated this behaving to the greater surface tension, viscosity and low conductivity present on the higher concentration solutions that consequently resulted in larger drops formations during the process.

![Figure 5-4](image_url)

**Figure 5-4.** The average of PGI-MS particles size obtained with different concentrations and keeping the same flow rate at 200µL/min, distance of the nozzle 15 cm and voltage of 20kV.
Figure 5-5. PGI-MS particles obtained with the optimum spraying parameters (PGI 20%wt. in DCM, voltage of 15 kV, flow rate of 200 μL/min and nozzle distance of 15 cm).

3.2 Electrospaying of PGI with simultaneous cross-linking

PGI-MS-XI was produced using the same crosslinker and photoinitiator as for the PGI-XI fibres. Initially the optimum parameters were used, however the solution with 20% wt. of PGI formed fibres during the process, so the solutions with 10% wt. of PGI in DCM were used. The samples produced were dried overnight and their morphology were evaluated by SEM (Figure 5-6). The SEM images show that the electrospaying parameters influenced the morphology of the particles. In some samples pore formation on the particles was observed (Figure 5-6, condition MS-XI 9). This was correlated to solvent evaporation during the electrospaying process, such that fast evaporation could cause bubbles formation and consequently the porosity found on these particle. 9
In the literature, some papers defined that the polymer solution concentration and the applied voltage play an important role for the electrospraying results. The results of PGI-MS-XI optimization showed that the voltage influences the distribution of the droplets. Different samples were obtained with various conditions, as shown in Figure 5-6.

Figure 5-6. SEM images of PGI-MS-XI samples obtained during the optimization process.
of the particle size (Figure 5-7). Increasing the voltage, the particle sizes distribution tends to decrease slightly, which could be correlated to the stability of the liquid jet during the process. This result is in agreement with results presented by Faramarzi et al.\textsuperscript{10}, who studied the optimization of the poly(lactic-co-glycolic acid) (PLGA) electrospraying process. The authors described that the voltage directly influenced the charge on the droplet surface, and consequently cause Rayleigh effects, Coulomb fission, and the instability of the jet.

![Figure 5-7. PGl-MS-XI particles obtained varying the voltage and keeping the concentration of PGI 10%wt. in DCM, flow rate of 200 µL/min and nozzle distance of 15 cm.](image)

The optimum process conditions for PGI-MS-XI electrospraying was reached using solutions of PGI 10% wt. in DCM, distance of the nozzle of 15 cm, voltage of 19kV and a flow rate of 200 µL/min. To evaluate the success of crosslinking process, the samples obtained under these conditions were suspended in DCM, and some drops of this suspension were added to the SEM stubs and dried under vacuum overnight. The particle morphology was evaluated by SEM (Figure 5-8). DCM was used due to the high solubility of PGI in this solvent. The SEM images show that after the suspension
the spherical morphology of the particles was still intact, confirming the success of the in-situ crosslinking process. Some surface imperfections can be observed, however.

![SEM images of PGl-MS-XI particles before (A) and after being suspend in DCM.](image)

**Figure 5-8.** SEM images of PGl-MS-XI particles before (A) and after being suspend in DCM.

The crystallinity of the samples was measured by XRD. The results demonstrate that the electrospraying process altered the crystallinity of the PGl (Figure 5-9). As observed for the PGl electrospinning results, the electrospraying process increase the crystallinity of PGl. For both spectra of PGl-Bulk and PGl-MS, respectively, reflections peaks at 20 values at 21.4º and 20.2º were observed, which indicates a $d$-spacing 0.415 and 0.440 nm.\(^{15}\) As for the electrospinning, the electrospraying process tends to induce chain alignment and crystallinity.\(^{16}\)

![XRD diffraction spectra of microspheres (PGl-MS) and bulk (PGl-bulk) of poly(globalide).](image)

**Figure 5-9.** XRD diffraction spectra of microspheres (PGl-MS) and bulk (PGl-bulk) of poly(globalide).
Figure 5-10. DSC thermograms of first heating cycle of samples PGI-Bulk (black) and PGI-MS (red).

The thermograms of the PGI-bulk and PGI-MS samples (Figure 5-10) corroborate the XRD results (Figure 5-9). The melting enthalpies obtained from DSC are in agreement with the higher crystallinity of the particles ($\Delta H_m = 199.5$ J/g; melting point: 41.2°C) compared to the PGI-bulk ($\Delta H_m = 107.2$ J/g; melting point 43.3°C). These results illustrate that the electrospinning conditions enhance the uniformity on PGI crystallinity.\(^{17}\)

Initial experiments were carried out to evaluate the release profile of the PGI-MS, using Rhodamine B as a model drug. The dye was added directly to the spraying formulation. The release profile (Figure 5-11) displays an initial burst release in the first 480 minutes, which is assumed to be the non-encapsulated rhodamine B physically sorbed on the surface of the particles as well as diffusion of dye near the surface. Over the observed time of 1500 min the maximum released rhodamine B was 0.078 mg/mL, corresponding to a total of 15.6% of the rhodamine B encapsulate. While the results demonstrate the potential of the particles, further extensive release and complementary degradation studies of these specific PGI-MS particles are necessary before any firm conclusions regarding biomedical applications can be made.
3.3 Electrospraying of core-shell particles (PGI-CS)

Amphiphilic core-shell particles are promising materials for drug delivery systems as they combine a hydrophobic core and a hydrophilic shell. The possibilities of PGI-CS particle formation were investigated using coaxial electrospraying process. The coaxial nozzle has a double needle with different diameters, that enable the processing of two polymer solutions creating samples with core-shell structures. For this step, the PTFALL\textsubscript{80} was chosen to compose the shell of the particles due its hydrophilicity, biodegradability and low toxicity. The results of these initial tests were evaluated by SEM (Figure 5-12). The SEM images show that the coaxial electrospinning resulted in particles with different morphologies and with imperfections on their surface. In addition, increasing the voltage it was observed that the particles started to agglomerate and only few particles kept a spherical morphology (Figure 5-12, condition CS 6). As mentioned above, this result could be correlated to the instability of the liquid jet during the process, which result in a non-spherical droplet. The PGI-B, PTFALL\textsubscript{80}
and PGI-CS FT-IR spectra confirm the presence of both polymers on PGI-CS samples (Figure 5-13). The results showed an amide I band at 1649 cm\(^{-1}\) for PGI-CS and PTFALL\(_{80}\) that correspond the peptide backbone C=O, while for PGI-B this band was not observed.

Figure 5-12. SEM images of PGI-CS samples obtained during the optimization process.

Figure 5-13. Section of the FT-IR spectra for PGI-B, PTFALL\(_{80}\) and PGI-CS.
To evaluate the core-shell structure, the PGI-CS samples were suspended in ethanol:water (30:70%, V/V) solution and kept immersed during 20 min. Subsequently, the drops of this suspension were put on a glass slide and optical microscopy images were taken (Figure 5-14). The obtained images show that the polar shell, probably hydrated, and a denser core is present. This suggests that indeed a core-shell structure was obtained in this process.

![Figure 5-14. Optical images of PGI-CS particles.](image)

4. Conclusion.

The results presented on this chapter demonstrated that PGI is versatile material for electrospraying process to obtain particles with spherical morphology, which can also be cross-linked in-situ. The latter offers the advantage of solvent stability for example for drug loading. As observed in the electrospinning experiments, the electrospraying process alters the crystallinity of the material. Initial loading experiments demonstrate the potential of the particles to deliver active ingredients, but further studies are needed to generate more data. Finally, core-shell particles were obtained in a process combining two polymers, which could be a convenient way to render the surface properties of PGI particles.
5. References.


(7) Luo, C.; Okubo, T.; Nangrejo, M.; Edirisinghe, M. Preparation of polymeric


v.103, p. 3205–3216.


Chapter 6

Conclusion
Poly(e-caprolactone) (PCl) has been widely studied as a polymer for biomedical devices, including scaffolds for tissue engineering and particles for drug delivery. Polymers from macrolactones have been discussed as a good alternative to biomedical applications due to their mechanical and thermal properties, potential biodegradability and biocompatibility. In addition, polymer from unsaturated macrolactones such as poly(globalide) (PGl) would allow polymer backbone modification so as to adapt it for specific application. The aim of this project was to evaluate the possibilities of processing PGl into functional materials by electrospinning for fibrous scaffolds for tissue engineering and electrospraying to produce particles for drug delivery system.

Initially, the results obtained during the PGl electrospinning optimization, confirmed the influence of the processing parameters on the fibre morphology as described in the literature for other polymers. Increasing the polymer concentration, more homogeneous fibres were obtained. A critical choice was the spinning solvent as process performed using THF resulted in fibres not completely leading to some fusion points on the fibres mats. This was improved by using DCM as a solvent. Finally, optimum parameters for PGl spinning were obtained resulting in homogeneous PGl scaffolds (PGl-F). Cell viability tests confirmed that PGl fibres were a non-toxic and feasible for biomedical application.

As the next step, the possibilities of producing crosslinked PGl fibres (PGl-XI) were evaluated using thiol-ene photo-chemistry. For these studies, UV-lamp was positioned inside of the electrospinning cabinet to promote the UV-light emission onto the liquid jet and the fibres during the process to initiate the crosslinking reactions. During the optimization, it was observed that the type of photo-initiator as well as the cross-linker played an important role for the fibres morphology. In addition, it was observed that by using a dish plate collector with methanol the fibre morphology was improved and the samples were more homogenous and with fewer surface imperfection. This was correlated to the removal of the unreacted materials in the methanol during the fibre precipitation. Upon crosslinking improved mechanical properties and a decrease in crystallinity was observed. Most importantly, fibre swelling in organic solvents promoted drug-loading and subsequent release from the scaffolds. These results confirm the versatility of PGl-XI mats and highlight
opportunities of producing scaffolds that could simultaneously promote cells proliferations and a drug-release.

Aiming to further improve the cell viability of the fibrous PGI scaffolds, in the fourth chapter of this project possibilities of the surface functionalization were evaluated. For these studies, a sequence of amino-acids (RGD) was covalently attached to the fibre surface. Initially, using thiol-ene photo-chemistry 2-(Boc-amino) ethanethiol was added to the PGI-Xl surface, and after the deprotection the presence of amino-groups on the fibres (PGI-NH$_2$) was confirmed by ninhydrin test and XPS. Subsequently, RGD was reacted with the amino groups. Cell viability analysis confirmed that the presence of RGD promoted an increase in cell attachment and DNA content of PGI-RGD was 9.88 times higher than PGI-F on day 1. In addition, after 8 days the DNA content in PGI-RGD was 3.57 times higher than PGI-F samples, confirming the improvement on the cell viability on the PGI scaffolds.

Lastly, electrospraying was performed using PGI solutions aiming to produce particles for drug delivery system. The initial results confirm the versatility of the PGI and microspheres (PGI-MS) with good morphology and homogeneous diameters were obtained. Release studies suggest potential for application in drug delivery systems. Based on the success of fibre crosslinking process, the same principals were applied to produce PGI cross-linked particles (PGI-MS-Xl). The results confirm that the in-situ crosslinking process could be applied for the PGI-MS-Xl production, and the particles retain their shape obtained even after being suspended in DCM. Finally, aiming to increase the hydrophicity on the microspheres surface, a coaxial electrospray was performed. For this study, poly($\varepsilon$-trifluoroacetyl-L-lysine) (PTFALL$_{80}$) was used in the outer solution (shell) and PGI in the inner solution (core). The core-shell structure was confirmed by optical microscope images and also FT-IR analysis, confirming the possibilities of production of PGI microspheres with a hydrophilic shell.

In this project, it was demonstrated that PGI is a versatile material for biomedical applications. For TE, the crosslinking process was a key element to the PGI fibrous scaffolds, because it improves the mechanical properties and also enable the bioconjugation of biomolecules on the fibres surface. In addition, the PGI particles can perform drug realising and the core shell structure combining the hydrophobic and hydrophilic properties can increase the bioactivity of the drugs as well as to decrease
their side effect. Concluding, PGI can be processed by different techniques and the structured samples shows a great range of different properties confirming the possibilities of its adaptations for a specific application.
Appendix
Appendix

Appendix 2-1. Optimization of the electrospinning process: PGI solutions in DCM collected on aluminium foil.
Optimization of the electrospinning process: PGI solutions in DCM collected on aluminium foil. (continuation)
Appendix 3-1. Calibration curve of indomethacin using HPLC.

\[ y = 2\times 10^6 x - 881921 \]
\[ R^2 = 0.99965 \]

Appendix 5-1. Calibration curve of rhodamine B using UV-vis spectroscopy.
Publication.

Conferences.

Research Brazil and Ireland (RBI) 2015, Dublin - Ireland, February-2015. The enzymatic synthesis of poly(globalide) and its application in electrospinning process. Fernando Cabral Salles de Oliveira and Andreas Heise (Poster presentation)

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