Functionalization of synthetic biodegradable electrospun matrices for biomedical applications

António Castro
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Colophon

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Functionalization of synthetic biodegradable electrospun matrices for biomedical applications

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Doctoral Thesis

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To all my family and friends, who always supported me on this amazing journey.
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Chapter 1

General Introduction
1. Tissue engineering and regenerative medicine

During the last decennia, the field of biomaterials has shifted its emphasis from achieving exclusively a bioinert tissue response to developing bioactive materials that can elicit a controlled action and reaction in the physiological environment. This shift has given rise to a new tissue repair strategy: tissue engineering and regenerative medicine. This strategy comprises the use of bioactive materials or scaffolds to guide or support endogenous or transplanted stem cells to regenerate the damaged tissues. Biomolecules are also added to scaffolds, promoting cellular growth and differentiation.

2. Biodegradable polymers

Ceramic, metallic and polymer-based biomaterials have been developed and are regularly used for tissue engineering applications. Composites constituted from the combination of these materials have also been developed. Polymers are the most popular class of biomaterials used in the tissue engineering and regenerative medicine field due to their availability and ease to synthesize, process and modify, resulting in materials with diverse physicochemical properties. The development of degradable biocompatible polymers was an important breakthrough, as it allowed the creation of materials that can be implanted in the body and thereafter fully degrade. Biodegradable polymers are widely used in the development of biomaterials for the most diverse clinical and biomedical applications. The main advantage related to the use of such materials is the fact that they degrade in the body, avoiding the need for a second surgical procedure to remove the implant.

Such polymers can be divided into two categories: degradable natural polymers and degradable synthetic polymers. Natural polymers, which include proteins (e.g. collagen, gelatin, and silk), polysaccharides (e.g. starch, cellulose, and chitosan) and deoxyribonucleic acid (DNA), have high biocompatibility, ability to initiate cellular adhesion and proliferation, and the degradation products are non-toxic. However, natural polymers might suffer from poor mechanical performance, fast and variable degradation rate, and ethical and safety concerns, the latter specifically when obtained from animal sources.

Degradable synthetic polymers, on the other hand, overcome these disadvantages. Their physicochemical properties and degradability can be easily tailored. Synthetic polymers can be cost-effectively synthesized, processed or modified to present good mechanical properties, higher thermal stability, and desired degradability. The most widely used synthetic biocompatible polymers include poly(anhydrides), poly(acrylics), poly(α-hydroxy) acids (e.g. poly(lactic acid), PLA; poly(glycolic acid), PGA), poly(ε-caprolactone) (PCL), and poly(hydroxyalkanoates) (e.g. poly(β-hydroxybutyrate), PHB). However, synthetic polymers have a major limitation: they generally lack biofunctionality.
This limitation is related to their inherent hydrophobicity and lack of reactive functional groups, leading to lower levels of cellular adhesion, proliferation, and differentiation.

Figure 1. Structure of polymers commonly used in electrospinning.

3. Electrospinning: A leading technique in the processing of biomaterials

Biodegradable (synthetic) polymers can be processed using diverse techniques, enabling the development of scaffolds with different shapes, morphologies, and physicochemical properties. Commonly applied techniques include solvent casting\(^{12, 13}\), thermally induced phase separation (TIPS)\(^{14, 15}\), freeze-drying\(^{16, 17}\), lithography\(^{18, 19}\), three-dimensional (3-D) printing\(^{20, 21}\), and electrospinning.\(^{22}\) Studied and patented by Formhals in the 1930s\(^{23, 24}\), electrospinning is an efficient technique to produce ultrafine polymeric fibers by applying an electric force to polymeric solutions.\(^{25}\) A typical electrospinning setup consists of a pump to push the polymeric solution, a nozzle through which the solution is extruded, a high voltage power supply connected to the nozzle to generate the electric force, and a grounded collector onto which the fibers are deposited. Several parameters can affect the formation and properties of electrospun fibers, mainly the applied voltage, flow rate, the viscosity, and conductivity of the polymeric solution.\(^{26, 27}\) Electrospun fibers have a diameter of 50 nanometers to a few microns, possessing a high surface area-to-volume ratio and a high porosity.\(^{28, 29}\) Due to these proper-
ties, electrospun scaffolds morphologically resemble the fibrous components of the native extracellular matrix (ECM). This feature makes electrospun membranes extremely attractive to be used as tissue engineering scaffolds.

4. Biomedical applications of biodegradable synthetic electrospun membranes

Electrospun membranes can be used for tissue engineering purposes. Electrospun non-woven membranes, which are considered to be two-dimensional (2-D) constructs, show potential as barrier membranes, keeping a soft tissue (skin, gingiva) wound clean and avoiding bacterial infiltration. Therefore, 2-D electrospun scaffolds seem specifically applicable in guided bone regeneration (GBR) and guided tissue regeneration (GTR) procedures.[4, 9, 30] Electrospun membranes can then be used as a physical barrier, preventing the migration of fibroblasts and the formation of fibrous tissue, and hence creating in such a way a secluded space around the bone defects, in which osteogenic cells can migrate and proliferate toward the formation of new bone.[11]

In addition, drugs can be incorporated in electrospun membranes. Due to their high surface area-to-volume ratio, electrospun membranes represent ideal drug carriers. Electrospun membranes can be produced or modified to achieve either an immediate release of the pharmaceutical compounds or a more sustained and slow release. Antibacterial and pharmaceutical compounds can be loaded in the electrospun fibers both by blending and coaxial electrospinning or incorporated
using post-spinning functionalization methodologies. Membranes containing tetracycline\textsuperscript{[31]}, gentamicin\textsuperscript{[32]} or vancomycin\textsuperscript{[33]} have been produced and their antibacterial activity has been evaluated. Pharmaceutical compounds as anti-cancer drugs\textsuperscript{[34-37]}, osteoinductive compounds\textsuperscript{[38, 39]} or anti-resorptive drugs\textsuperscript{[40-43]} have also been incorporated. The delivery of DNA and gene therapy using electrospun membranes as a carrier is currently under investigation.\textsuperscript{[44, 45]}

A disadvantage of the use of biodegradable polymers for the manufacturing of electrospun membranes is the final mechanical properties of the membranes. Frequently, the membranes are not stiff enough to maintain the space to allow ingrowth of bone. Consequently, polymers are combined with ceramic components (e.g. calcium phosphate precursors\textsuperscript{[46, 47]} or silicon-based components\textsuperscript{[48, 49]}) in order to create composite materials with enhanced mechanical properties. Ceramics are inherently brittle, and by incorporating polymeric fibers in their matrix, an improvement of the tensile properties and toughness can be achieved, due to resistance to crack propagation. Further, surface mineralization of electrospun polymeric synthetic fibers has been studied. By this strategy, the polymeric fibers have been coated with a mineral layer, leading to an improvement of their strength\textsuperscript{[50, 51]} and an increase in cellular adhesion and proliferation.\textsuperscript{[50]}

![Figure 3. Biomedical applications of electrospun fibers.](image-url)
5. Functionalization of electrospun biodegradable synthetic fibers

Many types of biodegradable synthetic polymers can be used for electrospinning. However, due to the lack of bioactivity of these polymers, the electrospun fibers need to be modified to render them biological functionality and improve their performance. The incorporation of relevant compounds by blending is a simple and popular strategy. As the name indicates, during blending the compounds are mixed with the polymeric solution before electrospinning. This strategy has the advantage of being simple and straightforward, enabling an easy incorporation of compounds in the electrospun fibers. However, the dissolution of the polymers is performed mostly by using strong organic solvents, limiting the incorporation of biomolecules. Additionally, a non-homogeneous distribution of the compounds in the polymeric fibers can occur, leading to unpredictable release phenomena. Coaxial electrospinning was developed to address such drawbacks. By this technique, fibers constituted of a core and a shell are formed. In the formation of the core, dissolution of biomolecules or pharmaceutical compounds is performed using mild organic solvents or aqueous solutions, while the shell is composed of the electrospun synthetic polymeric component. As such, the compounds are more homogeneously incorporated in the fibers and their release is limited by an initial diffusion from the core to the shell and lastly to the medium, leading to a more sustained and controlled release. Post-electrospinning strategies are also largely applied. Adsorption is a common strategy used to render electrospun membranes biofunctionality, by their immersion in solutions containing the compounds of interest. The interaction between the electrospun fibers and the compounds is based on physical entrapment and Van der Waals bonding. Due to this weak interaction, this method is often not efficient, showing a low incorporation rate. Layer-by-layer (LbL) deposition is a more refined adsorption technique, based on the sequential deposition of polyelectrolytes with an opposite charge. Due to electrostatic interactions and hydrogen bonding, polyelectrolyte multilayers (PEMs) can be deposited at the surface of the fibers. Charged compounds can also be incorporated due to their electrostatic interaction with these polyelectrolytes. Several studies demonstrated a higher incorporation of compounds via this technique and the possibility to control their deposition and release by altering several parameters including the pH, the ionic strength of the solutions, or the temperature. Covalent functionalization is a more permanent strategy. Reactive molecular groups (e.g. amine, hydroxyl and carboxylic groups) can be attached to the molecular structure of the polymers. The incorporation of peptides or proteins in the electrospun materials can be achieved by these methods. Covalent modifications can have a major effect on the physicochemical properties of the fibers, modifying
their hydrophilicity\textsuperscript{[74]}, degradability\textsuperscript{[75]} or mechanical properties.\textsuperscript{[76]} Further, cellular adhesion, proliferation, and differentiation can be directed with such modifications.\textsuperscript{[77, 78]}

6. Objectives of this thesis

Polymer-based synthetic biodegradable electrospun fibers and devices are widely studied for biomedical applications. However, as highlighted in this introduction, functional limitations are still largely present. The main goal of the work described in this thesis was the development of synthetic electrospun fibers or matrices with new physicochemical properties, which could address and overcome the limited biofunctionality presented by the current synthetic electrospun materials. In this thesis, GBR electrospun membranes possessing new functional properties were developed and evaluated. Further, techniques of post-spinning functionalization were applied to create electrospun materials with new chemical attributes. Finally, electrospun fibers were used for the formation of micro-cylindrical building blocks for biomedical applications.

Taken into account the challenges previously described, this thesis focused on the following research questions:

1- What is the current state-of-the-art with respect to the functionalization of electrospun membranes for biomedical applications? (Chapter 2)

2- Can mechanical and biofunctional properties of PCL-based electrospun membranes be improved by the incorporation of silica nanoparticles? (Chapter 3)

3- Can a thermal treatment lead to a more sustained release of the simvastatin incorporated in PLLA electrospun membranes, enabling their use as GBR electrospun membranes? (Chapter 4)

4- Can a layer-by-layer (LbL) strategy, based on the deposition of PAA and PLL, be used to functionalize PLLA electrospun fibers, facilitating the incorporation of small molecules? (Chapter 5)

5- Can functionalized microcylinders be developed by a top-down modification of electrospun fibers? (chapter 6)

6- Does the incorporation of PLLA microcylinders in calcium phosphate cements (CPCs) have a positive effect on their mechanical properties? (Chapter 7)
References

Chapter 1


[41] D. Puppi, A. M. Piras, F. Chiellini, E. Chiellini, A. Martins, I. B. Leonor, N. Neves, R. Reis, Optimized electro- and wet-spinning techniques for the production of polymeric fibrous scaffolds loaded with bisphosphonate and hydroxyap-
Chapter 1


[53] W. G. Cui, Y. Zhou, J. Chang, Electrospun nanofibrous materials for tissue engineering and drug delivery, Science and Technology of Advanced Materials,


Chapter 2

Biofunctionalization of electrospun fibers for tissue engineering and regenerative medicine

This chapter is based on:
A. G. B. Castro, F. Yang, J. J. J. P van den Beucken, J. A. Jansen,
Handbook of Intelligent Scaffolds for Tissue Engineering and Regenerative Medicine, Chapter 18, 2nd Ed, p 479-510.
Biofunctionalization of electrospun fibers for tissue engineering and regenerative medicine
## List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCNU</td>
<td>1,3-Bis(2-chloroethyl)-1-nitrosourea</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenetic Protein</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>Fe$_3$O$_4$</td>
<td>Iron (III) Oxide</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>GRGDY</td>
<td>Glycine-arginine-glycine-aspartic acid-tyrosine</td>
</tr>
<tr>
<td>LbL</td>
<td>Layer-by-layer</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal Stem Cells</td>
</tr>
<tr>
<td>NR$_4^+$</td>
<td>Quaternary Ammonium</td>
</tr>
<tr>
<td>PDA</td>
<td>Polydopamine</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived Growth Factor</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>PLLA</td>
<td>Poly($\epsilon$-lactic acid)</td>
</tr>
<tr>
<td>PCL</td>
<td>Poly($\epsilon$-caprolactone)</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-glycine-aspartic acid</td>
</tr>
<tr>
<td>TMCh</td>
<td>$N,N,N$-trimethylchitosan iodide</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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</tbody>
</table>
1. Introduction

Nowadays nanotechnology is gaining increasing relevance in scientific research, given that nanomaterials have shown improved mechanical, conductive, optical and biofunctional properties.\(^1\) In the field of tissue engineering, the development of nanofibrous mats is one of the most promising approaches. The morphological resemblance to the structure of extracellular matrix (ECM) and high surface area-to-volume ratio make nanofibers very appealing to be used as scaffolds or drug carriers.\(^2\)

Nanofibers can be produced using several techniques, such as template\(^3\), self-assembly\(^4\), melt-blown\(^5\), phase separation\(^6\), or electrospinning methods.\(^7\) Among these techniques, electrospinning is widely used as it is a low-cost process, the setup is simple and the technique can be applied to a broad range of materials, including polymers. In most of the cases, electrospun polymeric nanofibers are based on synthetic polymers rather than naturally derived polymers due to their well-controlled physiochemical properties, abundant resources, and relatively low cost. Such fibers are easily processed and present appropriate reproducibility.\(^8\) However, they also present a hydrophobic nature and lack cell recognition signals. Such characteristics can lead to rejection of the implanted material, low connection with the surrounding tissues, and abnormal tissue regeneration and growth.\(^9\)

Functionalization can be used to improve biocompatibility, cellular adhesion, tissue connection, and bioactivity. Furthermore, functionalization of electrospun fibers with clinically relevant compounds, such as drugs or therapeutic agents for antibacterial or anticancer purposes, is also widely studied due to their high loading capacity and mild process conditions. Functionalization techniques or post-spinning surface modification.
2. Electrospinning Process

The apparatus used to generate electrospun fibers consists in its essence of a high-voltage generator, a nozzle through which a high voltage can be applied to the solution of interest, and a collector. When the voltage is applied to the polymeric solution and the consequent electrostatic charge overcomes the surface tension of the solution, a jet is created. This jet elongates (at the same moment solvent evaporation occurs) and the resulting fibers in a non-woven form are deposited in the collector.[7, 25, 26] Although the concept behind electrospinning is rather simple, the obtained fibrous structure can be controlled via several factors, including the viscosity/concentration of the solution, the applied voltage, the solution flow rate, the type of collector, and others.[26, 27] Solution viscosity/concentration is a major parameter that has to be taken into account when developing electrospun fibers. Polymer concentration determines the entanglement between the polymer chains and consequently the viscosity of the solution. If the concentration is too low, a jet cannot be produced due to the lack of entanglement and only droplets are sprayed. Likewise, if the concentration is too high, the viscosity of the solution hinders the flow of the solution and a constant jet cannot be developed.[27, 28] Another important parameter is the applied voltage. Several authors observed a direct relation between the augment in fiber diameter and the increase of the voltage field.[7, 29] However, in some studies the opposite behavior has been reported.[30] It can be concluded that the voltage effect is closely related to the nature of the polymer used. The type of collector also affects the final structure of the fibers produced. Rotating collectors have
been employed to produce fibers with a higher alignment. Such fibers presented an improvement of mechanical and functional properties.\cite{4, 31, 32}

There are other technical aspects, which can influence the electrospinning process, not being addressed in this work. For a more detailed review of these subjects, articles by Agarwal, Pham, Sill, Huang, and other authors can be consulted.\cite{3, 7, 19, 25, 28}

3. Functionalization of Electrospun Fibers

As aforementioned, electrospun fibers based on synthetic polymers can be functionalized in order to improve their biocompatibility and obtain an appropriate biological response when implanted. Such functionalization, as summarized in Table 1, can be achieved either by co-electrospinning, meaning mixing the polymer with other compounds prior to/during electrospinning process, or by surface modification of already prepared electrospun fibers.

3.1. Co-electrospinning

An approach developed to functionalize electrospun fibers is the incorporation of compounds in the fiber structure. The main advantages of such procedures are twofold: allowing the modification of the structural properties of the fibers and a longer time of release of the compounds incorporated. Co-electrospinning can be performed by several ways, including blending, coaxial electrospinning, or emulsion-based methods.

Blending

Blending of compounds with the polymeric solution and posterior electrospinning is a common strategy to perform functionalization.\cite{36, 40, 71} Because most of the synthetic polymers are easily dissolved in organic solvents, this method is especially effective for the incorporation of hydrophobic compounds.\cite{72, 73} However, some drugs and other compounds have to be dissolved in an aqueous medium in order to maintain their functionality, making their incorporation difficult only by blending methods. Another very common phenomenon observed when blending is an initial burst release of the compounds, due to their presence on the surface of the fibers and diffusional aspects.\cite{19, 72, 74}

Coaxial electrospinning

In order to overcome the drawbacks present in blending, core-shell fibers have been developed. One of the main techniques used to produce such structures is coaxial electrospinning. Coaxial electrospinning is based on the simultaneous electrospinning of two separated solutions, using an inner and an outer nozzle. The resulting fibers are composed of a core, where commonly the compounds to be released are present, and a shell. In most cases, the core consists of a hydrophilic polymer, such as poly(ethylene glycol) (PEG)\cite{75, 76}, which allows a suc-
cessful incorporation of hydrophilic compounds. The use of a polymeric shell not only provides mechanical stability but also results in a more sustained and prolonged release of the compounds, making their action more efficient.\textsuperscript{[77–79]}

However, some disadvantages are present using coaxial electrospinning, including the complexity of the experimental setup and procedure, and a moderate burst release of the compounds.\textsuperscript{[53, 74]}

**Emulsion-based fibers**
An alternative approach to produce core-shell fibers is emulsion electrospinning. As the name indicates, this method is based on the development of water-in-oil or oil-in-water emulsions. Ideally, during the electrospinning process, micelles present in the solution elongate and unify, forming a core, while the dissolved polymer forms a surrounding shell.\textsuperscript{[20, 80]} The main advantage of this method is that it is a one-step method, allowing the use of a single-needle apparatus while still enabling the incorporation of hydrophilic compounds difficult to spin or to blend with the polymeric solution.\textsuperscript{[81, 82]}

**3.2. Surface Modification**
In the previous section, the incorporation of compounds into the fiber structure was described. However, in some cases surface functionalization is desirable, allowing the maintenance of the structural properties of the fibers, avoiding the use of a second polymeric solution and allowing the immobilization of different compounds (ECM proteins, growth factors, DNA, or drugs) on the surface of the fibers.

**3.2.1. Adsorption**
Adsorption methods are a popular and straightforward way to modify the surface of electrospun fibers, being a rapid, easy and versatile route. The fact that electrospun fibers have a high surface area-to-volume ratio makes their functionalization by adsorption very efficient. Physical adsorption and layer-by-layer (LbL) techniques will be addressed next.

**Physical adsorption**
Physical adsorption is performed by immersing the electrospun scaffolds in a solution or emulsion. Electrostatic forces, hydrophobic interactions, Van der Waals forces and physical entrapment all can lead to the attachment of molecules on the surface of the fibers, which can be rapidly released at the implantation site.\textsuperscript{[72, 83]} Although this burst release can be an advantage, for example in the rapid release of antibiotics on wound sites, such weak interactions can also be disadvantageous when a more controlled and sustainable release is desirable. Nowadays, more complex systems are under development, consisting of the attachment of nanoparticles or electrolytes mainly through the establishment of electrostatic interactions, such as using the LbL technique. In the next section,
such a technique will be described in more detail, for reasons of its importance as an adsorption based surface modification method for tissue engineering applications.

**Layer-by-layer surface functionalization**

The LbL technique is widely used in the development of multilayer modifications, in an attempt to overcome the limitations present in single-layer modifications. Multilayer techniques enable the attachment of molecules with different properties on the same substrate and allow the formation of more complex structures and the incorporation of different biomolecules, making it possible to mimic to some extent the complex structure of the ECM.[12, 60, 84] Traditionally, the LbL technique is based on the electrostatic interactions between species with opposite charges, although Van der Waals and hydrophobic interactions can play an important role.[83, 85, 86] Covalent LbL methods have also been reported, being based on covalent bonding between carbonyl, aromatic, or organometallic groups present in the reactive molecules.[86] The LbL technique presents several advantages, including its simplicity, versatility, high efficiency, and low cost. However, there are also some disadvantages, including the need for intermediate washing steps to remove excess residues and the fact that due to weak interactions a high initial release of compounds might occur.

### 3.2.2. Covalent functionalization

The establishment of covalent bonding is an important aspect when considering surface modification of electrospun fibers. Such bonds are stronger than the ones present in the techniques already described, resulting in a higher stability and immobilization of the compounds incorporated or in a more prolonged release of the same. In most of the cases, covalent functionalization of polymeric fibers with biological compounds cannot be performed immediately. The main reason for this is the lack of reactive groups, such as hydroxyls, carboxyl, carbonyl, amine, or sulfhydryl groups on the surface of the polymer with which new molecules can react and establish new covalent bonds. Next, some of the major techniques used to perform covalent-based surface functionalization will be addressed.

### 3.2.2.1. Energy-mediated surface modification

For several techniques, non-thermal energy is used in order to induce chemical reactions at the surface of the electrospun fibers. In such methods, reactive species are subject to high-energy exposure, which leads to the formation of radicals and others. These species react with the surface of the fibers, inducing the formation of functional groups. Plasma surface modification and photo-mediated modifications will be addressed next as examples of energy-based surface functionalization techniques.
Plasma surface modification

Plasma surface modification is widely used in the modification of metallic, ceramic and polymeric materials. It consists of the incorporation of molecular groups (hydroxyl, carboxyl, carbonyl, or amine groups) at the material surface or of the cleavage of already present groups. During plasma treatment, a sample is inserted in a vacuum chamber, where a low-pressure gas is injected and energized. Several types of gases are used for plasma treatments, being divided into non-polymerizing gases (argon, oxygen, nitrogen, or air) and polymerizing gases (fluorocarbons, silicon monomers, or hydrocarbons). Modification of the surface occurs when the created groups (e.g., ions, radicals, and intermediate species) are accelerated and bombard the surface of the material, leading to etching, activation and/or crosslinking phenomena. In the case of polymeric fibers, such modifications greatly affect their wettability, functional properties, and biocompatibility as well as enable the incorporation of relevant compounds. Recently, atmospheric plasma has been studied as an advantageous approach when compared to more traditional plasma techniques, because of its low cost, the non-use of toxic reagents, and high efficiency. Although plasma treatments are commonly used for the modification of polymeric surfaces, they also have some drawbacks, including the possibility of the materials degradation and a limited actuation depth.

Photo-mediated modifications

Surface modification of polymers can also be obtained by employing light-based energy. The increased use of photo-based techniques is explained by the high selectivity of the chemical reactions, processing under mild conditions, no need for catalysts or special solvents, and high versatility. UV light is widely used to modify the surface of fibers. In most cases, such processes involve the use of light-sensitive reagents called photo-initiators. Such compounds react with the UV radiation, creating reactive species that lead to reversible or irreversible chemical processes on the surface of the fibers, including photo-degradation, photo-functionalization, photo-initiated polymerization, and photo-isomerization. Photo-initiated polymerization is used for the functionalization of biodegradable polymers, as in the case of PEG, polyesters, and others. Although photo-induced reactions present several advantages, they are also associated with some drawbacks. For example, in photo-degradation reactions, a loss of polymer molar mass can occur. For a more detailed description of these processes, the following reviews can be consulted.

3.2.2.2. Immobilization of organic groups

Functionalization can be performed through the covalent immobilization of organic groups. In such cases, the covalent attachment of a reaction product or the chemical scission of the polymer molecular chains occurs, allowing the
generation of functional groups. Hydrolysis and aminolysis are the most common chemical reactions performed for the functionalization of electrospun fiber surfaces with organic residues.

**Hydrolysis**

Hydrolysis is based on the cleavage of chemical bonds exposed at the surface of the polymers, resulting in the creation of new hydroxyl and carboxyl reactive groups. Hydrolysis can occur at acidic or alkaline pH. Acidic conditions, although used during hydrolytic surface modification, require a very low pH, leading to a high degree of polymer degradation and resulting in a more difficult control over the chemical process. Alkaline conditions, on the other hand, are commonly performed using sodium hydroxide and are preferred due to relatively mild conditions, allowing superior control over functionalization and reducing the degradation of the fibers. [29, 104–106]

**Aminolysis**

Aminolysis is a known technique to modify the wettability and adhesion properties of polymeric materials. By this technique, the amine precursors used induce chain scission reactions and the attachment of amine groups. Such amine groups can further act as linking points for the incorporation of other compounds, for example proteins, peptides and polysaccharides. [61, 107] Aminolysis can also be used to alter the structure of electrospun fibers, making it possible to obtain a controlled degradation of the fibers. [108, 109]

### 3.2.2.3. Functionalization with polymeric linkers

Covalent functionalization can be achieved by the incorporation of macromolecules. In such cases, the structural properties of the fibers are extensively modified affecting their wettability, adhesion properties, and cellular proliferation or differentiation. In this section, surface graft techniques for the attachment of macromolecules and the use of polydopamines for covalent immobilization will be described as examples.

**Surface graft polymerization**

A common technique to surface functionalize fibers with polymeric linkers is graft polymerization. Through this method, macromolecular branches are covalently attached to the end groups of polymeric fiber chains, functioning as anchor points for the attachment of biomolecules and active agents present in the ECM or cellular membrane. [62, 110–113]

The most common methods used to perform graft polymerization are known as grafting-to, grafting-from, and grafting-through. Grafting-to consists of the direct attachment of macromolecules to the surface of the polymer backbone. This method, although being widely used, has some disadvantages, for example, the occurrence of steric hindrance phenomena. An alternative approach is the graft-
Biofunctionalization of electrospun fibers for tissue engineering and regenerative medicine

ing-from technique, which consists of the initiation of the branching polymerization from the reactive sites present at the surface of the polymer backbone. Through this method, it is possible to achieve a higher extension of the branches during the grafting process. Grafting-through starts with a combination of reactive monomers and acrylate functionalized macromolecules. The monomers react with each other, forming the polymeric backbone, and attach to the acrylate branches, which can then be further functionalized.[114–116]

Polydopamines
A recent approach to modify electrospun fibers is the functionalization of their surface with polydopamines (PDAs). PDAs result from the polymerization of dopamine at neutral to alkaline pH, due to oxidation of the catechol group present in the dopamine molecule.[117–119] PDAs present strong adhesive properties, attaching to glass, metals or polymeric surfaces due to the establishment of covalent or coordination bonds.[120] PDAs, besides granting a hydrophilic character to the materials where immobilized can also be used for the attachment of proteins or peptides.[121, 122]

Table 1 - Compounds and methods used in the functionalization of electrospun fibers for tissue engineering applications

<table>
<thead>
<tr>
<th>Functionalization methods</th>
<th>Compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blending</td>
<td>Gelatin[^33^]</td>
</tr>
<tr>
<td></td>
<td>Hyaluronic acid[^34^]</td>
</tr>
<tr>
<td></td>
<td>Chitosan[^35^]</td>
</tr>
<tr>
<td></td>
<td>Silk fibroin[^36^]</td>
</tr>
<tr>
<td></td>
<td>Collagen[^36^]</td>
</tr>
<tr>
<td></td>
<td>Laminin[^37^]</td>
</tr>
<tr>
<td></td>
<td>Elastin[^38^]</td>
</tr>
<tr>
<td></td>
<td>VEGF[^46^]</td>
</tr>
<tr>
<td></td>
<td>BCNU[^40^]</td>
</tr>
<tr>
<td></td>
<td>Paclitaxel[^41^]</td>
</tr>
<tr>
<td></td>
<td>Chlorhexidine[^42^]</td>
</tr>
<tr>
<td></td>
<td>Tetracycline hydrochloride[^43^]</td>
</tr>
<tr>
<td>Co-electrospinning</td>
<td>Gelatin[^44^]</td>
</tr>
<tr>
<td></td>
<td>Hyaluronic acid[^45^]</td>
</tr>
<tr>
<td></td>
<td>Chitosan[^46^]</td>
</tr>
<tr>
<td></td>
<td>Collagen[^47^]</td>
</tr>
<tr>
<td></td>
<td>Laminin[^48^]</td>
</tr>
<tr>
<td></td>
<td>VEGF[^46^]</td>
</tr>
<tr>
<td></td>
<td>Doxorubicin[^49^]</td>
</tr>
</tbody>
</table>
### 4. Applications

In this section, some of the current applications in the field of tissue engineering and drug delivery for functionalized electrospun fibers will be summarized (Table 2).

#### 4.1. Cell Adhesion

As already mentioned, many of the polymeric fibers produced by electrospinning techniques are based on synthetic polymers, which lack specific cell recognition and binding sites. A common approach to improve the cellular ad-
hesion to synthetic polymeric fibers is the incorporation of natural polymers, for example, gelatin, heparin, hyaluronic acid or chitosan.\textsuperscript{39,123–125} A more target-controlled approach is the functionalization of fibers with components of the ECM, for example, collagen, fibronectin, or laminin, which are recognized by receptors present on the cellular membrane and can trigger signaling pathways activated upon cellular adhesion.\textsuperscript{55, 63, 67, 126, 127} Recently, peptide sequences have been used to improve the adhesive properties of electrospun fibers as well. The use of these short sequences has as main advantage that they can be incorporated in a higher number, presenting a lower steric hindrance when compared to larger macromolecules. Also, an optimal molecular orientation can be more easily achieved and peptides present a higher resistance to degradation. A widely studied peptide sequence to enhance cellular adhesion is the RGD sequence.\textsuperscript{128–131} Other sequences, such as GRGDY or E7, have also been studied.\textsuperscript{129, 132, 133} Inorganic compounds can be incorporated on the surface of polymeric fibers in order to improve the adhesion of cells. A common class of inorganic compounds is represented by calcium phosphate minerals. PLLA fibers covered with hydroxyapatite particles have shown an increase in hydrophilicity.\textsuperscript{134, 135} The surface mineralization of PCL fibers with hydroxyapatite has further shown to enhance the immobilization of fibronectin-osteocalcin fusion protein and consequently to improve the adhesion and proliferation of mesenchymal stem cells (MSCs).\textsuperscript{136}

The development of fibers with magnetic properties for tissue engineering applications is a new research topic. Magnetic fibers are commonly prepared using cobalt (Co), nickel (Ni), iron (Fe), or metal oxides, for example, Fe\textsubscript{3}O\textsubscript{4}. Electrospinning techniques can help to overcome some of the problems regarding the use of such compounds, including cytotoxicity or particle aggregation.\textsuperscript{137, 138} The development of magnetic fibers for bone regeneration has been recently undertaken. It is anticipated that by subjecting bone cells to oriented magnetic fields, it becomes possible to enhance cell proliferation and differentiation. \textit{In vitro} tests performed by seeding osteoblasts in magnetic electrospun fibers showed reduced cytotoxicity, increased adhesion, and increased proliferation rates.\textsuperscript{137, 139}

\section*{4.2. Growth Factors and Gene Delivery Systems}

Growth factors play an essential role in tissue regeneration. These proteins directly modulate cell behavior by attaching to receptors present on the cellular membrane, affecting cell proliferation, adhesion, migration, differentiation, metabolic functions, and survival. The application of growth factors to induce \textit{in vivo} tissue regeneration or physiological responses can be simply achieved by local injection. However, the direct injection of these biomolecules has not shown a high efficiency and when applied in high concentrations resulted in adverse effects. Therefore, the incorporation of such compounds within a carrier, for example electrospun fibers, allowing a more localized and controlled release,
is desirable for certain cases. Bone morphogenetic proteins (BMP-2, BMP-7, or their derivates) have been successfully immobilized on the surface of electrospun fibers, showing to induce osteogenic differentiation of human mesenchymal stem cells and in vivo bone regeneration. Vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) have also been studied in order to induce blood vessels formation and regeneration. An effective proliferation and adhesion of vascular endothelial cells and vascular smooth muscle cells in in vitro cell culture with the electrospun fibers and in vivo implantation of vascular grafts was achieved.

Although the use of growth factors has shown great potential, many challenges still exist due to the complexity of the cellular signaling pathways involved in their action, and the absence, in most cases, of a single mechanism of action for these biomolecules.

A similar approach is the immobilization and release of nucleic acid sequences (i.e. containing genetic information). These biomolecules present some advantages compared to proteins, being more stable and less susceptible to degradation from enzymes when released. Also, the genes being taken up by the cells are integrated into their genome, leading to more prolonged and higher protein expression levels. An interesting approach is the possibility to incorporate genes, which lead to the expression of growth factors. Poly(D,L-lactide)–poly(ethylene glycol) electrospun fibers have been functionalized with the plasmids pVEGF and pFGF in order to enhance vascularization. Higher cell viability, a sustained release of the plasmids, and a high density of mature blood vessels was achieved.

4.3. Drug Delivery Applications

Another important application for electrospun fibers is the development of drug delivery systems. Nanofibrous systems allow not only a high load of these compounds but also, due to the fact that such biomaterials are locally implanted, a more localized action of the therapeutic agent with high efficiency. In the following sections, the most recent developments in antibacterial and anticancer therapies are addressed.

Antibacterial strategies

Infections due to bacterial proliferation are a serious concern when considering the implantation of biomedical devices, especially orthopedic implants. Bacteria can develop biofilms on the surface of these devices, being in many cases resistant to the traditional topical or intravenous treatments. Electrospun fibers are preferred candidates for the development of more efficient strategies, being the incorporation of antibacterial compounds currently under investigation. An interesting approach is the surface functionalization of electrospun fibers with quaternary ammonium (NR₄⁺) compounds. These molecules present strong antimicrobial properties, binding to the bacterial cell membrane and

40
causing its disruption. Surface modification of polyurethane electrospun fibers with NR\textsuperscript{+} has shown an efficient reduction in the number of viable *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) bacteria.\textsuperscript{148} PCL fibers surface-functionalized with N,N,N-trimethylchitosan iodide (TMCh) have also shown antibacterial activity, inhibiting the proliferation of *E. coli* and *S. aureus* bacteria and also the suppression of *S. aureus* adhesion to the fiber's surface.\textsuperscript{149}

### Anticancer therapy

Electrospun fibers loaded with anticancer drugs are under investigation in order to achieve a more efficient release, diminish side effects, and prolong the half-life of the used drugs. Traditionally, blending or emulsion-based techniques were developed in order to incorporate anticancer drugs in polymeric fibers. Doxorubicin, BCNU, and paclitaxel were incorporated by these methods showing anti-tumor activity.\textsuperscript{40, 52, 53, 150} However, non-uniform distribution of the drugs in the fibers and burst release profiles were observed. A very recent and target-oriented approach is the stimuli-dependent functionalization of polymeric fibers. By this method, drug release is dependent on an external stimulus, such as pH, temperature, or enzyme activity.\textsuperscript{151, 152} Jiang et al. successfully incorporated doxorubicin in PCL fibers by a polydopamine-mediated surface modification method. The authors managed to obtain a pH-dependent release of the drug into the medium and an effective reduction of the number of H1299 cancer cells.\textsuperscript{70}

### Table 2 - Tissue engineering and drug delivery applications of electrospun fibers

<table>
<thead>
<tr>
<th>Electrospun fiber application</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Improvement of cellular adhesion</td>
<td>Gelatin</td>
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<tr>
<td></td>
<td>Hyaluronic acid</td>
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<td></td>
<td>Silk Fibroin</td>
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<td></td>
<td>Collagen</td>
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<tr>
<td></td>
<td>Fibronectin</td>
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<tr>
<td></td>
<td>PDGF</td>
</tr>
<tr>
<td>Cellular proliferation</td>
<td>Heparin</td>
</tr>
<tr>
<td></td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td></td>
<td>Collagen</td>
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<tr>
<td></td>
<td>Fibronectin</td>
</tr>
<tr>
<td></td>
<td>FGF</td>
</tr>
<tr>
<td></td>
<td>PDGF</td>
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<tr>
<td>Cellular migration</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td></td>
<td>Fibronectin</td>
</tr>
<tr>
<td></td>
<td>VEGF</td>
</tr>
</tbody>
</table>
5. Conclusion

Electrospun fibers present characteristics that make them ideal for tissue engineering applications. However, some challenges are still present. Issues concerning mass production, reproducibility, and the use of toxic solvents are still major challenges for the successful development of electrospun fiber-based materials. Nowadays, the development of multi-nozzle systems, melt electrospinning, and computer-assisted electrospinning are underway in order to allow an effective up-scaling of the technique. The so-called green electrospinning techniques, which consist of the use of non-toxic solvents (e.g., water), are currently possible and show very promising results. Another important aspect that is under development and certainly will be addressed in the future is the production of biodegradable three-dimensional systems incorporating electrospun fibers. Such materials could be a great improvement for the development of ECM-mimicking structures, allowing a more efficient attachment of macromolecules, for example, proteins and growth factors, and helping to improve tissue regeneration. Functionalization of fibers with drugs presents some further challenges, includ-
ing uniform distribution of the drug, bioactivity maintenance, or controlled release profiles. The development of hybrid particle-fiber systems or physical, chemical, and biological stimuli-based release systems is under investigation and can help to realize such challenges.
Chapter 2

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Biofunctionalization of electrospun fibers for tissue engineering and regenerative medicine

Development of a PCL-silica nanoparticles composite membrane for guided bone regeneration

This chapter is based on:
A. G. B. Castro, M. Diba, M. Kersten, J. A. Jansen, J. J. J. P van den Beucken, F. Yang,
Development of a PCL-silica nanoparticles composite membrane for guided bone regeneration

1. Introduction

Guided Bone Regeneration (GBR) is a common therapy used for the treatment of lesions in the alveolar or mandible bone caused by infections or trauma. The principle underlying GBR is to create a secluded space in the wound site, which favors the proliferation of bone-forming cells and consequently results in new bone formation. An essential step in such a strategy is the use of a physical barrier, mainly in the form of a membrane, preventing the fast-growing fibroblasts from migrating into the wound site and keeping a space for the slow-growing bone tissue to regenerate.\textsuperscript{[1–2]}

Electrospinning is an ideal technique to produce polymeric GBR membranes. The application of an electric current to the polymeric solution leads to the creation of a jet and consequently to the production of polymeric fibers, due to the evaporation of the solvent.\textsuperscript{[3, 4]} Electrospun membranes share the advantages of possessing a high surface area-to-volume ratio and a microstructure which resembles the extracellular matrix structure (ECM).\textsuperscript{[5, 6]} Electrospun GBR membranes are made of natural or synthetic polymers.\textsuperscript{[7, 8]} The use of natural polymers is justified by their great biocompatibility, possessing protein motifs that enhance cell adhesion and proliferation. However, these natural materials lack the ideal tensile properties for GBR applications and present a high degradation rate.\textsuperscript{[9, 10]} Synthetic membranes are often made of polyesters, e.g. polyglycolide (PGA) and polylactide (PLA), which possess easily controllable and tunable physicochemical properties. However, PGA or PLA release acidic degradation products, potentially leading to local inflammatory reactions.\textsuperscript{[11]}

Poly(ε-caprolactone) (PCL) is a known biocompatible polyester, having the advantage that its degradation products are not acidic. A major disadvantage of synthetic membranes is their lack of osteoconductive capacity to allow bone growth along their surface and promote bone healing or regeneration.\textsuperscript{[12–14]}

A known strategy for introducing functional properties in synthetic GBR membranes is the incorporation of compounds that lead to improved mechanical properties and/or trigger an osteogenic response. These additives can be organic, for example, proteins and peptides\textsuperscript{[15, 16]}, or inorganic, such as calcium phosphate (e.g. hydroxyapatite) or silicate bioceramic particles.\textsuperscript{[17–19]} The conjugation of inorganic particles and polymeric components: polymeric hydrogels\textsuperscript{[20]}, polymeric membranes\textsuperscript{[21]} and polymeric molecular branches\textsuperscript{[22, 23]} are a current strategy used in the development of materials with new functional properties. Such new hybrid materials are used in the most diversified areas\textsuperscript{[24]}, including in the biomedical field: cancer therapy\textsuperscript{[25–27]}, gene therapy\textsuperscript{[28]}, development of new antibacterial therapies\textsuperscript{[29, 30]}, optogenetics\textsuperscript{[31]}, drug delivery systems\textsuperscript{[23]} among others. Previously, we prepared GBR membranes based on PCL and nano-apatite rod-like particles.\textsuperscript{[18]} The addition of nano-apatite particles led to improved mechanical properties and osteoblast-like cells differentiation. However, uncontrollable aggregation of the particles inside the fibers occurred,
which negatively affected the mechanical properties of the membranes. Calcium biosilicates have shown in the past to induce osteogenic cellular differentiation and bone tissue formation. More recently has been shown that silica nanoparticles (Si-NPs) can be an alternative type of bioceramic particles for osteoconductive purposes. Their application is based on their reported positive effect on the mechanical properties of polymeric membranes and promotion of an osteogenic response from osteoblastic progenitor cells. However, despite promising results, Si-NPs weight percentages above 30 wt% frequently result in adverse effects, e.g. particle aggregation, poor interaction between the polymeric components and the silica particles or non-ideal mechanical properties. The aim of this study was to develop a PCL-based GBR membrane, in which Si-NPs are more homogeneously dispersed inside the electrospun fibers at amounts above 30 wt%. To this end, Si-NPs were produced by a sol–gel Stöber method to achieve mono-sized nanoparticles. Membranes with a series of PCL : silica weight ratios (100:0, 100:25, 100:50 and 100:75) were fabricated by an electrospinning methodology. Morphology, structure, and chemical composition were analyzed via scanning electron microscopy (SEM), transmission electron microscopy (TEM), attenuated reflectance-infrared spectroscopy (ATR-IR) and X-ray diffraction (XRD). Mechanical properties were evaluated using a tensile test. Stability of the membranes in wet conditions was evaluated by immersion in phosphate buffered saline (PBS) solution at 37 °C. Osteoblastic cell response was determined by in vitro culture of MC3T3-E1 cells on the membranes and by assessing cellular morphology, proliferation, and osteogenic differentiation.

2. Materials and methods

2.1. Preparation of silica nanoparticles
Si-NPs were prepared using a Stöber method. Briefly, a 6% v/v mixture of ammonia (ammonia solution 25%; Merck; Germany) in ethanol was prepared. Afterward, tetraethyl orthosilicate (TEOS; reagent grade, 98%; Sigma-Aldrich; USA), was added to the mixture, under continuous stirring, and left to react at room temperature for 30 minutes. The solution was kept at 4 °C overnight. Finally, the nanoparticles were washed first with ethanol, second a mixture of ethanol-water (50:50) and third water, with intermediate centrifugation and redispersion steps.

2.2. Preparation of the polymeric solutions and fabrication of the membranes
Four solutions were prepared by firstly dissolving PCL (average Mn 80,000; Sigma-Aldrich; USA) in a 80% v/v 2,2,2-trifluoroethanol (TFE; Sigma-Aldrich; USA)/water mixture with sodium dodecyl sulfate (SDS; Fluka; Germany) (Table 1). Afterward, a specific amount of Si-NPs was added and the dispersion
Development of a PCL-silica nanoparticles composite membrane for guided bone regeneration

was left to mix overnight. Table 1 describes the Si-NPs : PCL weight ratio and silica weight percentages.

Table 1. Si-NPs : PCL membranes composition

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Si-NPs : PCL weight ratio</th>
<th>Si-NPs weight / %</th>
<th>SDS / %</th>
</tr>
</thead>
<tbody>
<tr>
<td>S0</td>
<td>0 : 100</td>
<td>0</td>
<td>0.05</td>
</tr>
<tr>
<td>S25</td>
<td>25 : 100</td>
<td>20</td>
<td>0.05</td>
</tr>
<tr>
<td>S50</td>
<td>50 : 100</td>
<td>33</td>
<td>0.05</td>
</tr>
<tr>
<td>S75</td>
<td>75 : 100</td>
<td>43</td>
<td>0.05</td>
</tr>
</tbody>
</table>

A commercially available electrospinning setup was used for the fabrication of the membranes (Advanced Surface Technology, The Netherlands). The prepared solutions were fed into a glass syringe, controlled by a pump (KD Scientific Inc.; USA), and connected by a Teflon tube to a blunt-end nozzle with an inner diameter of 0.5 mm. The electrospinning process was performed with a distance between the nozzle and a roll-drum collector of 20 cm, a voltage between 18–22 kV and a feeding rate of 2.0 mL h\(^{-1}\).

2.3. Morphological and structural evaluation

The morphology of the membranes was observed using scanning electron microscopy (SEM; Zeiss, SIGMA 300; The Netherlands) and transmission electron microscopy (TEM; 1101, JEOL; Japan). Images were taken at 1,000× magnification and 10,000× magnification for SEM and 20,000× for TEM.

The chemical profile of the membranes was determined by attenuated total reflectance-infrared spectroscopy (ATR-IR; UATR two, PerkinElmer, The Netherlands) with a resolution of 4.0 cm\(^{-1}\) and a scanning range from 400 cm\(^{-1}\) to 4000 cm\(^{-1}\).

X-ray diffraction (XRD; pw1830, Philips; The Netherlands) was performed to determine the crystallographic profile of the samples. Electrospun membranes as a thin planar layer were placed in a glass holder and scanned. XRD spectra were registered at 40 kV, 30 mA (Cu-Kα radiation with a wavelength of 1.54 Å) and a 2θ between 10 and 40°, at a step size of 0.005°.

2.4. Mechanical evaluation

To determine the effect of silica nanoparticles on the mechanical performance of the membranes, 6 samples of 10 mm in width and 50 mm in length were cut with a scalpel and tested for each group. The thickness of the membranes
was measured with a caliper having a precision of 0.01 mm. The samples were attached to a tensile test machine (LS1; Lloyd instruments; Ametek; USA) using 10 mm of the sample on both ends. The tensile properties of the membrane were tested with a 100 N load cell under a cross-head speed of 10 mm min\(^{-1}\). The following mechanical properties were recorded or calculated from the stress–strain (\(\sigma–\varepsilon\)) curves: (1) the tensile modulus was calculated from the slope of the initial linear part of the curve, consisting of 10% of the initial strain values (2) the maximum strain at break was defined as the maximum strain that the samples could reach immediately before break and (3) the tensile strength was defined as the maximum stress at break.\[^{[43]}\]

2.5. Stability of the membranes in a wet environment
Circular membranes with a diameter of 15 mm were punched from the electrospun sheets. Each sample was immersed in 10 mL of phosphate buffered saline (PBS) solution in a test tube. All tubes (n=3) were placed in a water bath at 37 °C under continuous shaking. After immersion periods of 14, 21 and 28 days the membranes were washed with \(\text{dd}_w\) water and freeze-dried. The membranes, before and after PBS immersion, were examined by SEM.

2.6. In vitro cell culture
MC3T3-E1 cells (ATCC, USA) were maintained in \(\alpha\)-MEM medium (Gibco\(^*\), Life Technologies; USA) supplemented with 10% v/v fetal bovine serum (FBS; Gibco\(^*\)) and a mixture of penicillin (100 Units ml\(^{-1}\)) /streptomycin (100 μg ml\(^{-1}\)) (Gibco\(^*\)) in an incubator with 5% CO\(_2\) at 37 °C.

Electrospun membranes were disinfected using a plasma cleaning procedure, consisting of exposing the membranes for 5 minutes to charged argon and followed by immersion in ethanol 70% v/v for 1 hour. Afterward, the membranes were washed with PBS three times and incubated with non-osteogenic culture medium overnight. Cells were seeded at a density of 10,000 cells cm\(^{-2}\) on top of the membranes and cultured with osteogenic medium: \(\alpha\)-MEM supplemented with 10% FBS, 0.2 mM ascorbic acid, 10 mM sodium β-glycerophosphate and 10 nM dexamethasone (supplements were purchased from Sigma-Aldrich).

Samples used for cellular morphology evaluation were collected at 3, 14 and 28 days. A 2% v/v solution of glutaraldehyde (glutaraldehyde solution 25%; Merck; Germany) was used to fixate the cells and a series of dehydration steps, using ethanol at different concentrations, (70%, 80%, 90%, 96% and 100% v/v) were performed. Lastly, samples were covered with tetramethylsilane (TMS; \(\geq99.0%\) GC; Sigma-Aldrich; USA). Afterward, samples were prepared for SEM observation.

DNA quantification and ALP (alkaline phosphatase) activity measurements were performed as described in previous studies.\[^{[44]}\] Membranes cultured with cells were collected and immersed in 1 mL of \(\text{dd}_w\) water. Afterward, three freeze-thaw cycles were performed, in order for cellular lysis to occur. Cellular
DNA content was measured at 3, 7, 14, 21 and 28 days using a Quant-IT™ PicoGreen® ds-DNA Assay Kit (Invitrogen; The Netherlands) according to the manufacturer’s instructions. In a 96-well plate 100 μl of sample or standard were added to a working solution. The plates were incubated at room temperature in the dark for 5 minutes and fluorescence read (excitation: 485/20 nm; emission: 530/25 nm). ALP activity was analyzed at 3, 7, 14, 21 and 28 days of culture. ALP activity was measured by a p-nitrophenyl phosphate (4-NP; Sigma-Aldrich, USA) colorimetric assay, according to the manufacturer’s instructions. 80 μL of samples or standards were added to 20 μL of buffer and 100 μL of substrate. The mixture was incubated for 60 minutes at 37 °C. Afterward, 100 μL of 0.5 M NaOH was added, in order to stop the reaction. Absorbance at 405 nm was read and ALP activity normalized according to the DNA content for each sample.

2.7. Statistical analysis
Measurement of fiber diameter was performed based on SEM images (n = 100 for each composition) and diameter distribution was calculated using Origin Pro 8.0 software (OriginLab Corporation, USA). Analysis of the mechanical properties of the membranes was performed using Prism 6.01 software (GraphPad Software, Inc., USA). Data were reported as mean ± standard deviation (SD). Significance was evaluated using a one-way ANOVA significance test combined with a post-hoc Tukey’s multiple comparison test. Differences were considered significant at p < 0.05. DNA quantification and ALP activity values (n= 6) were analyzed using Prism 6.01 software performing a two-way ANOVA significance test combined with a post-hoc Tukey’s multiple comparison test. Differences were considered significant at p < 0.05.

3. Results
3.1. Morphological evaluation of the membranes
The SEM images in Figures 1a–d show that the PCL membranes with different amounts of Si-NPs consisted of smooth fibers. For membranes S50 and S75, noticeable nanoparticle agglomerates were present in between the fibers as indicated by the yellow arrows (Figures 1c and 1d). Figures 1e–h present the fibers diameter and size distribution and show that with increasing amounts of Si-NPs, the diameter of the fibers and the fiber size distribution decreased (1.4 ± 1.68 μm for S0, 0.4 ± 0.25 μm for S25; 0.1 ± 0.04 μm for S50 and 0.2 ± 0.05 μm for S75).
Figure 2 presents TEM images for the different electrospun fibers. S0, the pure PCL fiber, presented a uniform morphology. Dark dots were observed in the fibers of S25, S50, and S75 compositions, indicating that the Si-NPs were incorporated inside of the electrospun fibers. No signs of aggregation were observed for S25 and S50 fibers. However, for S75 fibers, a low degree of aggregation of the Si-NPs seemed to occur.

Figure 1. SEM images of membranes prepared by electrospinning; 10,000× magnification a) S0 b) S25 c) S50 d) S75. Images highlighted in red 1,000× magnification. Yellow arrows indicate nanoparticles agglomerates. Fibers diameter and size distribution for e) S0 f) S25 g) S50 h) S75.
3.2. Structural characterization

ATR-IR and XRD were performed and the results are shown in Figures S1 and S2 in Supplementary Information. ATR-IR spectra display peaks at 1725 cm\(^{-1}\) and 1185 cm\(^{-1}\) for all compositions, associated with C=O and C-O-C groups in PCL (blue lines in Figure S1). For the compositions containing Si-NPs, peaks related to Si-O-Si stretching were also detected at 475 cm\(^{-1}\), 807 cm\(^{-1}\) and 1070 cm\(^{-1}\) (red lines in Figure S1). With increasing amounts of Si-NPs, a decrease in intensity for the peak at 1185 cm\(^{-1}\) was observed. XRD spectra were similar for all compositions, presenting a first peak at 21.25° and another peak at 23.70°; both peaks are assigned to PCL lattice planes, \textit{i.e.} (110) and (200) respectively (Figure S2).

3.3. Mechanical evaluation

Membranes were subjected to a tensile test. Figure 3a presents a typical stress (\(\sigma\)) - strain (\(\varepsilon\)) curve for each group. Tensile modulus, strain at break and tensile strength were calculated based on the stress-strain curves. A significant increase in tensile modulus was observed for S50 when compared with S0, with the values of 13.5 ± 1.27 MPa and 9.5 ± 1.75 MPa respectively. However, when higher amounts of Si-NPs were incorporated (S75) a decrease in tensile modulus occurred (8.9 ± 1.32 MPa). The incorporation of Si-NPs also had an effect on the strain at break. Figure 3c demonstrates that with increasing amounts of silica the strain at break decreased. S25 and S50 presented a significant increase in tensile strength compared to pure PCL membranes (2.9 ± 0.31 MPa for S0, 6.5 ± 1.55 MPa for S25 and 5.8 ± 0.19 MPa for S50). However, S75 presented a tensile strength similar to S0 membranes (3.7 ± 0.99 MPa).
3.4. Stability of the membranes in a wet environment

In order to evaluate the stability of the electrospun fibers in a wet environment and silica release, the membranes were immersed in PBS at 37 °C for 14, 21 and 28 days. Figure 4 shows the SEM images for the immersed membranes after 14 days. S0 electrospun fibers did not show significant signs of degradation, as fissures or cracks (Figure 4a). Membranes containing Si-NPs presented spherical holes (Figures 4b–d, red arrows) in the surface of the electrospun fibers, indicating release of Si-NPs present in the outer regions of the fibers.

Figure 3. a) Stress-strain curves of the electrospun membranes. Tensile test results b) tensile modulus c) maximum strain at break d) tensile strength. *p < 0.05, **p < 0.01, ***p = 0.0001, ****p < 0.0001.
3.5. *In vitro* cell culture

Figure 5 presents the membranes after *in vitro* culture with MC3T3-E1 cells. After 3 days of culture, cells were observed on the surface of all membranes. Cells possessed a “star-shaped” morphology, indicating adherence to the electrospun fibers.
Chapter 3

Figure 5. SEM images of membranes after in vitro culture with MC3T3-E1 cells; 1,000× magnification. Images highlighted in red 10,000× magnification a) S0 3 days b) S0 28 days c) S25 3 days d) S25 28 days e) S50 3 days f) S50 28 days g) S75 3 days h) S75 28 days.

DNA quantification demonstrated an increase in DNA content with time for all groups. For S0 higher amounts of DNA were measured compared to membranes with Si-NPs from 14 days of culture (Figure 6a). ALP-activity was evaluated at day 3, 7, 14, 21 and 28. Figure 6b shows that ALP activity was only detected after 14 days of culture.

Figure 6. a) DNA quantification of MC3T3-E1 cells cultured with membranes. b) ALP activity of MC3T3-E1 cells cultured with membranes. *p < 0.05, **p < 0.01, ****p < 0.0001.
4. Discussion

The addition of bioceramic nanoparticles in electrospun membranes is a straightforward strategy for the fabrication of functional GBR membranes with improved properties. However, aggregation of nanoparticles and a weak interaction between the ceramic and polymeric components can occur during the fabrication process, which in turn weakens the mechanical properties of such materials. Here, we developed a composite GBR membrane with significantly enhanced mechanical properties by incorporating high amounts of Si-NPs (i.e. >30 wt %) without substantial aggregation. A Stöber method was selected to prepare the Si-NPs, since previous studies have shown that the physical and chemical properties of the nanoparticles could be precisely tuned by this method. Nanometric particles were successfully produced and adequate control over size and shape of the nanoparticles was achieved. The nanoparticles presented a clear spherical shape and homogenous dimensions. Our results showed that Si-NPs were successfully incorporated in the electrospun fibers and aggregation starts to occur for composition S50, although being only substantial for composition S75 (Si-NPs content of 43 wt %). Mechanical properties of the membranes were highly affected by the incorporation of Si-NPs. Tensile strength for composition S25 and S50 and tensile modulus for composition S50 increased significantly with the incorporation of the nanoparticles. In vitro cell culture with MC3T3-E1 osteoblastic cells demonstrated that none of the compositions were cytotoxic, allowing cell adhesion and proliferation. Osteogenic differentiation occurred for cells cultured on all the membranes.

The major goal of this work was to incorporate a high number of nanoparticles in the electrospun fibers, achieving a more homogeneous distribution of the nanoparticles and averting aggregation. A major cause for such aggregation are the particles concentration in solution, particles diameter and attractive interactions between the particles, being these stronger when considering anisotropic particles. In order to overcome this issue, two strategies were adopted. First, Si-NPs were developed using a Stöber sol–gel method, which enabled adequate control over nanoparticle diameter and size distribution. A second strategy was the use of a mixture of TFE and water, conjugated with a surfactant, as already described by Yang et al. TFE is a fluorinated alcohol commonly used for the dissolution of polypeptides and polymers, such as PCL. Water was added to increase the dielectric constant of the solution, enabling the production of electrospun fibers without beads or defects. Homogenous electrospun fibers with a smooth surface were produced and, as observed by TEM, Si-NPs are well dispersed in the interior of the fibers up to a Si-NPs weight content of 43 wt %, which is remarkable, especially when compared with similar recent studies. TFE interacts strongly with the PCL polymeric chains, resulting in their swelling and a looser polymeric matrix. This effect allows for an improved dispersion of the silica nanoparticles in the solution. Addition of
the anionic surfactant SDS likely also contributed to the appropriate dispersion of the nanoparticles in the solution. SDS is an amphiphilic molecule, interacting with both hydrophobic and hydrophilic compounds. Karlson et al. explain such effect by a surrounding of the nanoparticles by the hydrophilic group of the surfactant molecules, exposing at the same time their hydrophobic region to the polymeric chains. This way, the surfactant worked as a “bridge” between PCL and Si-NPs. The presence of a high number of particles in composition S75, combined with their hydrophilic nature led to the hindrance of their dispersion in the PCL solution, resulting in phase separation between silica-rich and PCL-rich regions within the fibers and the formation of aggregates in the interior of the PCL fibers. Incorporation of Si-NPs had a significant effect on the morphology of the electrospun fibers. When comparing the diameter of the electrospun fibers for the different compositions, a decrease in fiber diameter with increasing Si-NPs content was observed (Figure 1). As apparent from Tables S1 and S2 in the Supplementary Information, the addition of Si-NPs resulted in an increase of the conductivity and viscosity of the solutions. As postulated previously, an increase in conductivity leads to a greater tension in the polymeric jet during electrospinning, resulting in an easier formation of fibers and hence in a decrease in fiber diameter. According to several studies, an increase in viscosity leads to an increase in fiber diameter. However, this effect was not observed in our work. A possible explanation is that when Si-NPs were incorporated the increase in conductivity overbalanced the increase in viscosity, leading to the rapid formation of the fiber jet and the production of fibers with a smaller diameter.

The mechanical properties of the membranes were significantly affected by the incorporation of Si-NPs. The tensile modulus of S50 membranes was significantly higher than pure PCL membranes (S0), showing that the incorporation of the Si-NPs was beneficial up to this amount. The actual values of tensile moduli can be explained by the properties of PCL and silica. PCL presents a glass transition temperature ($T_g$) of approximately -60 °C and polymers subjected to electrospinning have shown to present a lower crystallinity. The reduction in crystallinity can affect the elastic behavior of the membranes, making them become easily deformed when subjected to tensile forces. S0 membranes are composed solely by PCL, presenting the lowest value of tensile modulus. The incorporation of Si-NPs compensated the low modulus of PCL for compositions S25 and S50. The homogeneous distribution of the nanoparticles inside the electrospun fibers can explain the increase in tensile modulus. Allo et al. claim that the presence of silica could promote cross-linking phenomena among the PCL fibers, due to the bonding between the silanol groups (Si-OH) present in the Si-NPs and the carbonyl groups (C=O) present in PCL. In our study, the formation of these bonds could not be confirmed by ATR-IR, although specific peaks attributed both to PCL and Si-NPs were detected. When membranes S25 and S50 were subjected to tension, the Si-NPs inside the electrospun fibers likely
“hold” the polymeric chains, hindering fiber deformation and increasing the elastic behavior of these membranes.\textsuperscript{[60]} The addition of Si-NPs led to a significant increase of the tensile strength, achieving more than double the value of S0 for S25 and S50 membranes. Furthermore, compared to several studies focused on the development of PCL-silica based composite membranes, it is noticeable that the values obtained in this study are superior or equal to those reported previously.\textsuperscript{[34, 40, 61–63]} This increase in tensile strength can be explained by the fact that Si-NPs were distributed along the electrospun fibers for compositions S25 and S50, being mostly separated from each other. Such dispersion of the nanoparticles inside the electrospun fibers resulted in an alignment of the nanoparticles in the interior of the fibers, when these were subjected to tensile forces.

Upon immersion of the membranes in PBS the presence of holes in the fibers from S25, S50, and S75 membranes was observed (Figure 4). Such results demonstrate that dissolution of the Si-NPs occurs through time.\textsuperscript{[34, 37]} The release of Si-NPs in the PBS potentiates the clinical relevance of such membranes by the increase of silica levels in the medium.\textsuperscript{[64, 65]} To obtain better insight into the effect of PCL : Si-NP membranes on osteoblastic differentiation, an \textit{in vitro} culture with osteoblastic MC3T3-E1 cells was performed. An increase in DNA content could be observed for all compositions when comparing earlier times of \textit{in vitro} culture (3 and 7 days) and later time points (14, 21 and 28 days). Cells were cultured in osteogenic medium and consequently, osteogenic differentiation was expected for all groups. In this study, it was intended to evaluate if the presence of membranes containing silica would affect the osteogenic differentiation rate and levels. ALP levels and activity could be detected for all the compositions. However, a direct effect from the Si-NPs on osteoblastic differentiation could not be determined.

5. Conclusion

The present study aimed to develop electrospun membranes for GBR with improved mechanical and biofunctional properties. Nanometric spherical Si-NPs were produced and successfully incorporated in the PCL fibers, being the nanoparticles well dispersed in their interior up to a PCL : Si-NPs ratio of 100:75 (43 wt%). Tensile modulus and tensile strength improved when Si-NPs were added. Membranes have shown to be cytocompatible although a direct action of the membranes on osteoblastic differentiation could not be observed.
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References


derived silica hybrid scaffolds and PHB/PCL/fumed silica composite scaffolds, Colloids and Surfaces B: Biointerfaces, 2015. 136: p. 93–98.


[65] Y. Xing, L. Yuanyuan, L. Xujie, H. Qianli, H. Wei, Z. Ranran, F. Qingling,
Development of a PCL-silica nanoparticles composite membrane for guided bone regeneration

## Supplementary Information

Table S1. Conductivity of the prepared compositions

<table>
<thead>
<tr>
<th>Composition</th>
<th>Conductivity / μS cm⁻¹</th>
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<tr>
<td>S0</td>
<td>5.7±0.28</td>
</tr>
<tr>
<td>S25</td>
<td>34.1±0.73</td>
</tr>
<tr>
<td>S50</td>
<td>31.4±0.26</td>
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<tr>
<td>S75</td>
<td>23.4±0.05</td>
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Table S2. Viscosity of the prepared compositions

<table>
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<th>Composition</th>
<th>Viscosity / cP</th>
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</thead>
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<tr>
<td>S0</td>
<td>891±28.1</td>
</tr>
<tr>
<td>S25</td>
<td>1400±16.7</td>
</tr>
<tr>
<td>S50</td>
<td>2227±20.6</td>
</tr>
<tr>
<td>S75</td>
<td>5693±66.0</td>
</tr>
</tbody>
</table>
Development of a PCL-silica nanoparticles composite membrane for guided bone regeneration

Figure S1. ATR-IR spectra for electrospun membranes. Blue lines delimit the bands assigned to PCL groups. Red lines delimit the bands assigned to silica groups.

Figure S2. XRD spectra for electrospun membranes. a describes the peaks assigned to PCL.
Chapter 4

Incorporation of simvastatin in PLLA membranes for guided bone regeneration: effect of thermal treatment on simvastatin release

This chapter is based on:
A. G. B. Castro, D. W. P. M. Löwik, M. J. van Steenbergen, J. A. Jansen, J. J. J. P. van den Beucken, F. Yang,
Incorporation of simvastatin in PLLA membranes for guided bone regeneration

1. Introduction

Dental implants are a common treatment option for replacing missing teeth due to diseases, like periodontitis, or trauma. Successful implant placement requires adequate alveolar ridge dimensions, which are essential to house the implant and provide esthetics and function. Un fortunately, this is usually not the case due to bone resorption or damage. Therefore, a ridge augmentation technique is often required to regenerate enough bone for successful implant placement. Guided bone regeneration (GBR) is an established strategy for this purpose and is based on the creation of a secluded space, in which pluripotent and osteogenic cells are allowed to proliferate and form new bone, whilst soft tissue formation (by e.g. fibroblasts and epithelial cells) is inhibited. This can be achieved by a physical barrier, mainly in the form of a membrane. Electrospinning is an optimal technique to create polymeric membranes. Via electrospinning, polymer solutions are ejected under an electrical force; the fluid jet is elongated and solidifies to ultrafine fibers before reaching a collector; those randomly deposited ultrafine fibers form non-woven membranes. The electrospun membranes have a high porosity and surface area-to-volume ratio, resembling the extracellular matrix (ECM) structure. More importantly, the pore size of the electrospun membranes, in general, is less than the average cell size and previous studies have shown that such small pores do not allow cell penetration.

GBR membranes do not possess an active role in local bone regeneration, and specifically synthetic membranes are inherently hydrophobic and biologically inert. Different strategies can be adopted to overcome these drawbacks. The development of new materials based on polymeric composites or graphene/graphene oxide composites are promising approaches, leading to the proliferation and osteogenic differentiation of osteoblastic cells. An alternative strategy is the incorporation of osteostimulatory compounds. The incorporation of growth factors, such as bone morphogenetic proteins (BMPs) is a first choice due to their potent osteoinductive effect. However, their incorporation is limited by their inactivity when in contact with organic solvents or at physiological conditions, uncontrolled release which can lead to supraphysiologic concentrations and high cost. Small osteogenic pharmaceutical drugs have shown to be a promising alternative, showing higher chemical stability and a lower cost. Simvastatin (SV) is used to treat hypercholesterolemia and hyperlipidemia. Recently, it has also been reported that SV directly promotes osteoblastic differentiation and improves new bone formation by increasing the expression of bone morphogenetic protein-2 (BMP-2), alkaline phosphatase (ALP), osteocalcin and collagen type 1 (COL1) mRNAs in osteoblast-like cells and stem cells. The mechanism associated with the influence of simvastatin on the osteogenic differentiation is not fully understood. However, recent studies point out to a direct action of simvastatin in the activation of the Wnt/β-catenin pathway.
signaling pathway, which is involved in the differentiation of mesenchymal stem cells (MSCs) and the proliferation, differentiation, mineralization and apoptosis of osteoblastic cells.\[^{19}\]\ SV has been incorporated in different materials, including calcium-phosphate particles and granules,\[^{20, 21}\] metallic substrates\[^{22, 23}\] and polymeric particles,\[^{24}\] micelles\[^{25}\] and scaffolds.\[^{26, 27}\] Electrospun polymeric membranes containing SV have been previously developed\[^{28, 29}\] but SV release from these membranes occurred as fast as within 8 days,\[^{20, 21, 24, 26}\] which does not match the pace of bone regeneration (~12 weeks).\[^{30}\] Consequently, the need for a system achieving prolonged SV release is apparent.

Thermal treatments such as annealing,\[^{31}\] quenching,\[^{32}\] or aging\[^{33}\] are commonly used to modify the physicochemical properties of polymers. By heating a polymer to a temperature slightly above its cold crystallization temperature ($T_{\text{CC}}$), it is possible to increase its overall crystallinity. Here, our goal was to develop an electrospun poly($\ell$-lactic acid) (PLLA) membrane for prolonged SV release. PLLA was chosen due to its biocompatibility, biodegradability, and appropriate mechanical and physicochemical properties. More specifically, PLLA showed no obvious degradation within 3 months, which ensures the mechanical stability of the GBR membranes during the treatment period.\[^{34, 35}\] PLLA is a semi-crystalline polymer and it has been reported that the change of crystallinity of a polymeric drug carrier significantly affects the release of drugs.\[^{36–40}\] Consequently, we utilized thermal treatment of the electrospun PLLA membranes to increase its crystallinity and control the drug release. The morphological properties of the membranes were evaluated by scanning electron microscopy (SEM), physicochemical properties were analyzed by attenuated total reflectance-infrared spectroscopy (ATR-IR), X-ray diffraction (XRD) and differential scanning calorimetry (DSC). The release profile of simvastatin from the membranes with or without thermal treatment was evaluated by in vitro incubation in near-physiological conditions at 37 °C. The cellular osteogenic response to the membranes and biological activity of SV was determined by in vitro culture of the different membranes with rat bone marrow stromal cells (rBMSCs) and evaluating cell morphology, proliferation, and osteogenic differentiation over culture time.

2. Materials and methods

2.1. Preparation of polymeric solutions and fabrication of electrospun membranes

Poly(\(\ell\)-lactic acid) (PLLA; Purasorb PL65\(^{\oplus}\); Corbion, The Netherlands) solutions were prepared by dissolving the polymer in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP; Fluorochem, UK) to a concentration of 3% w/v. Solutions containing simvastatin (SV; Sigma-Aldrich, USA) were prepared by dissolving the SV (5% w/w to PLLA) and PLLA in HFIP and left to be stirred overnight. Membranes
were prepared by electrospinning (Advanced Surface Technology BV, The Netherlands) and the following parameters were applied: needle diameter = 1.2 mm, feeding rate = 2.0 ml h⁻¹; distance between the needle and the collector = 20 cm; voltage = 19.25 kV. Thermal treatment of the membranes was performed in an oven at 80 °C for 16 hours. Four different membranes were produced: pure PLLA membranes (PLLA); PLLA membranes subjected to the thermal treatment (PLLA HT); PLLA membranes containing simvastatin (PLLA + SV); PLLA membranes containing simvastatin and subjected to the thermal treatment (PLLA + SV HT).

2.2. Morphological and structural evaluation
The morphology of the membranes was observed using scanning electron microscopy (SEM; SIGMA 300; Zeiss, The Netherlands). Samples were fixed into aluminum stubs with carbon tape and sputter coated with chromium (thickness of 20 nm). The chemical profile of the membranes was assessed by attenuated total reflectance-infrared spectroscopy (ATR-IR; UATR two; PerkinElmer, The Netherlands) with a resolution of 4.0 cm⁻¹ and a scanning range from 400 cm⁻¹ to 4000 cm⁻¹. X-ray diffraction (XRD; X'pert³ powder; PANalytical, The Netherlands) was performed to determine the crystallographic profile of the membranes. Electrospun membranes as a thin planar layer were placed on a glass holder and scanned. XRD spectra were registered at 40 kV, 30 mA (Cu-Kα radiation with a wavelength of 1.54 Å) and a 2θ between 10° and 40°, at a step size of 0.005°. Crystallinity was determined by modulated differential scanning calorimetry (DSC; Discovery®; TA instruments, The Netherlands). For DSC analysis, samples were placed in perforated aluminum pans, to avoid overpressure, and scanned from 20 to 200 °C at a heating rate of 2 °C min⁻¹. The degree of crystallinity (χc %) was calculated considering the ratio between the experimental heat of melting (ΔH_m) minus the cold crystallization heat (ΔH_cc) and the heat of melting of 100% crystalline PLLA (ΔH°_m = 93.6 J g⁻¹),[41] according to the following equation:

\[ \chi_c \% = \frac{[\Delta H_m - \Delta H_{cc}]/ \Delta H°_m}{\Delta H°_m} \times 100 \]

2.3. Release of simvastatin (SV) in an aqueous medium
Membranes containing SV with or without thermal treatment (= 0.8 mg each sample) were immersed in 1 ml of PBS at 37 °C. The supernatant was collected at predetermined time points, being the medium renewed with 1 ml of PBS. The amounts of SV released were determined by liquid chromatography-mass spectrometry (LC-MS). The conditions of analysis were the following:
LC-MS was performed with a mobile phase that consisted of acetonitrile (ACN) + 0.1% v/v formic acid and water (H₂O) + 0.1% v/v formic acid, with a gradient of acetonitrile from 5 to 100% for 30 minutes and a flow rate of 0.2 ml min⁻¹. Charged ions ([simvastatin + H]⁺ at m/z = 420.57 and [simvastatin acid + H]⁺ at m/z = 437.59) were analyzed in order to determine the amount of simvastatin present in the samples. The release of SV was fitted according to the Higuchi and Korsmeyer–Peppas mathematical kinetic models.

2.4. In vitro cell culture

rBMSCs were cultured with the membranes and their osteostimulatory potential evaluated. rBMSCs were isolated from healthy male Wistar rats with Radboud UMC Animal Ethics Committee approval (RU-DEC 2014-157). Cell culture medium and all supplements were purchased from Gibco (Gibco®, Life Technologies, USA), except the fetal bovine serum (FBS; Sigma-Aldrich, USA). rBMSCs were isolated and cultured using a method adapted from Maniotopoulos et al.[42] The tibia of male Wistar rats were extracted, and epiphyses were cut off. Cells were flushed out of the remaining diaphyses using cell culture medium consisting of α-MEM supplemented with 10% v/v fetal calf serum, 50 mg ml⁻¹ ascorbic acid, 10⁻⁴ M dexamethasone, 50 mg ml⁻¹ gentamycin and 10 mM sodium β-glycerophosphate. The flush-out of the two tibias was cultured for one day in three 75 cm² culture flasks in a humidified incubator (5% CO₂; 37 °C), after which the medium was refreshed to remove non-adherent cells. The osteogenic potential of the cells was assessed by culturing rBMSCs with an osteogenic medium for 28 days, presenting the cells mineralization capability.

A preliminary study was firstly performed to evaluate the cytotoxicity of simvastatin. Briefly, rBMSCs were cultured in a growth medium (α-MEM supplemented with 10% v/v FBS, 100 units per ml penicillin, and 100 mg ml⁻¹ streptomycin) containing different concentrations of simvastatin (4200 ng ml⁻¹ or ≈10⁻⁵ M, 420 ng ml⁻¹ or ≈10⁻⁶ M, 42 ng ml⁻¹ or ≈10⁻⁷ M, and 4.2 ng ml⁻¹ or ≈10⁻⁸ M) at a cell density equal to 2 x 10⁴ cm⁻² and incubated in a humidified incubator (37 °C, 5% CO₂). Cytotoxicity was evaluated both by visual examination with an optical microscope (DM IL LED, Leica, The Netherlands), for the cells cultured at 3 days, 14 days and 28 days, and by an AlamarBlue® viability test (Molecular Probes®, Invitrogen®, Thermo Fisher Scientific, The Netherlands) after 3, 7, 14, 21 and 28 days, according to the manufacturer's instructions to obtain quantitative data on cellular activity. Viability percentages are relative to a positive control, where rBMSCs were cultured in an osteogenic medium (α-MEM supplemented with 10% v/v FBS, 50 mg ml⁻¹ ascorbic acid, 10⁻⁸ M dexamethasone, 10 mM sodium β-glycerophosphate and 100 units per ml penicillin + 100 mg ml⁻¹ streptomycin).

For the cell culture experiments, electrospun membranes were first disinfected with Argon plasma for 5 minutes (plasma cleaner/sterilizer PDC-001; Harrick,
Incorporation of simvastatin in PLLA membranes for guided bone regeneration

rBMSCs were statically seeded in 24-well adherent culture plates with growth medium at a cell density equal to $2 \times 10^4$ cm$^{-2}$ and incubated in a humidified incubator (37 °C, 5% CO$_2$), allowing the cells to adhere to the bottom of the wells. After 24 hours, membranes were placed in cell culture inserts (Thincert™, Greiner Bio-one, The Netherlands) and indirectly cultured with the cells. The different groups and conditions of culture are described in more detail in Table 1.

Table 1 Groups for in vitro cell culture experiments

<table>
<thead>
<tr>
<th>Group</th>
<th>Composition</th>
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<tr>
<td>PLLA</td>
<td>Membrane + growth medium + 10 mM β-GP</td>
</tr>
<tr>
<td>PLLA+SV</td>
<td>Membrane + growth medium + 10 mM β-GP</td>
</tr>
<tr>
<td>PLLA+SV HT</td>
<td>Membrane + growth medium + 10 mM β-GP</td>
</tr>
</tbody>
</table>

rBMSCs DNA quantification and ALP (alkaline phosphatase) activity measurements ($n = 3$) were performed at 3 days, 7 days, 14 days, 21 days and 28 days of co-culture as described previously.$^{[43]}$ Briefly, cells were washed with PBS twice, collected and immersed in 1 ml of dd$_2$ water. Afterward, sonication and three freeze–thaw cycles were performed. DNA content in the supernatant was measured using a Quant-IT™ PicoGreen® ds-DNA assay kit (Invitrogen; The Netherlands) according to the manufacturer’s instructions. In a 96-well plate, 100 ml of sample or standard were added to a working solution. The plates were incubated at room temperature in the dark for 5 minutes and fluorescence read (excitation: 485/20 nm; emission: 530/25 nm). ALP activity of the same sample was measured by a $p$-nitrophenylphosphate (4-NP; Sigma-Aldrich, USA) colorimetric assay, according to the manufacturer’s instructions. 80 ml of samples or standards were added to 20 ml of buffer and 100 ml of the substrate. The mixture was incubated for 60 minutes at 37 °C. Afterward, 100 ml of 0.5 M NaOH solution was added to stop the reaction. Absorbance at 405 nm was read and ALP activity normalized according to the DNA content for each sample.

To observe the cellular morphology, cells were directly cultured on the membranes with the specific medium indicated in Table 1. Samples were collected at 3 days, 14 days and 28 days. A 2% v/v solution of glutaraldehyde (glutaraldehyde solution 25%; Merck; Germany) was used to fixate the cells and ethanol at graded concentrations (70%, 80%, 90%, 96% and 100% v/v) was used for dehydration. Finally, samples were covered with tetramethysilane (TMS; ≥99.0% GC; Sigma-Aldrich; USA) and prepared for SEM observation as referred previously.
2.5. Statistical analysis
Fiber diameter was estimated from the corresponding SEM images using ImageJ software (National Institutes of Health, USA). Twenty different measurements were taken from 5 different regions of each electrospun membrane (n = 100). Data were reported as mean ± standard deviation (SD). Statistical analysis was carried out by using one-way analysis of variance (ANOVA) combined with a Tukey’s multiple comparison post-hoc test. Differences were considered significant at p < 0.05. Statistical differences in crystallinity of the different samples (n = 3) were analyzed using an unpaired t-test with Welch’s correction, calculated with Prism 6.01 software (GraphPad Software, Inc., USA). Data were reported as mean ± standard deviation (SD). Differences were considered significant at p < 0.05. DNA content and ALP activity (n = 3) were analyzed using Prism 6.01 software and performing a two-way ANOVA significance test combined with a post-hoc Tukey’s multiple comparison test. Data were reported as mean ± standard deviation (SD). Differences were considered significant at p < 0.05.

3. Results

3.1. Morphological and structural evaluation of electrospun membranes containing SV
Figure 1 shows the morphology of the electrospun PLLA membranes with and without SV before and after thermal treatment (HT). All membranes are composed of micrometric fibers, with a small decrease of the diameter of the fibers when the drug was incorporated (1.4 ± 0.14 μm for PLLA; 1.3 ± 0.11 μm for PLLA HT; 1.0 ± 0.10 μm for PLLA+SV; 0.9 ± 0.07 μm for PLLA+SV HT). No noticeable changes in the morphology of the fibers were detected when SV was incorporated (Figure 1c and Figure 1d). Membranes subjected to the thermal treatment (PLLA HT and PLLA + SV HT) presented a similar morphology when compared with their non-thermal treated counterparts (PLLA and PLLA + SV), although some deformation of the fibers was visible (Figure 1b-red square and Figure 1d-red square).
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The chemical profile of the membranes was analyzed by ATR-IR (Figure 2). The spectra were similar for all membranes, showing the characteristic peaks of PLLA highlighted with red lines: 1751 cm\(^{-1}\) (carbonyl group); 1000–1450 cm\(^{-1}\) (ether groups) and 870 cm\(^{-1}\) (alkyl groups).\(^{[32, 44, 45]}\) No characteristic peaks related to the presence of SV were detected, either before or after thermal treatment. Further, thermal treatment did not affect the chemical composition of the membranes, with no significant changes in the polymeric backbone.

Figure 2. ATR-IR spectra of electrospun membranes. Red lines represent the main peaks belonging to PLLA.

Figure 3 presents the XRD profiles for the different membranes. Samples without thermal treatment showed no sharp peaks on the XRD spectra. Membranes with thermal treatment showed a peak at approximately \(2\theta = 16^\circ\) (red arrows, Figure 3), representing the characteristic peak of the crystal structure of the \(\alpha\)
Figure 3. XRD spectra of electrospun membranes. Red arrows indicate the peak of the α form of PLLA.

Figure 4 shows the crystallinity percentage of the different electrospun membranes. As expected, crystallinity increased after the membranes were subjected to thermal treatment, shown by XRD (42 ± 7.8 % vs. 58 ± 4.4 % for PLLA without SV and 38 ± 3.0 % vs. 62 ± 1.1% for PLLA with SV). On the other hand, the incorporation of SV did not affect the crystallinity of PLLA. The representative DSC spectra of each group are shown in Figure S1 and the correlated thermal properties are summarized in Table S1. The glass transition temperature \( T_g \) decreased when SV was incorporated (54 ± 1.1 °C vs. 46 ± 0.1 °C for PLLA and PLLA + SV, respectively). \( T_g \) of membranes subjected to thermal treatment was not detectable considering the total heat flow (Figure S1). However, when examining the data obtained with the reversing heat flow, the \( T_g \) for these membranes was determined (Figure S2). A substantial increase of the \( T_g \) values due to thermal treatment was detected (73 ± 0.3 °C and 73 ± 1.0 °C for PLLA HT and PLLA + SV HT respectively). The melting temperature \( T_m \) was not affected by the incorporation of SV or by the thermal treatment.

Figure 4. Crystallinity of electrospun membranes. * p < 0.05; ** p < 0.01.
3.2. Drug release from PLLA membranes

The release profiles of the membranes containing SV are presented in Figure 5. No initial burst release of SV was detected for both membranes. A higher SV release from PLLA without thermal treatment was detected. SV released at a fast speed within the first 5 days and continued to release at a constant slow speed up to 28 days, except that PLLA + SV showed a sudden increase in the amount of SV released at week 4. Not all the incorporated SV was released from PLLA membranes after 4 weeks: 11 ± 1.9% SV released from PLLA+SV and 3 ± 0.8 % from PLLA+SV HT.

![Figure 5. Cumulative release of simvastatin in PBS at 37 °C (a) cumulative mass (b) cumulative percentage.](image)

SV release data were fitted according to two well-established drug release models: the Higuchi model and the Korsmeyer–Peppas model. As shown in Figure S3, SV release correlated highly with both models, although the Korsmeyer–Peppas model presented a higher correlation for both membranes (R² = 0.98 vs. R² = 0.97 for Higuchi model). The Korsmeyer–Peppas model fitting showed different release exponents (n): n = 0.56 for PLLA+SV and n = 0.47 for PLLA+SV HT.

3.3. In vitro study with rBMSCs

rBMSCs were cultured in a proliferative medium containing simvastatin at different concentrations for 28 days, and cytotoxicity was evaluated using light microscopy and AlamarBlue assays (Figures S4 and S5). Cellular proliferation occurred mainly for the cells cultured with simvastatin concentrations of 42 ng ml⁻¹ (10⁻⁷ M) and 4.2 ng ml⁻¹ (10⁻⁸ M). Simvastatin concentrations above 420 ng ml⁻¹ (10⁻⁶ M) resulted in substantial cytotoxicity, leading to cellular death (Figure S4) and low viability percentages (Figure S5).

rBMSCs were directly seeded on the different electrospun membranes with the purpose to analyze the cellular morphology and response. Figure 6 shows the images obtained by SEM after 3, 14 and 28 days of culture. After 3 days of culture, cells showed adherence to the membranes. At 14 days of culture, the
number of cells increased significantly, covering almost the entire surface of the membranes. At 28 days, the membranes were completely covered by the cells and a thick cell layer on top of the membranes was observed.

Figure 6. SEM images of rBMSCs cultured on the membranes at 150x (red squares) and 1,000x.

Figure 7 shows the DNA amount and ALP-activity obtained upon indirect \textit{in vitro} culture experiments. An increase in the DNA amount with time was observed for all groups. At individual time points, no significant differences between groups were observed. PLLA + SV HT membranes evoked an increase in ALP-activity up to 7 days followed by a decrease. Further, PLLA + SV HT membranes induced the highest ALP-activity levels among all groups (Figure 7b).
4. Discussion

We here aimed to incorporate an osteostimulatory drug, simvastatin (SV), in a PLLA electrospun membrane and prolong its release, with the ultimate objective of creating a membrane with suitable biological properties for GBR applications. SV was chosen not only due to its well-documented promotion of in vitro osteogenic differentiation\(^47, 48\) and bone regeneration, both in vivo\(^49, 50\) and in clinical studies\(^51\), but also because it possesses a high thermal stability (\(T_m\) of 139 °C), and good solubility in organic solvents\(^52, 53\). The effect of PLLA crystallinity on the release of SV was investigated. In order to increase PLLA crystallinity, membranes were treated at 80 °C for 16 hours. SV release kinetics showed that variations in PLLA crystallinity can be used to control SV release. In vitro cell culture assays demonstrated a continuous proliferation of cells over time for all compositions, with a significant increase in ALP-activity levels of osteogenic cells by membranes containing SV.

PLLA membranes containing SV were produced by electrospinning. Morphological evaluation of the membranes showed the formation of micrometric electrospun fibers with a smooth surface and a decrease of the diameter of the fibers when SV was incorporated. The decrease in fiber diameter is probably related to an increase in the conductivity of the polymeric solution for the compositions containing SV, leading to a decrease in fiber diameter during electrospinning\(^54\). Thermal treatment did not affect the surface morphology or the diameter of the fibers, although limited fiber deformation could be noticed. Similar ATR-IR spectra were obtained for all the membranes due to the overlap of characteristic infrared bands of SV with those of PLLA\(^52, 55\). Further, the similar spectra of membranes with or without thermal treatment indicate that the thermal treatment did not change the chemical structure of the electrospun membranes.

XRD and DSC results confirmed that the thermal treatment used in this study increased the crystallinity of PLLA membranes. It has been shown previously...
that an increase in polymer crystallinity due to thermal exposure leads to a slower diffusion of the drugs because the crystalline domains function as a physical barrier and reduce polymeric degradation.\cite{56, 57} The results from our study corroborate these data. The amount of SV released from PLLA + SV membranes was approximately 2 times higher than the membranes subjected to the thermal treatment (PLLA + SV HT), demonstrating the primary role of crystallinity in controlling the release of SV. Release data of both membranes were fitted according to two kinetic models (i.e. the Higuchi model and the Korsmeyer–Peppas model) in order to understand the drug release mechanism(s).\cite{58, 59} The Higuchi kinetic model is an ideal model, which considers the release of a drug from a homogeneous solid matrix, where the diffusion of the drug is constant and phenomena like polymer swelling and dissolution are negligible. The Korsmeyer–Peppas kinetic model is more complex and different release mechanisms are considered. According to the Korsmeyer–Peppas model, for cylindrical structures an $n \leq 0.45$ corresponds to Fickian-based diffusion, an $0.45 < n < 0.89$ to non-Fickian (anomalous) diffusion, an $n = 0.89$ to case II (relaxational) transport, and an $n > 0.89$ to super case II transport, the two last mechanisms being related to polymer swelling and erosion. For both models, the correlation coefficient was high, although slightly higher for the Korsmeyer–Peppas kinetic model. The Korsmeyer–Peppas model was considered as more accurate to explain the mechanism(s) related to the in vitro release of SV. Considering that ideally the electrospun fibers possess a cylindrical geometry, the determined $n$ values demonstrate that PLLA + SV membranes exert an anomalous non-Fickian diffusion of SV to the medium ($n > 0.45$), likely due to fiber erosion phenomena. PLLA + SV HT membranes, on the other hand, present an $n$ value very close to 0.45, suggesting a diffusion of SV that follows the Fick’s law.\cite{60} rBMSCs were cultured with pure and SV-loaded PLLA membranes with or without thermal treatment (PLLA, PLLA + SV and PLLA + SV HT). The mass of the membranes utilized in culture was calculated based on the SV release profile so that the concentration of SV in the medium was within the reported range capable of inducing an osteogenic response.\cite{20, 25, 61, 62} Should be mentioned that the potential cytotoxicity of simvastatin was also considered. Viability tests, where rBMSCs were cultured in medium containing different concentrations of simvastatin, were performed on an early phase of this study (Figures S4 and S5) and the following assays were performed taking into account the viability results obtained.

The purpose of these experiments was to evaluate if (i) the membranes could induce an osteogenic response from the rBMSCs, and (ii) the differences in the amount of drug released from the membranes with or without thermal treatment would affect osteogenic differentiation. Morphological observations indicated that rBMSCs adhered and proliferated on all membranes over time. The results of the DNA assay were consistent with this finding. The DNA results further demonstrated that the tested membranes did not evoke a cytotoxic ef-
Incorporation of simvastatin in PLLA membranes for guided bone regeneration

Effect. Moreover, SV incorporation followed by thermal treatment induced an increased osteogenic response from rBMSCs, demonstrated by a significantly increased ALP-activity for the cells cultured on such membranes (PLLA + SV HT). A suppression of an initial burst-release, combined with the significantly lower amounts of SV present in the medium, due to its more sustained release from these membranes, appeared to be beneficial for the osteogenic differentiation of pre-osteoblastic cells.\textsuperscript{25, 60} In this study a non-osteogenic medium supplemented with β-glycerophosphate was used to solely evaluate the effect of SV on the osteogenic differentiation of rBMSCs, without the influence of known osteoinductive compounds as ascorbic acid or dexamethasone. Also, we focused on analyzing the ALP-activity levels, since SV is known to have a major effect in the expression of this protein and a significant impact on the early stages of the osteogenic differentiation of osteoblastic cells.\textsuperscript{50} Further studies should be performed in order to evaluate the effect of the systems developed on the mineralization capability of rBMSCs.

5. Conclusion

We developed electrospun PLLA membranes with enhanced osteostimulatory properties by incorporating simvastatin (SV) and modified the SV release profile by varying the PLLA crystallinity. Thermal treatment successfully increased the PLLA crystallinity for all compositions without significantly changing the chemical properties and morphology of the electrospun fibers. A markedly more sustained release of SV was observed upon the thermal treatment to increase the PLLA crystallinity. \textit{In vitro} culture of PLLA membranes with rBMSCs demonstrated that the membranes are cytocompatible, with an increase in cell proliferation over time. PLLA + SV HT induced an osteogenic response from rBMSCs, with significantly increased ALP-activity levels demonstrating both a preservation of biological activity of SV and an appropriate SV release profile. Thermal treatment to increase PLLA crystallinity in electrospun membranes can be used to control SV release and render these membranes biologically active.
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Supplementary Information

Figure S1. Modulated DSC spectra for the different electrospun membranes.

Figure S2. Modulated DSC spectra (reversing signal) for PLLA HT and PLLA + SV HT membranes.
As described in Table S1, SV incorporation led to a small decrease in the values of glass transition temperature ($T_g$) and cold crystallization temperature ($T_{cc}$), related to a plasticizing effect common when drugs are added to polymeric materials.\cite{1, 2} Membranes subjected to the thermal treatment showed an increase in $T_g$ values, which is related to a higher organization and rigidity of the polymeric chains, a consequence of the higher crystallinity present on these membranes.

Table S1. Thermal properties of the electrospun membranes

<table>
<thead>
<tr>
<th>Group</th>
<th>Crystallinity / %</th>
<th>$T_g$ / °C</th>
<th>$T_{cc}$ / °C</th>
<th>$T_m$ / °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLLA</td>
<td>42.0 ± 7.76</td>
<td>53.6 ± 1.07</td>
<td>66.2 ± 0.62</td>
<td>185.8 ± 0.15</td>
</tr>
<tr>
<td>PLLA HT</td>
<td>58.3 ± 4.40</td>
<td>73.1 ± 0.32</td>
<td>--------------</td>
<td>184.8 ± 0.21</td>
</tr>
<tr>
<td>PLLA + SV</td>
<td>38.4 ± 3.04</td>
<td>46.3 ± 0.12</td>
<td>60.6 ± 0.15</td>
<td>182.1 ± 0.06</td>
</tr>
<tr>
<td>PLLA + SV HT</td>
<td>61.5±1.06</td>
<td>73.3±1.04</td>
<td>--------------</td>
<td>178.8±0.10</td>
</tr>
</tbody>
</table>

Figure S3. Fitting of the drug percentage released for both membranes according to a) Higuchi model b) Korsmeyer-Peppas model.
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Figure S4. Optical microscope images of rat bone marrow stromal cells (rBMSCs) cultured with a proliferation medium containing different concentrations of simvastatin, after 3, 14 and 28 days of culture.

Figure S5. AlamarBlue test for rBMSCs cultured with proliferative medium containing different concentrations of simvastatin. * p<0.05, ** p<0.01, **** p<0.0001. Viability percentages (%) are relative to the positive control (rBMSCs cultured in an osteogenic medium).
References

Layer-by-layer functionalization of PLLA fibers in electrospun membranes and effect of surface charge on drug loading and release
1. Introduction

Electrospinning is a technique to produce ultrafine polymeric fibers that enables the development of non-woven membranes with a high porosity and surface area-to-volume ratio, allowing their use for a wide range of applications, including filtration systems\textsuperscript{[1, 2]} textile industry\textsuperscript{[3, 4]} and biological or clinical applications\textsuperscript{[5-7]}. Biomedical applications require the electrospun membranes to be not only biocompatible but also bioactive. In the case of synthetic membranes, bioactivity is a major issue since most of the synthetic polymers possess a high degree of hydrophobicity and do not have the chemical moieties to induce a positive biological response, having a passive role in the tissue healing and regeneration. Such obstacles led to the development of several strategies to chemically modify the surface of synthetic membranes, including adsorption\textsuperscript{[8]}, covalent functionalization\textsuperscript{[9]}, and surface grafting\textsuperscript{[10]}. Among adsorption techniques, layer-by-layer (LbL) is a popular strategy to functionalize inert substrates of irregular geometry. LbL techniques use adsorption of polyelectrolytes (surface charged oligomers or polymers) with opposite charges to the surface of substrates\textsuperscript{[11]}. The interaction between the polyelectrolytes results in the creation of a layered structure on the surface of the substrate, \textit{i.e.} polyelectrolyte multilayers (PEMs). Interactions between the polyelectrolytes and between the polyelectrolytes and the substrate are mainly governed by electrostatic forces due to the opposite charges present, although hydrogen bonding, covalent bonding, and hydrophobic interactions also might have a contribution. Despite of such interactions being normally weak, LbL possesses several features that makes it a desirable functionalization strategy: a wide range of polyelectrolytes are available\textsuperscript{[12]}; functionalization can be controlled by changes in pH, ionic strength or temperature; the majority of the systems can be developed in aqueous environments and at room temperature\textsuperscript{[11, 13, 14]}. These features allow the incorporation of a wide range of compounds. Substrates containing antibiotics\textsuperscript{[15, 16]}, peptides\textsuperscript{[17, 18]} and growth factors\textsuperscript{[19-20]} have been developed, showing an improved sustained release compared to traditional strategies (e.g. physical adsorption or blending). Although LbL has been proposed in the early 90s by Decher \textit{et al.}\textsuperscript{[21]} as a promising surface functionalization technique, to the best of our knowledge, only a few studies focused on the functionalization of electrospun membranes by LbL for drug delivery purpose\textsuperscript{[22-24]}. The aim of this study was to explore the possibility to functionalize poly(\textit{l}-lactic acid) (PLLA) electrospun membranes by an LbL methodology. Furthermore, we intended to investigate the influence of drug charge on the incorporation process during LbL and subsequent release profiles. In order to achieve these goals, poly(\textit{l}-lysine) (PLL) and poly(acrylic acid) (PAA) were chosen as polyelectrolytes. PLL and PAA have ideal properties that make them suitable to be used for the surface functionalization of electrospun membranes for biological purposes. PLL is positively charged at physiological
pH (polycation; pKa = 10.5).\textsuperscript{[25]} It is not only biocompatible but also supports high levels of cellular adhesion and proliferation.\textsuperscript{[26, 27]} Moreover, PLL molecular chains have a flexible structure, which contributes to the increased mobility of the compounds incorporated in the LbL system.\textsuperscript{[25]} On the other hand, PAA is negatively charged at physiological pH (polyanion; pKa = 5).\textsuperscript{[28]} It is also biocompatible and has been widely used for LbL applications.\textsuperscript{[28, 29]} Rhodamine B and clindamycin were chosen as two model drugs for this study, due to their close molecular weight but distinct surface charge. Rhodamine B has a small molecular weight (M.W = 479.02 g mol\textsuperscript{-1}), and is a “zwitterion”, having a neutral charge at physiological pH (pKa = 3.1).\textsuperscript{[30]} On the other hand, clindamycin, a widely used antibiotic, has a similar molecular weight (M.W = 424.98 g mol\textsuperscript{-1}) but presents a positive charge at physiological pH (pKa = 7.79).\textsuperscript{[31]} PLLA electrospun membranes were produced and subjected to different LbL experimental procedures. The membranes were then characterized morphologically by scanning electron microscopy (SEM), fluorescence microscopy and confocal microscopy. PEM growth was quantified by solid-state elemental analysis of nitrogen, an element only present in PLL. Drug immobilization and release profile were analyzed both by high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS).

2. Materials and methods

2.1. Preparation of electrospun membranes
Poly(l-lactic acid) (PLLA; Purasorb PL65\textsuperscript{®}; Corbion, The Netherlands) was dissolved in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP; Fluorochem, UK) to a concentration of 3% w/v. Electrospun membranes were prepared by an electrospinning method. Electrospinning was performed using a commercially available electrospinning apparatus (Advanced Surface Technology BV, The Netherlands) according to the following parameters: needle diameter = 1.2 mm, feeding rate = 2.0 mL h\textsuperscript{-1}; distance between the needle and the collector = 20 cm; voltage = 19.25 kV.

2.2. Layer-by-layer assembly
Electrospun membranes were functionalized by a layer-by-layer methodology. Prior to the development of the double-layers, the surface of the membranes was activated according to two different strategies: 1) the use of poly(ethyleneimine) (PEI, M.W = 25,000 g mol\textsuperscript{-1}, Sigma-Aldrich, USA) to produce a basal reactive layer; or 2) by the incorporation of reactive amine groups using an aminolysis approach (Figure 1).
2.2.1. Layer-by-layer using PEI
PLLA membranes were first treated with argon plasma for 5 minutes to increase their hydrophilicity. Afterward, the membranes were immersed in a solution of PEI (5 mg ml\(^{-1}\) in 10mM Tris Buffer with 150 mM sodium chloride; pH =7.4) for 30 minutes at room temperature, followed by immersion in a solution of poly(acrylic acid) (PAA; M.W = 60,000 g mol\(^{-1}\); Polysciences Inc., USA; 1 mg ml\(^{-1}\) in 150 mM sodium chloride solution; pH =5.5) for 10 minutes and subsequently by immersion in a poly(\(L\)-lysine) solution (PLL; M.W= 30,000-70,000 g mol\(^{-1}\); Sigma-Aldrich, USA; 1 mg ml\(^{-1}\) in 10 mM Tris Buffer with 150 mM sodium chloride; pH =7.4). Washing steps of 1 minute with dd\(_2\) water were performed between the immersion of the membranes on the polyelectrolyte solutions to remove the non-bonded PAA and PLL. To build up multi-layers, PAA-PLL immersion steps were repeated as shown in Figure 1. The created LbL system was named as PEI-[PAA-PLL]\(_n\) with \(n\) representing the number of multi-layers.

2.2.2. Layer-by-layer of aminolyzed samples
PLLA membranes were subjected to an aminolysis procedure prior to LbL assembly. Briefly, samples were incubated at 37 °C in a solution of ethylenediamine (EtDA; Sigma-Aldrich, USA) with different concentrations (0.1M, 0.5M, 0.75M EtDA in isopropanol and 0.75M in dd\(_2\) water) and for different incubation periods. Afterward, samples were alternatively immersed in PAA and PLL solutions as described in the previous section.
2.3. Incorporation of rhodamine B and clindamycin
Rhodamine B and clindamycin were added drop-wise to the membranes after the creation of a first PAA-PLL-PAA multilayer. Different amounts of rhodamine B or clindamycin (25 μg, 250 μg, and 2500 μg) were loaded onto the membranes according to the LbL methodology depicted in Figure 2. Membranes modified by aminolysis followed by LbL were loaded solely with 250 μg of clindamycin. The created systems were denoted as PAA-PLL-[PAA-Rhod.B-PAA-PLL]_n and PAA-PLL-[PAA-Clind.-PAA-PLL]_n.

2.4. Morphological evaluation
Morphology of the membranes was observed using scanning electron microscopy (SEM; SIGMA 300; Zeiss, The Netherlands). Samples were fixed into aluminum stubs with carbon tape and sputter coated with chromium (thickness of 20 nm).

2.5. Energy-dispersive X-ray Spectroscopy
Elemental analysis of the membranes was performed by Energy-dispersive X-ray Spectroscopy (EDS). Samples used for morphological evaluation by SEM were exposed to a voltage of 10 kV at a working distance of ≈ 10 mm and a magnification of 50,000x. Elemental analysis was performed considering the coating of chromium.

2.6. Fluorescence and confocal microscopy
For fluorescence assays, a fluorescein isothiocyanate (FITC) tagged PLL (M.W = 30,000–70,000 g mol⁻¹; Sigma-Aldrich, USA) was used to replace the pristine PLL during the LbL process. Membranes were posteriorly visualized by fluorescence microscopy and confocal microscopy. Fluorescence microscopy images were taken using an Alexa Fluor 488 (AF488) filter, with a magnification
of 20x and a 4 milliseconds exposition. Confocal microscopy images were taken also using an AF488 filter and a magnification of 63x. Three-dimensional (3-D) reconstruction was performed using Fiji software (National Institute of Health, USA).

2.7. Solid-state elemental analysis of nitrogen
Elemental analysis was performed using a carbon and nitrogen analyzer (NA1500; Carlo Erba - Thermo Fisher Scientific, The Netherlands). Briefly, membranes were ground, weighted in tin containers (= 3 mg for each sample) and loaded into an automatic sampler. A combustion occurs in the presence of oxygen at a temperature of 1800 °C. The gaseous combustion products are carried to a Cu-column where nitrogen oxides are reduced to elementary nitrogen. A thermal conductivity detector (TCD) produces an electrical signal proportional to the concentration of nitrogen. The amount of nitrogen was normalized according to the weight of the samples.

2.8. Determination of amine groups present in aminolyzed membranes
Quantification of amine groups density on the surface of the membranes subjected to aminolysis was performed using a TNBS colorimetric assay. Briefly, a known amount of sample (= 3 mg for each sample) was incubated with 400 μL of a 0.5% w/v picrylsulfonic acid solution (Sigma Aldrich, USA) for 2 hours at 40 °C. Samples were digested using 700 μL of HCl (37% w/v, 12.1M) and incubated at 60 °C until the solution was completely clear. Absorbance at λ = 405 nm was read with a UV-VIS microplate reader (Synergy® HTX; Biotek, The Netherlands). A 1:1 stoichiometry for the complex formed between the amine group and the dye was considered. Values were normalized according to the weight of each sample.

2.9. Release of rhodamine B and clindamycin in an aqueous medium
Membranes functionalized by LbL and containing rhodamine B and clindamycin (= 3 mg each sample) were immersed in 1 mL of PBS at 37 °C. The supernatant was collected at predetermined time points. The amount of rhodamine B released was determined by high-performance liquid chromatography (HPLC) and the amount of clindamycin released determined by liquid chromatography-mass spectrometry (LC-MS).

HPLC was performed according to the following method:
A reversed-phase hydrophilic column (AQ-C18; 2.1 by 150 mm; 3 μm particle size; GL Sciences, Japan) was used. Two eluents, acetonitrile (ACN) and water (H₂O), were used as the mobile phase in a gradient method. Assays were performed with a gradient of 60% to 100% ACN in H₂O over 10 minutes. A flow of 0.5 mL min⁻¹ was chosen. Rhodamine B detection was performed with a λ = 550nm.
LC-MS was performed following the described conditions:

LC-MS was performed with a mobile phase consisting of acetonitrile (ACN) + 0.1% v/v formic acid and water (H$_2$O) + 0.1% v/v formic acid, with a gradient of acetonitrile from 5 to 100% in 30 minutes and a flow rate of 0.2 ml min$^{-1}$. Charged ions ([clindamycin+H]$^+$) at m/z 425.98 were analyzed in order to determine the amount of clindamycin present in the samples.

2.10. Statistical analysis

Statistical significance of TNBS assay was performed using an unpaired $t$-test with Welch's correction, calculated with Prism 6.01 software (GraphPad Software, Inc., USA). Data were reported as the mean ± standard deviation (SD) and n = 3. Differences were considered significant at p < 0.05.

3. Results

Figure 3 presents the SEM results of the electrospun PLLA membranes subjected to the LbL technique with a PEI basal layer. Untreated electrospun fibers presented a homogeneous and smooth surface (Figure 3a). After the LbL treatment, the surface of the fibers became rough (Figure 3b and Figure 3c). The EDS analysis showed that nitrogen appeared in the membranes after LbL treatment and the amount was proportional to the number of double-layers: 3.94% for PLLA-PEI-[PAA-PLL]$_5$ and 7.96% for PLLA-PEI-[PAA-PLL]$_{10}$ (Figure 3b and 3c and, table S1).

Figure 3. SEM-EDS results of PLLA membranes, 20,000x. a) PLLA membrane b) PLLA-PEI-[PAA-PLL]$_5$ c) PLLA-PEI-[PAA-PLL]$_{10}$.

Figure 4 and Figure 5 show the results obtained with fluorescence and confocal microscopy, respectively. In Figure 4, an increase in fluorescence with the number of double-layers is noticeable, and the pristine PLLA membrane was not fluorescent, as expected (Figure 4a).
Layer-by-layer functionalization of PLLA fibers in electrospun membranes

By confocal microscopy was created a three-dimensional morphology of the functionalized membranes (Figure 5). Fluorescent PLL (PLL-FITC) could be detected in all membranes subjected to the LbL technique. It can also be seen that the amount of PLL increases with the number of double-layers.

In order to obtain quantitative data concerning the development of the double-layers, the nitrogen content present in the membranes was quantified by solid-state elemental analysis and the results are shown in Figure 6. It is clear that the nitrogen percentage (%) is almost directly proportional to the number of deposited double-layers. A higher increase rate of the nitrogen % up to 5 double-layers was observed and afterward a lower increase rate from 5 double-layers to 20 double-layers.
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Figure 6. Nitrogen content for samples subject to LbL functionalization.

Figure 7 represents the amount and percentage (%) of rhodamine B incorporated in the membranes subjected to the LbL procedure with a PEI basal layer. As expected, a higher amount of rhodamine B was detected in the membranes when a higher initial amount of drug was used. It can also be seen that the amount of incorporated rhodamine B was significantly lower than the amount used in the experimental procedure. In other words, the drug incorporation efficiency was extremely low (< 0.014%).

Figure 7. Amounts and percentage of rhodamine B incorporated in the membranes after LbL.

Figure 8 shows the release profile of rhodamine B for the prepared membranes. The amount of rhodamine B released from the membrane loaded with 25 μg was below the detection limit of HPLC. For the membranes loaded with 250 μg and 2500 μg of rhodamine B, ~ 40 to 50% of the drug was released within 8 hours of incubation. After that, no release could be detected.
Clindamycin was not detected by this first methodology both for the loading and release experiments. Considering the results described above, a second functionalization strategy, based on an initial aminolysis procedure was adopted. The membranes were first subjected to a preliminary study with different aminolysis experimental conditions, in order to evaluate the optimal time of incubation for the different concentrations of EtDA (data not shown). Figure 9 shows the SEM images of the membranes subjected to the optimal aminolysis conditions for the EtDA concentrations tested. All the electrospun fibers showed a similar morphology under all the tested aminolysis conditions, presenting a smooth and homogenous surface. No fissures or cracks in the fibers were observed for all the conditions tested.

Figure 9. SEM images of membranes aminolyzed, 10,000x. a) 0.1 M EtDA-2.5 hours b) 0.5 M EtDA-2 hours c) 0.75 M EtDA-1 hour d) 0.75 M EtDA+H2O-1.5 hours.
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Figure 10 presents the density of amine groups for the membranes subjected to aminolysis, determined by a TNBS assay. The membranes subjected to an aminolysis process using an aqueous solution of 0.75 M EtDA and an incubation period of 1.5 hours presented the highest average amines density ($3.7 \times 10^{-4} \text{NH}_2 \text{cm}^{-2}$). This condition was selected for further experiments.

![Graph showing amine groups density](image)

Figure 10. Amine groups density on electrospun membranes subjected to different conditions of aminolysis.

An LbL procedure was performed on the aminolyzed membranes using the PLL/PAA system. Figure 11 presents a morphological comparison between the membranes aminolyzed with the aqueous solution of 0.75 M EtDA and the membranes subsequently subjected to the LbL procedure. Unlike the aminolyzed membranes, the membranes subjected to a later LbL process presented fibers with a rough surface. EDS analysis also showed that the nitrogen content in the membranes increased from 4.27 % for aminolyzed membranes to 6.30 % after 10 LbL double-layer coatings (Table S2).

![Images showing SEM-EDS results](image)

Figure 11. SEM-EDS results of aminolyzed PLLA membranes, 10,000x and 50,000x (red square). a) aminolyzed PLLA membrane b) aminolyzed PLLA-[PAA-PLL]$_{10}$. 
Clindamycin was loaded on the membranes and subjected to the aminolysis-based second methodology. The in vitro release is shown in Figure 12. After 24 hours, 0.101 μg (0.004%) clindamycin was released. Furthermore, no clindamycin could be detected after 48 hours.

Figure 12. Cumulative release of clindamycin from functionalized PLLA membranes.

4. Discussion

An LbL methodology using two polyelectrolytes, poly(acrylic acid) and poly(L-lysine), was used for PLLA electrospun membrane functionalization and drug loading. This strategy was followed to create electrospun membranes with high biocompatibility that could additionally function as a drug delivery reservoir for the incorporation of small molecular weight compounds. The influence of drug charge on their loading and release was studied. Two model drugs were used, i.e. rhodamine B (neutrally charged) and clindamycin (positively charged). The results showed that the LbL process was successful for polyelectrolyte deposition. However, the drug-delivery capacity was compromised due to low loading efficacies and/or limited release.

Polyelectrolyte multilayer deposition on the electrospun PLLA membranes was successfully achieved by the LbL procedure. The deposition of the first 5 double-layers was more efficient than the later ones since the nitrogen percentage increment was higher during the first 5 double immersion steps. Previous studies have shown that the number of deposited layers influences the dissociation constant (pKa) of the polyelectrolytes, showing for PLL a decrease in its pKa value. A decrease in the base strength of PLL is related with the adoption of a secondary conformational order by the polymeric chains and formation of hydrogen bonds, resulting in a decrease of the number of functional groups available to interact with the polyelectrolytes in solution.\(^{[2]}\)

The other goal of this study was to improve our understanding of how the charge of small drugs influences their loading and release upon incorporation in the LbL systems. Two model drugs, rhodamine B and clindamycin, were selected
due to their similarity in molecular weight (479.02 g mol$^{-1}$ for rhodamine B and 424.98 g mol$^{-1}$ for clindamycin) but distinct charge at physiological pH (i.e. rhodamine B neutral and clindamycin slightly positive). We tested the loading efficiency of rhodamine B and found it was lower than 0.014%. The poor incorporation of rhodamine B was expected due to its neutral charge at physiological pH, which likely compromised the interactions between rhodamine B and the polyelectrolytes. The incorporation of rhodamine B was only governed by non-specific adsorption phenomena. Furthermore, the loading efficiency of rhodamine B decreased with increasing initial amounts of rhodamine B used. This inverse correlation is probably due to steric repulsion when a large amount of rhodamine B is present; thus, rhodamine B molecules could only be adsorbed to the surface of the electrospun fibers up to a certain amount. After a certain threshold, there was a saturation of the rhodamine B adsorbed on the surface of the fibers and most of the compound was washed out during the washing steps.

Clindamycin was also used as a model drug in this study. Due to a higher pKa, clindamycin presents positive charge at the physiological pH. Therefore, it was hypothesized that clindamycin would have a higher affinity to the polyanion PAA present in the LbL system. Surprisingly, no clindamycin was detected when the membranes functionalized by our first LbL strategy were immersed in PBS. A possible explanation for this result is a competitive effect between PLL (highly positive) and clindamycin to interact with PAA.$^{[25, 33]}$ Further, the LbL intermediate washing steps were performed using dd$^2$H$_2$O, which could lead to some level of deprotonation of the bound clindamycin, due to an increase in the pH of the solution and diffusion of the drug to the water.

A second strategy was attempted to enhance clindamycin incorporation. In this case, the LbL process was not initiated using a reactive basal layer of PEI. Instead, the electrospun membranes were initially functionalized by an aminolysis process. Several studies demonstrated that aminolysis can be used to incorporate amine groups on the polymer surface.$^{[34, 35]}$ Ethylenediamine (EtDA) was chosen as the amine donor; different concentrations of the reagent in isopropanol (IPA) at different times of incubation were prepared (0.1M EtDa-2.5 hours; 0.5M EtDa-2 hours and 0.75M EtDa-1 hour) according to preliminary experiments (data not shown). Furthermore, aminolysis using a solution of 0.75M EtDA in dd$^2$H$_2$O for 1.5 hours instead of IPA was included. Electrospun fibers presented a similar morphology for all the different conditions tested, suggesting that the used approach was mild and does not lead to chemical scission of the fibers. In order to select the optimal condition for further experiments, a TNBS test was performed in the aminolyzed samples and the density of amine groups calculated. The membranes incubated in a solution of 0.75 M EtDA in dd$^2$H$_2$O presented the highest number of amine groups per cm$^2$. As described in a previous study from our group the dissolution of EtDA in IPA has a synergistic effect, promoting the chemical scission of the fibers.$^{[36]}$ By using an aqueous solution the chemical scission activity of EtDA is controlled, allowing the incorporation of the
amine groups from the amine donor but avoiding the chemical degradation of the membranes. Membranes aminolyzed using the optimal conditions of 0.75M EtDA in \( \text{dd}_2\text{H}_2\text{O} \) for 1.5 hours were subjected to the same LbL protocol as before. Polyelectrolytes deposition occurred on the fibers. The percentage of nitrogen present after LbL was similar to the percentage measured on the membranes functionalized by the first strategy (Table S2). Clindamycin was loaded, and its release profile evaluated. Incorporation of clindamycin by the second approach was achieved, although the released amount was very limited, and no further release was detected after 2 days. A possible explanation for a higher incorporation of clindamycin using the aminolysis-based LbL is that the distribution of the polyelectrolytes is more efficient by this method, creating a double layer system where the PAA carboxylate groups are more exposed and can interact electrostatically with the positively charged clindamycin.\[16, 37\] The fast release of clindamycin is in accordance with other studies where small compounds were incorporated in LbL functionalized substrates.\[15, 16, 23-25, 38\] These results are related with the weak interaction between the drug and the multilayers at the pH of PBS, since clindamycin has a pKa value only slightly higher than the physiological pH. Further, PBS contain salts which compete with clindamycin for the interaction with PAA.\[15, 16\] When incorporated, clindamycin presented a more prolonged release than rhodamine B, which was released from the membranes in the first 8 hours of incubation. Although these results demonstrate that the interaction between the compounds and the multilayer systems is dependent on the methodology used, a clear understanding of the mechanisms involved in the loading and release of the compounds is still not established. Further studies are needed to have a clear understanding of the PEM build-up and how the interaction between the polyelectrolytes and the drugs occurs.

5. Conclusion

In the present study, two layer-by-layer (LbL) methodologies were attempted in order to successfully functionalize PLLA electrospun membranes. A first strategy using a basal layer of PEI and a PAA-PLL double-layer build-up was followed. A second strategy, in which the membranes were first functionalized by aminolysis followed by the same PAA-PLL double-layer build-up was used. LbL functionalization was achieved with both strategies and an increase in PAA-PLL double-layer build-up was observed. Two model drugs (rhodamine B and clindamycin), with a different charge at physiological pH were tested, presenting low loading efficiency and limited release within a short (i.e. hours to days) time span.
References

[14] A. C. A. Wan, and J. Y. Ying, Nanomaterials for in situ cell delivery and tis-
Layer-by-layer functionalization of PLLA fibers in electrospun membranes


and osteoblast behavior of multilayered films on TiO\textsubscript{2} nanotube surfaces assembled by the layer-by-layer technique, Chinese Chemical Letters, 2016. 27(7): p. 1091-1096.


**Supplementary Information**

Table S1 – Elemental analysis by EDS for pristine and functionalized PLLA membranes (first methodology)

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<th>C / %</th>
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<tbody>
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<td>PLLA membrane</td>
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<td>3.00</td>
<td>14.92</td>
</tr>
<tr>
<td>PLLA membrane + 5 double-layers</td>
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Table S2 – Elemental analysis by EDS for pristine and functionalized PLLA membranes (second methodology)

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<tbody>
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Chapter 6

A top-down approach for the preparation of highly porous PLLA microcylinders

This chapter is based on:

*These authors contributed equally to this work
1. Introduction

Microsized particles have been widely used in the development of drug delivery systems. These particles can be fabricated from organic compounds like natural and synthetic polymers or inorganic minerals and metallic oxides. Each composition presents unique characteristics and opportunities to create particles of different sizes and shapes. Particles with a spherical geometry have been most commonly studied, especially in the development of systems that require high levels of cellular internalization and long circulation times in vivo.\[^{1, 2}\] Compared to spherical structures, non-spherical particles such as micron-sized cylinders present several advantages for the development of drug or biomolecule delivery systems. First, higher drug loading levels can be achieved using non-spherical particles due to a higher surface area-to-volume ratio for particles with similar dimensions.\[^{3}\] Second, non-spherical particles are cleared less rapidly from the body.\[^{1, 4}\] This property is related to the different hydrodynamic forces to which these non-spherical particles are subjected in the body, leading them to stumble and move across the vessel walls and to adhere to endothelial cells, rather than following the more linear pattern in the circulatory system shown by spherical analogs.\[^{1, 4}\] Finally, non-spherical micrometric particles have shown to be less susceptible to phagocytosis in vitro because their internalization is highly dependent on the particles’ geometry and length-normalized curvature (Ω).\[^{5, 6}\]

Although several physical methods have been developed to fabricate non-spherical structures, such as lithography\[^{7}\], stretching techniques\[^{8}\], and sectioning\[^{9}\], chemical-based strategies present appealing advantages, including lower costs and mild experimental conditions. Also, when compared, for example, to mechanical drawing techniques, a better control of the fiber diameter is achieved.\[^{10}\] In 2008, Kim and Park showed that poly(\(L\)-lactic acid) (PLLA) electrospun fibers could be shortened by a chemical transverse fragmentation method, leading to the development of cylindrical microstructures.\[^{11}\] A wet-chemical process called aminolysis was used to induce this transverse fragmentation. Aminolysis comprises a reaction between a reactive species and an amine or ammonia group donor, resulting in chemical scission of the reactive species and the incorporation of amine or amide groups to its molecular structure.\[^{12, 13}\] Alternatively, aminolysis is commonly used for the functionalization of polymers with amine groups, which can increase the material’s biocompatibility or facilitate the conjugation of other compounds to the polymer.\[^{13−15}\]

PLLA is one of the most extensively studied polymers for the development of new biomaterials, which is justified by its high biocompatibility, biodegradability, good mechanical properties, and typical degradation rates, which are compatible with clinical applications and result in lactic acid, a nontoxic degradation by-product.\[^{16, 17}\] However, as other polyesters, PLLA is in its pristine form hydrophobic, having a reduced wettability and consequently cellular adhesion. This drawback can be overcome by chemically modifying the surface or the molecular backbone...
of the polymer with specific functional groups, for example, amine groups, hydroxyl groups, or peptidic sequences.\textsuperscript{[18, 19]} As mentioned above, aminolysis renders the polymer with amine groups and can be used to functionalize PLLA. The use of electrospinning has been well established in the production of fibers with micro- and nanoscale diameters.\textsuperscript{[20]} Established by Formhals, this technique produces polymeric fibers by the application of an electric current to a polymeric solution. An electrospinning apparatus is essentially constituted by an electrical power source, a nozzle, and a collector. When an electrical potential between the nozzle and the collector is generated, the polymeric solution's droplet is stretched, creating a jet. Due to solvent evaporation, polymeric fibers are then generated and collected.\textsuperscript{[21, 22]} Electrospun scaffolds of biodegradable polymers, such as polylactic acid (PLA), poly(ε-caprolactone) (PCL), polyglycolic acid (PGA), and their copolymers have found wide-spread applications in various biomedical fields.\textsuperscript{[23–25]} Furthermore, the high surface area-to-volume ratio and the possibility to incorporate biomolecules within and/or on the surface of the electrospun fibers encourages the use of these structures as a platform for the delivery of therapeutic drugs and growth factors.\textsuperscript{[26]} Importantly, electrospun fibers with different morphologies and surface porosity can be obtained in a simple manner using solvents with different volatilities.\textsuperscript{[27–29]} Surface porosity has an important effect on the functional properties of micro-sized particles, especially in the development of drug delivery systems. Porous particles have shown higher loading efficiencies compared to dense particles, largely due to their increased surface area-to-volume ratio.\textsuperscript{[30]} Further, the porosity of a particle affects the drug release kinetics; by creating macroporosity, a higher initial release can be achieved\textsuperscript{[31, 32]}, while the presence of interconnected micropores leads to larger diffusion pathways, resulting in a more sustained release and diminished burst release profile.\textsuperscript{[33, 34]}

Considering the advantages of electrospinning and aminolysis for the preparation of micro-sized particles, we combined both techniques in a top-down approach for (i) production of PLLA-based electrospun fibers and (ii) generation of PLLA based micro-sized cylinders that feature high specific surface area and with or without surface porosity. To this end, PLLA polymeric fibers were electrospun and subsequently subjected to an aminolysis-based scission procedure. The morphology and chemical composition of the resulting dense or porous microcylinders were characterized by scanning electron microscopy (SEM), X-ray diffraction (XRD), differential scanning calorimetry (DSC), thermogravimetric analysis (TGA), and infrared spectroscopy. Additionally, their biocompatibility was assessed by evaluation of the viability levels of human fibroblasts cultured in vitro.
2. Materials and methods

2.1. Electrospinning of PLLA
Medical-grade poly(\(L\)-lactic acid) (Purasorb PL65; Corbion, The Netherlands) was dissolved at a concentration of 3% w/v in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP; Fluorochem, UK) or in a 4:1 v/v mixture of dichloromethane (DCM; Merck, Germany) and trichloromethane (TCM; Merck). A commercially available electrospinning apparatus (Advanced Surface Technology BV, The Netherlands) was used for the preparation of PLLA membranes. Electrospinning of PLLA in HFIP was performed using the following parameters: volume = 8 mL; needle diameter = 1.2 mm; feeding rate = 2.0 mL h\(^{-1}\); distance between the needle and the collector = 35 cm; and voltage = 19.25 kV. Electrospinning of PLLA in a DCM/TCM mixture was performed using the following parameters: volume = 8 mL; needle diameter = 1.2 mm; feeding rate = 2.0 mL h\(^{-1}\); distance between the needle and the collector = 35 cm; and voltage = 35.00 kV. Fibers were collected in a stationary flat collector, covered with aluminum foil. Electrospinning terminated after 4 hours. The obtained membranes of about 0.2 mm thick were left overnight in a fume hood and freeze-dried for 3 days to remove the residual solvent.

2.2. Preparation of PLLA microcylinders
PLLA membranes were subjected to an aminolysis process based on a previously described method, with some modifications.\(^{[11]}\) Briefly, electrospun fibers were immersed in a 5% v/v solution of ethylenediamine (EtDA; Sigma-Aldrich, USA) in isopropanol (IPA; Merck) for two different time points (3 and 5 hours). EtDA was chosen as a reactive amine donor, and IPA was used in order to promote the aminolysis reaction.\(^{[12]}\) After aminolysis, four cycles of centrifugation/washing with \(\text{dd} \) water were performed to remove the diamine solution in excess. Samples were finally resuspended in \(\text{dd} \) water, subjected to 40 seconds of ultrasonication at 100% amplitude (UP50H; Hielscher - Ultrasound Technology, Germany) to prevent aggregation of the microcylinders, freeze-dried, and stored at room temperature for further use.

2.3. Characterization of PLLA microcylinders
The morphology of the electrospun membranes and microcylinders was observed by SEM (SM3010; JEOL, Japan) under an acceleration voltage of 5 kV. Electrospun fibers as a mat and microcylinders as powder were fixed with carbon tape on aluminum holders and sputter-coated with gold (thickness = 10 nm). XRD (pw1830; Philips, The Netherlands) was performed to determine the crystallographic profile of the samples. Electrospun membranes as a thin planar layer or microcylinders in powder form were placed in a copper holder and scanned. XRD spectra were registered at 40 kV, 30 mA (Cu–Ka radiation with
a wavelength of 1.54 Å), and 2θ between 10 and 30°, at a step size of 0.005°. The crystallite sizes of PLLA for the main (110) and (203) reflections were determined according to the Scherrer equation:\[35\]

\[ D = \frac{K \lambda}{\beta_c \cos \theta} \]

where K is a dimensionless shape factor (0.9 for the spherulite structure), λ the wavelength of Cu−Kα radiation, and βc the full width at half-maximum value of the diffraction peak at 16.7° for PLLA, given in radians. Accordingly, θ is 16.7° for all calculations in this study.

The degree of crystallinity (χc) of the PLLA as received and after electrospinning and aminolysis processes was determined by DSC (Discovery®; TA Instruments, The Netherlands). For DSC analysis, samples were sealed in an aluminum pan and scanned from 20 to 200 °C at a heating rate of 5 °C min\(^{-1}\). The degree of crystallinity (χc) was calculated considering the ratio between the experimental heat of melting (ΔH\textsubscript{m}) minus the cold crystallization heat (ΔH\textsubscript{cc}) and the heat of melting of 100% crystalline PLLA (ΔH\textsubscript{m}° = 93.7 J g\(^{-1}\)), according to the following equation:\[35\]

\[ \chi_c \% = \frac{\Delta H_m - \Delta H_{cc}}{\Delta H_m^\circ} \times 100 \]

TGA (TA Instruments, The Netherlands) was carried out in a nitrogen atmosphere with a temperature range from 20 to 400 °C and a heating rate of 5 °C min\(^{-1}\).

The specific surface area of the different samples was determined by nitrogen gas adsorption/desorption isotherms (Gemini V, Micrometrics; USA) and based on the Brunauer−Emmett−Teller (BET) model:\[36\]

\[ \frac{1}{W((P_0/P) - 1))} = \frac{1}{W_m C + (C - 1)/W_mC(P/P_0)} \]

where W is the weight of the gas adsorbed, W\(_m\) is the weight of the adsorbate as a monolayer, P\(_0\)/P is the relative pressure, and C is the BET constant. The specific surface area (S) can be obtained from the following equation:\[36\]

\[ S = \frac{W_m N A_{cs}}{M} \]

where N is the Avogadro’s number, A\(_{cs}\) is the adsorbate cross-sectional area (16.2 Å\(^2\)/molecule for N\(_2\)), and M is the weight of the sample.

The presence of amine groups was determined by attenuated total reflectance-infrared spectroscopy (ATR-IR; UATR two; PerkinElmer, The Netherlands) with a resolution of 4.0 cm\(^{-1}\) and a scanning range from 400 to 4000 cm\(^{-1}\).

A fluorescamine assay was used in order to qualitatively detect the presence of amine groups. Briefly, the materials were incubated for 1 hour in a 1:1 (v/v)
mixture of \text{dd} water and a 1 mg mL\(^{-1}\) fluorescamine (Sigma-Aldrich) solution in acetone (Lab-Scan, Poland) at room temperature. Afterward, three centrifugation steps with \text{dd} water were performed to remove the excess dye. Fluorescence was assessed by confocal microscopy. Fluorescence intensity heat maps were created using ImageJ software (National Institutes of Health, USA).

In order to quantify the amount of amine groups in the samples, a 2,4,6-trinitrobenzenesulfonic acid (TNBS) colorimetric assay was performed. Briefly, a known amount of sample was incubated with 400 μL of a 0.5% w/v picrylsulfonic acid solution (Sigma-Aldrich, USA) for 2 hours at 40 °C. Afterward, 700 μL of pure hydrochloric acid (HCl, 37% w/v, 12.1M) was added in order to dissolve the samples, and the samples were incubated at 60 °C until the solution was completely clear. Absorbance at λ = 405 nm was determined with a UV−vis microplate reader (Synergy HTX; Biotek, The Netherlands). The amines concentration was calculated assuming a 1:1 stoichiometry of the complex between the amine groups and the dye. Values were normalized for the weight of each sample.

2.4. \textit{In Vitro} cytotoxicity of PLLA microcylinders

Adherent human foreskin fibroblasts were grown and maintained in DMEM medium (Gibco*, Life Technologies, USA) with 10% fetal bovine serum (FBS; Sigma-Aldrich, USA) and a mixture of penicillin (100 Units mg\(^{-1}\)) / streptomycin (100 μg ml\(^{-1}\)) (Gibco*). Cells were cultured in an incubator with 5% CO\(_2\) at 37 °C. For cytotoxicity analyses, fibroblasts were seeded at a density of 30,000 cells cm\(^{-2}\). A LIVE/DEAD assay (Molecular Probes, Invitrogen, Thermo Fisher Scientific, The Netherlands) was performed at 72 hours, according to the instructions of the manufacturer. A suspension of porous microcylinders in culture medium was prepared (0.5 mg mL\(^{-1}\)) and added to the cells 24 hours after seeding. Prior to LIVE/DEAD assay, samples containing the microcylinders were placed in an incubator overnight, in order for the microcylinders to settle in the bottom of the wells. Cells cultured in the same conditions without microcylinders served as a positive control. For the negative control group, cells were maintained at the same condition as the positive control except that the culture medium was changed to 70% v/v methanol in \text{dd} water 30 minutes prior to performing the LIVE/DEAD assay. Additionally, cells were cultured with PLLA porous microcylinders (0.1, 0.5, and 1.0 mg mL\(^{-1}\)) in 24-well culture plates, and cell viability was evaluated by performing an AlamarBlue viability test (Molecular Probes, Invitrogen, Thermo Fisher Scientific, The Netherlands) per the manufacturer’s instructions to obtain quantitative data on cellular activity. Positive and negative controls were prepared as described for LIVE/DEAD assay.

2.5. Statistical Analysis.

Prism 6.01 software (GraphPad Software, Inc, USA) was used to perform all statistical calculations. Fiber diameter and microcylinders dimensions (length
and diameter) were estimated from the corresponding SEM images using ImageJ software (National Institutes of Health, USA). Twenty different measurements were taken from 5 different regions of each electrospun membrane or each microcylinders group (n = 100 for the fiber diameter and n = 100 for the microcylinders' length and diameter). Data were reported as mean ± standard deviation (SD). Statistical analysis was carried out by using two-way analysis of variance (ANOVA) combined with a Sidak’s multiple comparison post-hoc test. The time of aminolysis and the morphology of fibers or microcylinders were considered as independent variables. Crystallite size data obtained by XRD from three samples (n = 3) and TNBS amines quantification data in triplicate (n = 3) were analyzed by a two-way ANOVA test combined with a Sidak’s multiple comparison post-hoc test, using the same variables. AlamarBlue data were assayed in triplicate (n ≈ 3) and analyzed using a one-way ANOVA test combined with a Tukey’s post-hoc comparison test. Differences were considered significant at p < 0.05.

3. Results and discussion

The electrospinning process resulted in the fabrication of two distinct types of fibers (Figure 1a and 1b). Morphologically, the use of HFIP (1,1,1,3,3,3-hexafluoro-2-propanol) as a solvent resulted in fibers with a smooth surface. In contrast, PLLA fibers with a porous surface were obtained using a mixture of DCM and TCM as a solvent. The differences between these fibers have been described previously[27, 37] and are related to the volatility of the solvent used; HFIP is a less volatile solvent (vapor pressure of 16 kPa at 20 °C), resulting in uniform, dense, homogeneous fibers. DCM is a highly volatile compound (vapor pressure of 47.3 kPa at 20 °C) and evaporates quickly, leading to the presence of polymer-rich and polymer-poor phases during electrospinning and resulting in the formation of surface porosity.[28, 29] The addition of TCM, which is less volatile than DCM (vapor pressure of 21.2 kPa at 20 °C), helped to avoid clogging of the nozzle during electrospinning due to drying of the polymeric solution. The fibers prepared with HFIP and the DCM/TCM mixture will be referred to as “smooth” and “porous”, respectively. Figure 1c shows that smooth fibers have a smaller diameter compared to the porous fibers (1.3 ± 0.20 versus 3.7 ± 1.14 μm, respectively). As DCM is a solvent with lower electrical conductivity than HFIP, it was necessary to apply a higher voltage in order to achieve a stable mode of electrospinning. It is likely that this higher voltage increased the size of the electrospinning jet and hence the fiber diameter.[38] Figure 1d and 1e show the morphology of the smooth and porous microcylinders obtained using a 3 hours aminolysis procedure. Significant morphological changes to the fibers’ surface were not observed during the aminolysis procedure; smooth fibers formed smooth microcylinders, and porous fibers formed porous microcylinders. However, the microcylinders’ length showed differences; porous microcylinders
were longer than the smooth microcylinders (14.6 ± 5.77 vs 6.6 ± 3.24 μm after 3 hours of aminolysis and 6.6 ± 3.57 vs 1.1 ± 0.19 μm after 5 hours of aminolysis, respectively), and aminolysis time had an inverse effect on length (Figure 1f). The differences in length between the two types of microcylinders are likely related to the different distribution of amorphous and crystalline regions in the developed fibers. In contrast, aminolysis time did not affect dramatically the diameter of the microcylinders, although a slight decrease of the diameter with the time of aminolysis could be observed (Figure 1c).

After 3 and 5 hours of aminolysis, the smooth microcylinders had average specific surface areas of 9.89 and 11.81 m² g⁻¹, respectively, while the porous microcylinders had average specific surface areas of 22.53 and 20.75 m² g⁻¹, respectively. As expected, the creation of a porous surface resulted in an increase in the specific surface area of the microcylinders.

DSC was used to study the thermal properties of the fibers and microcylinders. The curves obtained for the smooth and porous fibers and microcylinders were similar (Figure 2a and 2b, respectively). For both morphologies, the thermograms of untreated fibers presented a glass transition temperature (T_g) of PLLA at around 60 °C, consistent with results previously reported.[39] Also, the thermograms presented an exothermic peak at around 80 °C that can be attributed to a cold crystallization process, which accounts for the crystallinity developed during heating. Furthermore, the melting peak (T_m) at ~181 °C was preceded by a small exothermic signal, probably representing a second cold crystallization event.[40] The microcylinders showed the presence of a very small shift corresponding to the T_g and a second endothermic band related to the melting point of the polymer.
The degree of crystallinity ($\chi$) for both smooth and porous fibers and microcylinders was calculated based on the DSC results (Table 1). For electrospun fibers, the crystallinity was lower than 40%, which was lower than the crystallinity of the original polymer ($\chi_0 = 59\%$). This significant reduction in crystallinity is primarily due to the quick solidification of the polymer during jet formation in the electrospinning process, hindering the development of an ordered crystalline structure in the resulting fibers.\cite{41} However, when the microcylinders were formed, the crystallinity increased to approximately 60%, which can be attributed to aminolysis-induced crystallization.\cite{11} These results explain the absence of a peak related to the cold crystallization process at 80 °C in the DSC scans of the microcylinders. During aminolysis, the reactive amines group donor attacks the amorphous regions of the fibers, leading to the formation of microsized fragments that possess a high degree of crystallinity and do not crystallize further upon heating during DSC.

Both the melting temperature (as measured by DSC; Table 1) and decomposition temperature (as measured by TGA; Figure S1) decreased with increasing times of aminolysis. This may be caused by the polymer molecular weight decrease during the aminolysis procedure.\cite{11} As the aminolysis time increased, small fissures started to appear in the samples, as seen in Figure S2, although the main morphological features (a dense or porous surface) were maintained.

Figure 2. DSC curves for smooth fibers and microcylinders (a) and porous fibers and microcylinders (b). Well-defined $T_g$ and $T_m$ peaks are observed for the untreated fibers. The microcylinders do not exhibit the same transitions, indicating a fundamental shift in crystallinity during aminolysis.
A top-down approach for the preparation of highly porous PLLA microcylinders

To develop a more thorough understanding of the effects of aminolysis on the crystalline properties of the microcylinders, XRD analyses were performed (Figure 3a and 3b). Independent of surface morphology, untreated fibers did not exhibit any peaks throughout the 2θ range of 10–30°, showing only a broad bump of low intensity. This demonstrates the amorphous nature of the fibers. For the smooth and porous fibers that underwent aminolysis for 3 or 5 hours, distinctive peaks at 2θ = 16.5 and 18.9° were observed. These peaks are characteristic of the α-form of PLLA crystals. The size of the crystallites was calculated according to the Scherrer equation. The crystallites showed similar sizes for both types of microcylinders, with a size ranging from 12 to 22 nm for porous microcylinders and from 12 to 21 nm for smooth microcylinders (Table 1 and Figure 3c).

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<th>Specific surface area (m² g⁻¹)</th>
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<th>Degree of Crystallinity (%)</th>
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Table 1. Structural Properties of the Electrospun PLLA Fibers and the Microcylinders.
These results demonstrate that although the length of the microcylinders was dependent on aminolysis time, this process did not affect the crystallite units. Because there was no significant difference with respect to the size of the crystallites between 3 and 5 hours of aminolysis, it can be concluded that after 3 hours of aminolysis the PLLA crystallites seem not to be substantially affected by the chemical scission process and that the differences in length observed at 3 and 5 hours of aminolysis were predominantly caused by the degradation of the amorphous regions of the fibers.

Figure 3. XRD spectra for smooth (a) and porous (b) fibers and microcylinders. (c) Crystallite dimensions for smooth and porous microcylinders at 3 and 5 hours of aminolysis; no statistical differences were observed across groups.

To further elucidate the mechanism of aminolysis, we studied the contributions of IPA and EtDA, the two components used to prepare the reactive solution, to the microcylinders’ formation. Electrospun PLLA fibers were immersed for 1 hour in pure IPA, a 5% v/v solution of EtDA in \( \text{dd}_\text{H}_2\text{O} \), or a 5% v/v solution of EtDA in IPA (standard aminolysis solution). XRD spectra of the fibers after these treatments indicated that immersion in pure IPA or in a 5% v/v solution of EtDA in IPA substantially increased the crystallinity of the resulting microcylinders, presenting characteristic peaks of PLLA crystals (Figure 4). This observation corroborates previous work, demonstrating a tendency of PLLA to crystallize when it is immersed in a wide range of organic solvents.\[43\] However, EtDA alone appeared not to contribute to polymer crystallization. Interestingly, the combined use of EtDA and IPA showed a synergistic effect on polymer crystallization, as evidenced by the high intensity of the PLLA peak in the XRD spectra.
A top-down approach for the preparation of highly porous PLLA microcylinders

(Figure 4). Further, when both the smooth and the porous fibers were immersed in IPA or 5% v/v EtDA in ddwater for 1 hour, no fiber degradation was observed in comparison with the cleavage of the fibers when immersed in 5% v/v EtDA in IPA (Figure S3). Therefore, the aminolysis-based preparation of microcylinders can be regarded as an interplay between solvent induced crystallization and chemical cleavage of the amorphous regions of the fibers.

The DSC and XRD results suggest that during the microcylinders formation initially a solvent-induced crystallization occurs, which is followed by the EtDA preferential “cutting” of the amorphous regions within the polymer, resulting in an increase of the crystallinity. This mechanism is correlated with the theory proposed by Kim and Park.\textsuperscript{[11]}

\textbf{Figure 4.} XRD spectra for porous (a) and smooth (b) fibers subject to aminolysis for 1 hour.

Besides the fiber “cutting” effect, aminolysis is known to introduce amine groups at the surface of treated polymers.\textsuperscript{[15]} These free amine groups render the microcylinders suitable for further functionalization with therapeutic drugs or biomolecules. To monitor the amine groups at the microcylinders’ surface, ATR-IR analysis, a fluorescamine assay, and a TNBS colorimetric assay were performed.

As expected, infrared analysis of the untreated fibers did not show any bands related to amine groups (Figure S4). However, the aminolysis procedure induced two new bands for microcylinders that can be assigned to an amide II N–H bond (~1540 cm\textsuperscript{−1})\textsuperscript{[13]} and an amide I C=O bond (~1690 cm\textsuperscript{−1}).\textsuperscript{[13, 14]} Figure 5 shows the results obtained with the fluorescamine assay. This method is based on the specific attachment of the fluorescamine dye to primary amine groups. Microcylinders prepared via aminolysis showed an intense blue fluorescent signal at their surface (Figures 5b and 5d). Although the presence of amines can be assessed over the entire surface of the microcylinders, it is interesting to note that the edges present a higher fluorescence intensity (red arrows in Figures S5d and S5h). The untreated fibers did not show any fluorescent signal (Figures S5b and S5f). Such differences can be assessed more clearly when a fluorescence intensity map is created, showing that the extremities present a higher fluorescence intensity when compared with other regions of the microcylinders (Figure 5c and
5f). The results obtained with the fluorescamine assay support our hypothesis of a transversal sectioning of the electrospun fibers and degradation of their more amorphous areas, presenting the extreme regions a more intense fluorescence.

To quantify the amount of amine groups present at the surface of the microcylinders, a TNBS colorimetric assay was performed. This test is based on the attachment of free amine groups to the TNBS reagent, resulting in the formation of a yellow chemical complex. A direct correlation between the aminolysis time and the number of the amine groups grafted to both smooth and porous microcylinders could be observed (Figure 6). Porous microcylinders showed a larger average amount of amine groups compared to the smooth microcylinders that were subjected to similar aminolysis times, although there were no statistical differences between the groups.
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On the basis of these results, porous microcylinders present themselves as ideal candidates for local drug delivery purposes. The results obtained by DSC and XRD demonstrate that such structures are thermally stable and crystalline, increasing their chemical and physical stability. Furthermore, the increased specific surface area with the successful grafting of amine groups makes these microcylinders suitable for future use as therapeutic vehicles through the immobilization of, for example, anticancer agents for tumor therapy.\textsuperscript{[44, 45]} Alternatively, immobilization of growth factors\textsuperscript{[46, 47]} or biomolecules that mediate cellular adhesion\textsuperscript{[48]} are options for the application of these microcylinders in tissue engineering and regenerative medicine.\textsuperscript{[30]}

Biological evaluation of the microcylinders was performed by examining cell viability and cellular metabolic activity upon culture of human fibroblasts with the porous microcylinders (Figure 7). The LIVE/DEAD assay showed that human fibroblasts cultured with 0.5 mg mL\textsuperscript{−1} porous microcylinders presented viable green fluorescent cells (Figure 7c). These data indicate that during LIVE/DEAD assay only calcein was incorporated in the fibroblasts cultured with the porous microcylinders, which allows one to conclude that the microcylinders developed did not affect the integrity of the fibroblasts cellular membrane and hence no cytotoxic effect was observed. Figures 7d and 7e present the results obtained for the AlamarBlue viability test after 24 and 72 hours of incubation with the microcylinders, respectively. The fibroblasts viability was not affected by the presence of 0.1 mg mL\textsuperscript{−1} microcylinders. However, when fibroblasts are cultured with 1.0 mg mL\textsuperscript{−1} of microcylinders viability is significantly affected after both 24 and 72 hours of culture. Such results are related to the large number of microcylinders present at this concentration. Such a large number affect cell activity not only due to a possible high concentration of PLLA by-products in the medium but mostly due to the physical occupation of the well by the microcylinders, affecting cellular proliferation over time (Figure S6).
4. Conclusion

Electrospinning combined with an aminolysis chemical scission process led to the production of microcylinders of controllable dimensions, increased specific surface area, and with or without a surface porous morphology. The dimensions, crystallinity, specific surface area, and thermal stability of the structures were dependent on the experimental conditions used during fabrication, for example, the solvent chosen in electrospinning and the duration of aminolysis. In addition, amine groups were successfully introduced on the microcylinders’ surface, which can be used as reactive sites for the chemical immobilization of relevant compounds, such as therapeutic agents. The microcylinders showed biocompatible properties related to cell viability in vitro. This feature together with their increased specific surface area makes these structures appealing for future biomedical applications as local drug-delivery systems.
A top-down approach for the preparation of highly porous PLLA microcylinders

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A top-down approach for the preparation of highly porous PLLA microcylinders


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A top-down approach for the preparation of highly porous PLLA microcylinders

Supplementary Information

Figure S1. a) TGA curve for smooth fibers and microcylinders after 3 hours and 5 hours of aminolysis b) TGA curves for porous fibers and microcylinders after 3 hours and 5 hours of aminolysis.

Figure S2. a) Smooth micro-cylinders after 5 hours of aminolysis b) Porous micro-cylinders after 5 hours of aminolysis. Red arrows highlight the presence of small fissures in the structures.

Figure S3. Smooth electrospun fibers immersed for 1 hour in a) isopropanol (IPA) b) 5% ethylenediamine (EtDA) in water c) 5% ethylenediamine (EtDA) in isopropanol. Porous electrospun fibers immersed for 1 hour in d) isopropanol (IPA) e) 5% ethylenediamine (EtDA) in water f) 5% ethylenediamine (EtDA) in isopropanol. Cleavage of the fibers can be observed when immersed in 5% EtDA in IPA.
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Figure S4. ATR-IR spectra for porous untreated fibers and microcylinders after 3 hours and 5 hours of aminolysis.

Figure S5. Smooth electrospun fibers (a-bright field; b-fluorescence) and microcylinders after 3 hours of aminolysis (c-bright field; d-fluorescence). Porous electrospun fibers (e-bright-field; f-fluorescence) and microcylinders after 3 hours of aminolysis (g-bright-field; h-fluorescence). Red arrows indicate the areas with higher fluorescence.

Figure S6. Images obtained by optical microscope for fibroblasts cultured with a) 0.1 mg ml\(^{-1}\) of porous microcylinders b) 0.5 mg ml\(^{-1}\) of porous microcylinders and c) 1.0 mg ml\(^{-1}\) of porous microcylinders. Magnification of 10x.
Incorporation of PLLA microfillers for mechanical reinforcement of calcium-phosphate cement

Incorporation of PLLA microfillers for mechanical reinforcement of calcium-phosphate cement
1. Introduction

A calcium phosphate cement (CPC) is prepared from at least one type of calcium phosphate salt and a liquid phase. Mixing of these two phases triggers a precipitation reaction and results in the entanglement of precipitated calcium phosphate crystals to achieve the hardening of the cement, also known as setting. CPCs are prepared from various calcium phosphate precursors, e.g. α-tricalcium phosphate (α-TCP), β-tricalcium phosphate (β-TCP), tetracalcium phosphate (TTCP) or combinations thereof. After completion of the precipitation reaction, either an apatite-rich CPC or a brushite-based CPC are formed, depending on the pH of the setting reaction. CPCs are highly attractive for bone regenerative applications due to their biocompatibility, similarity to the mineral phase of bone, possibility to be applied in an injectable form, and osteoconductivity. During the past few years, CPCs have shown great potential for vertebroplasty procedures as well as treatment of osteoporosis-related fractures, maxillofacial defects and deformities. Despite these advantageous properties, CPCs possess a major drawback: their mechanical properties are inferior to native bone. One of the main disadvantages of CPCs is their brittleness. CPCs can be deformed plastically to a very limited extent due to their low intrinsic resistance to crack propagation, which ultimately can lead to catastrophic failure. This drawback inhibits the application of CPCs at load-bearing anatomical sites. Several strategies have been explored toward the enhancement of the mechanical properties of CPCs, for example by incorporation of titania, silica, carbon nanotubes and polymeric fibers. The addition of stiff and strong fibers for mechanical reinforcement of CPCs has become increasingly popular. In this way, loads can be transferred from the brittle matrix to the fibers, thereby improving the toughness and ductility of the resulting composite by mechanisms such as crack deflection, fiber bridging and frictional sliding. Synthetic polymeric fibers are widely used for reinforcement purposes due to their low cost, abundant availability and suitability for post-production modification. However, it is a challenge to incorporate long fibers into CPCs in a homogeneous manner. From a mixing perspective, short fibers are preferable. These fillers can be produced through several physical methods such as lithography, stretching or sectioning as well as chemical synthesis routes. Recently, we reported the preparation of surface-porous micrometric fillers from poly(L-lactic acid) (PLLA) electrospun fibers. This procedure is based on the incubation of polymeric fibers with a reactive amine donor that initiates a chemical scission process, resulting in transverse segmentation of the fibers. Apart from the fiber cutting effect, aminolysis functionalizes the surface of the polymer with amine groups, hence increasing the hydrophilicity and enabling subsequent functionalization of the polymer. Based on the aforementioned, we hypothesize that the introduction of
polymeric fillers can improve the toughness of CPCs provided that the fibers are mixed homogeneously throughout the cement matrix. Furthermore, we hypothesize that the presence of surface porosity on the polymeric microfillers improves the mechanical properties of the resulting reinforced cement by means of micromechanical interlock. To test these hypotheses, we prepared PLLA micron-sized fillers (microcylinders) with either a smooth or porous surface. We incorporated the PLLA microcylinders into CPCs containing (or not) poly(lactic-co-glycolic acid) (PLGA) microparticles to render the CPC degradable. Subsequently, the microcylinders distribution, as well as the morphology, cohesiveness, setting times and mechanical properties (in terms of flexural strength, flexural modulus and work-of-fracture) of the microcylinders reinforced cements were evaluated. PLLA microcylinders morphology and distribution together with CPCs morphology, cohesion, and initial and final setting times were examined. For mechanical evaluation, CPCs were subjected to a three-point flexural bending test to determine their flexural strength, flexural modulus, and work-of-fracture.

2. Materials and methods

2.1. Materials
Medical-grade PLLA (Purasorb PL65®) was obtained from Corbion-Purac (The Netherlands). 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) was purchased from Fluorochem (United Kingdom). Dichloromethane (DCM), trichloromethane (TCM), isopropanol (IPA), acetone and disodium hydrogen phosphate dihydrate (Na$_2$HPO$_4$·2H$_2$O) were obtained from Merck (Germany). Ethylenediamine (EtDA) was obtained from VWR chemicals (France). Fluorescamine was obtained from Sigma-Aldrich (USA). CPC powder (α-tricalcium phosphate) and milled PLGA particles (Purasorb PDLG 5002A; average diameter of 48.65 ± 22.07 μm) were provided by CAM Bioceramics (The Netherlands). For convenience, the samples made solely from CPC were named as CPC1 while the samples formed from CPC and milled PLGA particles were named as CPC2.

2.2. Electrospinning of PLLA
Continuous electrospun PLLA fibers were prepared according to a previously described method. PLLA was dissolved at a concentration of 3% w/v in HFIP or in a 4:1 v/v mixture of DCM : TCM. A commercially available electrospinning apparatus (Advanced Surface Technology BV, The Netherlands) was used to create the electrospun fibers. Electrospinning of PLLA in HFIP was performed using the following parameters: feeding rate = 2.0 mL h$^{-1}$; distance between the needle and the collector = 35 cm; voltage = 19.25 kV. Electrospinning of PLLA in the DCM : TCM mixture was performed using the following parameters: feeding rate = 2.0 mL h$^{-1}$; distance between the needle and the collector = 35 cm; voltage = 35.00 kV. Fibers were collected on a stationary flat collector covered
with aluminum foil. Electrospun membranes were left to dry overnight in a fume hood to evaporate the solvent.

2.3. Production of PLLA microcylinders
PLLAs were fabricated as described previously. First, the two different PLLA electrospun fibers were incubated in a 5% v/v EtDA solution in IPA at 37 °C for 3 hours. Subsequently, 4 cycles of centrifugation/washing with dd water were performed to remove the EtDA solution in excess. Finally, samples were resuspended in dd water, subjected to ultrasonication at 100% amplitude for 40 seconds (UP50H; Hielscher - Ultrasound Technology; Germany) to disperse the PLLA micro-fillers, freeze-dried, and stored at -20 °C for further use.

2.4. Morphological evaluation of PLLA fibers and microcylinders
Scanning electron microscopy (SIGMA 300; Zeiss, The Netherlands) was performed at an acceleration voltage of 3.0 kV to study the morphology of PLLA fibers and microcylinders. The fiber mats and discrete microcylinders powders were fixed on aluminum stubs with carbon tape and coated with chromium (thickness≈10 nm) for subsequent observation.

2.5. Preparation of calcium phosphate cements
The cement samples were prepared by mixing the calcium phosphate base powder phase with an aqueous phase. The different compositions are described in Table 1. For the groups including PLLA microcylinders, CPC1 or CPC2 powders were first mixed manually with 5% wt PLLA microcylinders. For the groups without PLLA microcylinders, only CPC1 or CPC2 were used as the powder phase. A 4% w/v Na₂HPO₄·2H₂O aqueous solution was then added to initiate the setting reaction. The liquid to powder (L/P) ratio for CPC1 and CPC2 based samples was 0.50 and 0.42, respectively.

<table>
<thead>
<tr>
<th>Sample</th>
<th>α-TCP/ wt%</th>
<th>PLGA/ wt%</th>
<th>Smooth microcylinders/ wt%</th>
<th>Porous microcylinders/ wt%</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPC1</td>
<td>100.0</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>CPC1ₜ</td>
<td>95.0</td>
<td>-----</td>
<td>5.0</td>
<td>-----</td>
</tr>
<tr>
<td>CPC1ₚ</td>
<td>95.0</td>
<td>-----</td>
<td>-----</td>
<td>5.0</td>
</tr>
</tbody>
</table>
2.6. PLLA microcylinders distribution

The spatial distribution of PLLA microcylinders throughout the CPCs was monitored using the fluorescent marker fluorescamine, which reacts with primary amines on the surface of aminolyzed fibers to form highly fluorescent products. To this end, 0.5 g PLLA microcylinders were incubated with 2 ml fluorescamine solution (50% v/v acetone/dd water) for 1 hour at room temperature, conferring them a blue color when excited at λ=401 nm. The labeled PLLA microcylinders were freeze-dried and used for cement preparation by following the method described in the previous section. Samples were cross-sectioned with a scalpel for observation under a fluorescence microscope (Axioimager Z1; Zeiss, The Netherlands). Backscatter SEM was performed at an acceleration voltage of 10.0 kV. Cements were broken, fixed to aluminum stubs with carbon tape, and examined as previously described.\textsuperscript{[31]}

2.7. X-ray diffraction analysis

X-ray diffraction (XRD; pw1830; Philips; The Netherlands) was performed to determine the crystallographic profile of the samples. Cements were incubated in SBF for 3 days, freeze-dried and grinded. Subsequently, as a powder, samples were placed in a copper holder and scanned. XRD spectra were registered at 40 kV, 30 mA (Cu-Kα radiation with a wavelength of 1.54 Å), 2θ between 10° and 60° and a step size of 0.005°.

2.8. Determination of porosity

Porosity was determined by a burn-out assay. First, cements were prepared, incubated in SBF for 3 days, freeze-dried and weighted. Subsequently a heating protocol was used to eliminate the organic components present in the cements. Briefly, samples were heated at a rate of 1.67 °C min\textsuperscript{−1} until the temperature reached 650 °C. Afterward, samples were maintained at this temperature for 3 hours, and the temperature was decreased at a rate of 1.67 °C min\textsuperscript{−1} until reaching room temperature. Total porosity (1) and macroporosity (2) percentages were calculated according to the following formulas:\textsuperscript{[32]}

\[
e_{\text{tot}} = \left(1 - \frac{m_{\text{macro}}}{V_{\text{HAp}}} \right) \times 100 \quad (1)
\]

\[
e_{\text{macro}} = \left(1 - \frac{m_{\text{macro}}}{m_{\text{micro}}} \right) \times 100 \quad (2)
\]
where є<sub>tot</sub> is the total porosity (%), є<sub>macro</sub> the macroporosity (%), m<sub>macro</sub> is the mass of the samples after the thermal treatment and m<sub>micro</sub> the mass of the samples after setting and freeze-drying.

2.9. Cohesion evaluation
The cohesion of CPCs was evaluated by visual examination. Immediately after mixing, the cement paste was immersed in PBS at 37 °C and the amount of debris present in the fluid examined. A score from 0 to 5 was applied, according to the visual examination. A value of 0 corresponds to a totally cohesive cement and a value of 5 to a large amount of debris in the medium (Table 2). Experiments were performed in triplicate (n=3).

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Large amount of debris in the medium</td>
</tr>
<tr>
<td>4</td>
<td>Moderate amount of debris in the medium</td>
</tr>
<tr>
<td>3</td>
<td>Small amount of debris in the mediums</td>
</tr>
<tr>
<td>2</td>
<td>Moderate amount of CPCs particles in the medium</td>
</tr>
<tr>
<td>1</td>
<td>Small amount of CPCs particles in the medium</td>
</tr>
<tr>
<td>0</td>
<td>Absence of debris/particles</td>
</tr>
</tbody>
</table>

Table 2. Grading table for cohesion evaluation

2.10. Setting time
The initial and final setting times of the CPCs were assessed using a Gillmore apparatus. A bronze block containing six holes (6 mm in diameter, 12 mm in height) was used as a mold. The mold was placed in a water bath at 37 °C to mimic body temperature and afterward, the holes were filled with the different cement pastes. The initial/final setting times were defined as the times at which a light or heavy Gillmore needle failed to make a perceptible circular indentation on the surface of the cement (n=3).

2.11. Mechanical evaluation
After mixing, the cements were introduced into Teflon molds of 4×4×20 mm<sup>3</sup> and left to set overnight. The obtained samples were immersed in SBF for 3 days at 37 °C, to achieve a complete conversion of α-TCP to hydroxyapatite. Afterward, the samples were subjected to a three-point flexural bending test
using a universal testing machine (858 mini bionix II; MTS, USA) with a load
cell of 1 kN and a crosshead speed of 0.2 mm min$^{-1}$ to evaluate the flexural
strength, flexural modulus, and the work-of-fracture. The mechanical properties
were determined according to the following equations.$^{[14]}$

**Flexural Strength:**

\[
Sc = \frac{3P_{\text{max}}L}{2bh^2}
\]

where \(P_{\text{max}}\) is the maximum load on the load-displacement curve, \(L\) is the flexure
span, \(b\) is the specimen width and \(h\) the thickness.

**Flexural Modulus:**

\[
E = \frac{mL^3}{4bh^3}
\]

where \(m\) is the slope of the tangent to the linear portion of the load-displace-
ment curve.

**Work of fracture:**

\[
WOF = \frac{A}{bh}
\]

where \(A\) is the area under the load-displacement curve.

### 2.12. Morphological evaluation of CPCs

The set CPCs were cross-sectioned, fixed on aluminum stubs with carbon tape
and examined by scanning electron microscopy as previously described.

### 2.13. Statistical analysis

Prism 6.01 software (GraphPad Software, Inc., USA) was used to perform
all statistical calculations. PLLA microcylinders dimensions (length and
diameter) were estimated from the corresponding SEM images using ImageJ
software (National Institutes of Health, USA). Data were reported as mean ±
standard deviation (SD) with \(n=100\). Statistical analysis was effectuated by a
Student’s \(t\)-test. Porosity calculations were performed using 3 samples of each
composition (\(n=3\)) and data were reported as mean ± standard deviation (SD).
Significance was analyzed using one-way ANOVA with a *post-hoc* Tukey’s
multiple comparison test. Differences were considered significant at \(p < 0.05\).

For mechanical evaluation, ten specimens of each composition (\(n=10\)) were
used for each of the mechanical tests and data were reported as mean ± standard
deviation (SD). Results were statistically analyzed using one-way ANOVA
with a *post-hoc* Tukey’s multiple comparison test. Differences were considered
significant at \(p < 0.05\).
3. Results

3.1. PLLA fibers and micro-fillers visualization

Figure 1 shows the morphology of the smooth and porous fibers after electrospinning as well as the smooth and porous microstructures obtained after 3 hours of aminolysis. Morphologically, the use of HFIP as a solvent during electrospinning resulted in fibers with a smooth surface (Figure 1a). In contrast, porous PLLA fibers were obtained using a mixture of DCM and TCM (Figure 1b). Aminolysis resulted in micrometric cylindrical structures with two distinct morphologies, based on the surface morphology of the fibers: smooth microcylinders were created when smooth fibers were subject to aminolysis (Figure 1c), while aminolysis of porous fibers resulted in surface-porous microcylinders (Figure 1d).

Figure 2 shows that the size of PLLA microcylinders was dependent on the electrospinning conditions resulting into a smaller length and diameter for smooth microcylinders as compared to the surface-porous microcylinders (average length of 6.6 ± 3.24 μm and 14.6 ± 5.77 μm, respectively, and average diameter of 1.3 ± 0.20 μm versus 3.7 ± 1.14 μm respectively). The aspect ratio was 5:1 for PLLA smooth microcylinders and 4:1 for PLLA surface-porous microcylinders.
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3.3. PLLA microcylinders distribution in CPCs

PLLA microcylinders distribution in the α-TCP matrix was evaluated by first labeling these with fluorescamine and then mixing with the remaining components of the CPCs. Figure 4 shows the results obtained when the different CPCs were observed by fluorescence microscopy. CPCs without microcylinders (Figure 4a and 4d) did not present any fluorescence. In contrast, CPCs containing...
either smooth or porous microcylinders showed overall blue areas which were homogenously distributed throughout the sample. However, due to resolution limitations, it was not possible to observe the individual PLLA microcylinders.

Backscatter SEM was performed to observe the distribution of the PLLA microcylinders in the cement. Figure 5 presents the SEM images for CPC1, CPC1_s, and CPC1_p compositions. A homogenous distribution of the PLLA smooth microcylinders can be observed for all samples, represented as small dark points in Figure 5b. Similarly, porous PLLA microcylinders showed a homogeneous distribution. Due to their higher dimensions, these microcylinders were more easily identified (Figure 5c).

Fig. 4. Fluorescamine assay a) CPC1 b) CPC1_s c) CPC1_p d) CPC2 e) CPC2_s f) CPC2_p 10× magnification. Blue color indicates the presence of fluorescamine labeled PLLA micro-fillers.
Figure 5. Backscatter SEM a) CPC1 b) CPC1, c) CPC1, 1,000x magnification. Red arrows indicate the presence of smooth PLLA microcylinders. Yellow arrows indicate the presence of porous PLLA microcylinders.
3.4. X-ray diffraction analysis
Figure 6 presents the XRD spectra for the different cements. All compositions show main peaks at 2θ of 26.1°, 31.7°, 32.2°, 39.8°, 46.7°, 49.6° and 53.3°, typical for hydroxyapatite.\[34\]

![Figure 6. X-ray diffraction spectra for the prepared cements. Red lines delimited the regions where peaks are assigned to hydroxyapatite.](image)

3.5. Cohesion evaluation
When in contact with PBS at 37 °C, released particles could be observed for all the CPCs, as shown in Figure 7. Qualitative analysis of the different compositions did not reveal substantial differences (Figure 7g).

![Figure 7. Immersion of the calcium-phosphate cements in PBS at 37 °C. a) CPC1 b) CPC1c) CPC1d) CPC2 e) CPC2f) CPC2g) Qualitative evaluation of the cement's cohesion. Grading scale: 0=absence of debris, 1=Small amount of CPCs particles in the medium, 2=moderate amount of CPCs particles in the medium, 3=small amount of debris in the medium, 4=moderate amount of debris in the medium, 5=large amount of debris in the medium. (n=3).](image)
3.6. Setting time

Figure 8 presents the initial and final setting times as determined with a Gilmore apparatus. In general, CPC2 groups needed longer time to set (~4 minutes vs. 2 minutes of initial setting and ~18 minutes vs. 9 minutes of final setting for CPC2 and CPC1, respectively). On the contrary, the addition of PLLA microcylinders had a limited effect on the setting time. PLLA microcylinders slightly shortened the setting times except for CPC1_P and CPC2_S formulations which revealed a prolonged final setting time compared with their counterparts without PLLA fillers.

![Fig. 8. a) Initial setting times b) final setting times. One-way ANOVA. *p < 0.05, **p < 0.01.](image)

3.7. Mechanical evaluation

Load-displacement curves (Figure S1) of cements containing smooth or porous microcylinders (CPC1_S and CPC1_P or CPC2_S and CPC2_P) presented similar curves to the CPC1 or CPC2 compositions, respectively. The incorporation of PLGA microparticles resulted in a significant increase in the displacement values for compositions CPC2, CPC2_S, and CPC2_P.

Figure 9 shows the mechanical properties of the cements. Figure 9a represents the flexural strength for the different CPCs. No statistically significant differences were observed with average values ranging between 4 and 6 MPa for all groups. For both flexural modulus and work-of-fracture, the addition of either smooth or porous PLLA microcylinders did not show any effect. On the contrary, CPC2 groups either with or without PLLA microcylinders always presented significantly lower flexural moduli (1 vs. 3 GPa; Figure 9b) and significantly higher values for the work-on-fracture (25 vs. 12 J m⁻²; Figure 9c).
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Figure 9. Mechanical properties for the cements prepared a) flexural strength, b) flexural modulus and c) work-of-fracture. One-way ANOVA. **p < 0.01, ****p < 0.0001.

3.8. Morphological evaluation of the CPCs
The morphology of the CPCs sections was examined using SEM (Figure 10). The morphology of the calcium phosphate matrix was typical for this type of ceramic materials as characterized by the presence of micrometric calcium-phosphate grains. For compositions with PLLA fillers, smooth or porous PLLA microcylinders (red and yellow arrows) were randomly distributed throughout the matrix (Figure 10b, 10c, 10e and 10f). No strong interfacial proximity could be observed between the calcium phosphate grains and the fillers. Instead, there were several voids and gaps between the two components.

Fig. 10. SEM images for a) CPC1 b) CPC1 c) CPC1 d) CPC2 e) CPC2 f) CPC2, 10,000× magnification. Red arrows indicate the smooth microcylinders, yellow arrows indicate the surface porous microcylinders and blue arrows indicate the PLGA microparticles.

4. Discussion
The purpose of this study was to evaluate whether the addition of PLLA microcylinders could be an efficient strategy for the reinforcement of calcium-phos-
Incorporation of PLLA microfillers for mechanical reinforcement of calcium-phosphate cements (CPCs). To this end, 5% wt PLLA microcylinders with either a smooth or porous surface were incorporated into the CPC and their effects on handling (i.e., cohesion and setting times) and mechanical properties (flexural strength, flexural modulus, and work-of-fracture) were assessed. It was observed that the microcylinders were uniformly distributed in the CPC matrix. The initial setting time was significantly reduced with the addition of the microfillers. The mechanical properties were not significantly affected by the incorporation of PLLA microcylinders.

Prior to mechanical evaluation, the different cements were immersed in SBF at 37°C for 3 days. As observed in Figure 6, this led to a total conversion of the α-TCP to hydroxyapatite. This suggests that cements with the incorporation of the PLLA microcylinders with or without PLGA microparticles would evoke a similar biological response compared to regular apatitic CPCs. As already described previously, immersion of calcium-phosphate cements in phosphate containing buffers leads to a fast conversion of such materials into hydroxyapatite. Besides mimicking more closely the bone mineral constituent, this conversion has shown to enhance the mechanical properties through the formation of needle-like structures and to a decrease in the size of the residual pores. The lack of mechanical reinforcement efficacy for the produced PLLA microcylinders can be attributed to several factors. Calcium-phosphate cements are inherently porous materials, containing mostly micropores. Figure 3 confirms this, with compositions CPC1, CPC1\textsubscript{S}, and CPC1\textsubscript{P} possessing low values of macroporosity and approximately 60% of total porosity. When PLGA microparticles were added, there was a large increase in macroporosity and also an increase in total porosity. A higher percentage of macropores for CPC2, CPC2\textsubscript{S}, and CPC2\textsubscript{P} compositions was expected, since the PLGA particles are micron-sized and will occupy a large volume in the samples. After thermal degradation, this led to the creation of macropores. The addition of PLLA microcylinders increased the values of macroporosity for CPC1, CPC1\textsubscript{S} and CPC1\textsubscript{P} (Figure 3a). However, this increase did not have a deleterious effect on the mechanical properties of the cements. The fact that only 5% of the total weight is due to the presence of PLLA microcylinders can explain these results. Gaps and voids were observed between the microcylinders and calcium phosphate matrix, indicating that the interfacial bonding between these two components was poor. Figure 4 and Figure 5 demonstrate that the PLLA microcylinders were homogeneously dispersed throughout the cement. The micrometric size and hydrophilic nature (attributed to amine groups), when compared with non-treated PLLA materials, might have contributed to this effect. Due to the limit of magnification it was not possible to observe the individual PLLA microcylinders in the cement by the fluorescamine assay, although the intense blue color through the CPC matrix indicates a homogeneous distribution of the PLLA microcylinders. Backscatter SEM enabled a better understanding of the distribution of the PLLA microcylinders in the cements. Figure 5b and Figure 5c show that the microcylinders
are present through all the cement, being well dispersed in the ceramic matrix.
It can be concluded that aminolysis led to an increase of hydrophilicity of the polymer and facilitated the mixing of the microcylinders with the other CPCs components. This treatment however, did not improve the interfacial bonding between the microcylinders and precipitated apatite crystals. The aspect ratio of the fillers might be a justification for the lack of mechanical improvement. When considering fiber reinforcement, mechanical properties are closely related to the dimensions of the fibers used. Several studies have shown that mechanical reinforcement increases with increasing aspect ratio of the fibers, although miscibility has been reported to decrease with increasing fiber length.\[18, 41\] To obtain a load transfer from the matrix to the fibers, the length of the fibers needs to be above the so-called critical length ($l_c$). Possibly the PLLA microcylinders did not enhance the mechanical properties of CPCs because the fiber length was lower than the critical length, thereby not occurring load transfer upon tensile loading.\[42, 43\] An alternative approach to overcome the above-mentioned limitations entailed the incorporation of microfillers with a porous surface. Such porous surface, besides increasing the surface area of the PLLA microcylinders, could also create contact points for micromechanical interlock, thereby increasing the friction at the surface of the microcylinders and consequently improving the mechanical properties of the CPCs.\[44, 45\] This mechanism was obviously not observed for the porous PLLA microcylinders (Figure 9). A third factor contributing to the lack of reinforcing efficacy of PLLA microcylinders could be the weight fraction used. In this study a PLLA microcylinders amount of 5% wt was chosen since this amount did not affect the handling properties negatively. Increasing the amount of PLLA microcylinders used might have caused a more positive effect on the mechanical properties of the CPCs, albeit at the expense of the handling properties. The limitations discussed previously point out that different strategies need to be adopted in the future. A possible approach is the incorporation of residues which promote the mineralization or affinity to calcium of the PLLA microcylinders.\[46, 47\] Cohesion is another important aspect in the development of CPCs since debris will not flush out from cohesive cements when in contact with the body fluids, thereby confining the paste in the site of application.\[5\] The different cements prepared showed a similar cohesion to the pristine CPC1 composition (Figure 7), indicating that the incorporation of PLLA microcylinders did not have a negative effect on cement cohesion. CPCs are self-setting materials.\[30\] When an aqueous solution interacts with the calcium-phosphate powder precipitation and recrystallization processes are initiated, leading to the hardening of the cement. Setting time is an important parameter, affecting the handling and functionality of a cement: if set too rapidly, it will hinder the correct placement and reshape of the cement in the injured site, while in the case of a too slow setting the cement can be flushed out from the implanted site by blood and other body fluids. Figure 8 describes the results
obtained regarding initial and final setting times as measured using the Gillmore needle method. PLLA microcylinders decreased the initial setting time for all compositions. An explanation for this reduction in the initial setting time is the decrease in the liquid/powder (L/P) ratio: water from the cement paste can be absorbed by the PLLA microcylinders, thereby reducing the amount of water available for the cement hardening reaction. Incorporation of PLGA micro-particles delayed the initial and final setting times considerably. This phenomenon was expected since CPC2 formulations contain a substantial amount of non-setting PLGA microparticles (40 %wt). Nevertheless, for all compositions the initial and final setting times were within the acceptable range for clinical usage.

CPC2 cements exhibit a lower stiffness and higher toughness (expressed as work-of-fracture) when compared with CPC1 compositions (Figure 9). CPC2, CPC2S, and CPC2P cements contain 40% wt of PLGA microparticles. The decreased flexural modulus was related to the lower stiffness of PLGA when compared with α-TCP. The increased toughness was attributed to energy dissipation mechanisms associated with the more flexible nature of the PLGA microparticles.

In the current work, homogeneous distribution of microcylinders in calcium-phosphate based cements was achieved, resulting in the maintenance of the handling properties characteristic of these biomaterials. The introduction of the PLLA microcylinders did not lead to improved mechanical properties, and so the clinical use of these materials remains limited to bone defects with only marginal load. However, the authors consider that by applying surface-functionalization strategies, which promote the connection between the calcium phosphate matrix and the PLLA microcylinders, or by using tougher polymers, the presented strategy is a promising approach for the reinforcement of calcium-phosphate cements.

5. Conclusion

PLLA microcylinders were incorporated into apatite-forming cements as a possible strategy to overcome their brittleness. The incorporation of the PLLA microcylinders resulted into a homogeneous dispersion throughout the cement and decreased initial setting times. However, no positive effects were observed on the mechanical properties of the cements, possibly due to their limited aspect ratio and lack of interaction with the ceramic matrix. Future functionalization strategies incorporating moieties able to promote a higher affinity for the calcium-phosphate matrix might lead to a more significant effect on the mechanical properties of CPCs.
Incorporation of PLLA microfillers for mechanical reinforcement of calcium-phosphate cement

References

Chapter 7

Incorporation of PLLA microfillers for mechanical reinforcement of calcium-phosphate cement

Chapter 7

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Incorporation of PLLA microfillers for mechanical reinforcement of calcium-phosphate cement


Supplementary Information

Figure S1. Load-displacement curves of the prepared cements.
Chapter 8

Summary, closing remarks and future perspectives
1. Summary

Electrospinning has been used for a wide range of applications, including the development of filtration systems, production of substrates for chemical catalysis, development of photoluminescent materials, the creation of new textile fabrics, and for biomedical applications. Biomedical applications of electrospinning include the development of antimicrobial membranes, drug delivery systems, new gene therapy approaches and development of scaffolds for tissue healing and regeneration. Biodegradable synthetic polymers, e.g. poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and poly(ε-caprolactone) (PCL), are prime candidate materials for the development of synthetic electrospun membranes. Biodegradable polymers have the advantage of avoiding a second surgery to remove the implant. However, the ideal scaffold, i.e. a “smart material” that is not only biocompatible, but also has an active role in cellular proliferation, cellular differentiation, and tissue regeneration or healing is still not available. Synthetic polymeric electrospun membranes are intrinsically hydrophobic and biologically inert, lacking a molecular structure that stimulates a desired cellular response. These drawbacks lead to the need to develop new methods for the functionalization of synthetic electrospun membranes, creating new biologically active materials.

What is the current state-of-the-art with respect to the functionalization of electrospun membranes for biomedical applications?

Chapter 2 entitled “Biofunctionalization of electrospun fibers for tissue engineering and regenerative medicine” focused on reviewing the most recent advances in the development, functionalization, and application of electrospun membranes for biomedical purposes. Different electrospinning techniques, including single-nozzle electrospinning, coaxial electrospinning, and emulsion electrospinning, were discussed, focusing on their advantages and limitations. The second part of the chapter focused on post-spinning functionalization of electrospun membranes. The main methodologies used for post-spinning functionalization were presented and discussed, being divided into non-covalent and covalent modifications. A wide range of techniques was described, reaching from physical adsorption and layer-by-layer to energy-mediated functionalization and wet-chemistry functionalization techniques, such as surface-graft polymerization. The last section of this chapter was dedicated to the different applications of electrospun membranes for biomedical purposes: development of scaffolds for cellular adhesion, proliferation, and differentiation; delivery of biomolecules; gene therapy; development of drug delivery systems.

Can mechanical and biofunctional properties of PCL-based electrospun membranes be improved by the incorporation of silica nanoparticles?
Chapter 3 described the development of a composite electrospun membrane for Guided Bone Regeneration (GBR). The pivotal step on GBR therapy is the insertion of a membrane for support and barrier functions. In Chapter 3, we studied the effect of silica nanoparticles (Si-NPs) incorporation in electrospun poly(ε-caprolactone) (PCL) membranes to improve the mechanical and osteoconductive properties of the membranes. To this end, Si-NPs were firstly synthesized and then suspended in PCL solutions containing a polar solvent (2,2,2-trifluoroethanol) and water with the addition of an anionic surfactant. Nanocomposite membranes were fabricated from the solutions through an electrospinning technique. Morphology, structure and chemical composition, and tensile properties of the membranes were analyzed. Membrane stability was determined by visual examination of the membranes after immersion in phosphate buffered saline (PBS). The effect of the materials on osteoblastic differentiation was evaluated by in vitro culture of the membranes with MC3T3-E1 osteoblastic cells. The results indicated that Si-NPs were successfully incorporated in the interior of the PCL electrospun fibers during the electrospinning process. Tensile modulus significantly increased for composition S50 and tensile strength significantly increased for compositions S25 and S50. Membranes containing Si-NPs showed to be cytocompatible. The results obtained demonstrate that the Si-NPs were homogeneously incorporated within the electrospun fibers, resulting in an improvement of the tensile properties of the prepared materials.

Can a thermal treatment lead to a more sustained release of the simvastatin incorporated in PLLA electrospun membranes, enabling their use as GBR electrospun membranes?

The incorporation of osteostimulatory compounds can improve the biofunctionality of electrospun membranes, making them active players toward bone regeneration. Simvastatin has shown to promote osteogenic differentiation both in vitro and in vivo. However, in most of these systems, simvastatin was quickly released, not matching the pace of bone regeneration. In Chapter 4 we developed poly(L-lactic acid) (PLLA) membranes containing simvastatin (SV) with an increased drug release rate, compatible with GBR applications. To this end, SV was mixed with PLLA and electrospun. The membranes were subjected to a thermal treatment to increase the crystallinity of PLLA. Morphological, structural and chemical properties of the electrospun membranes were characterized. The effect of the thermal treatment on the release profile of SV was evaluated by near physiological release experiments at 37 °C. The osteostimulatory potential was determined by in vitro culture of the membranes with rat bone marrow stromal cells (rBMSCs). The results confirmed that thermal treatment led to an increased polymer crystallinity and a more sustained release of SV. In vitro assays demonstrated cellular proliferation over time for all the membranes and a significant increase in ALP-activity expression for the cells.
Can a layer-by-layer (LbL) strategy, based on the deposition of PAA and PLL, be used to functionalize PLLA electrospun fibers, facilitating the incorporation of small molecules?

In Chapter 5, post-spinning functionalization of PLLA electrospun membranes by a Layer-by-Layer (LbL) strategy was investigated. Electrospun poly(l-lactic acid) (PLLA) membranes are widely used for biomedical applications. A major drawback for such systems is PLLA lacking biological functionality, leading to the need of developing functionalization strategies that alter the physicochemical properties of PLLA, and hence enable the incorporation of reactive molecular groups or macromolecules on its surface. The aim of this study was to functionalize the fiber surface of electrospun PLLA membranes by LbL, creating a system that potentially finds application as a highly biocompatible substrate with the capacity to function as a reservoir for small molecules (e.g. pharmaceutical compounds). To this end, biocompatible polyelectrolytes, poly(l-lysine) (PLL) and poly(acrylic acid) (PAA), were used to functionalize PLLA electrospun membranes. Morphological and elemental examination of the membranes was performed. LbL functionalization was achieved, with a proportional increase in the amount of polyelectrolytes deposited with the number of immersion steps. Furthermore, two model drugs with a different surface charge at physiological pH (rhodamine B and clindamycin) were incorporated and their loading efficiency and release evaluated. Both drugs were incorporated, albeit with low loading efficiency. Rhodamine B was released after 8 hours of incubation in phosphate buffered saline (PBS) at 37 °C, while clindamycin release occurred up to 48 hours of incubation. Our data demonstrated the feasibility of the approach, but more studies are needed to improve drug loading efficiency and control drug release.

Can functionalized microcylinders be developed by a top-down modification of electrospun fibers?

Electrospinning is a versatile technique, which can be used not only to produce scaffolds and membranes but also for the development of new structures and components for composite biomaterials. In Chapter 6, we described a top-down approach for the development of new functionalized micrometric structures based on electrospun PLLA fibers, which can be used for the development of drug delivery systems or as building blocks for new biomaterials. A wide range of particles has been developed for different applications in drug delivery, tissue engineering, or regenerative medicine. In contrast to traditional spherical particles, non-spherical (e.g. cylindrical) particles possess several structural and morphological advantages that make them attractive for specific applications. In
Chapter 6, electrospun fibers were processed into micron-sized cylinders (i.e. microcylinders) with a high specific surface area and with or without surface porosity. To obtain these microcylinders, PLLA solutions were subjected to electrospinning, followed by an aminolysis-based chemical scission procedure. The morphology, structure, and chemical composition of the microcylinders were then characterized. Specific surface area and surface porosity of the microcylinders were controlled by the volatility of the solvents and their length was dependent on the duration of the aminolysis reaction. During aminolysis, the microcylinders became functionalized with amine groups, enabling potential further modifications by grafting with compounds containing desired chemical groups, e.g. carboxyl, carbonyl or hydroxyl functional groups. Additionally, the microcylinders showed in vitro biocompatible properties related to cell viability. These data demonstrate that PLLA microcylinders with a high specific surface area, optional surface porosity, amine-based functional handles granting additional functionalization, and cytocompatible properties are candidate materials for future biomedical applications.

Does the incorporation of PLLA microcylinders in calcium phosphate cements (CPCs) have a positive effect on their mechanical properties?

Chapter 7 focused on the potential application of the structures described in Chapter 6 as polymeric microfllers to improve the mechanical properties of calcium phosphate cements (CPCs). CPCs are excellent biomaterials for bone regeneration being biocompatible, resorbable, injectable and osteoconductive. However, the CPCs brittle nature limits their application only to no load-bearing applications. The incorporation of long polymeric fibers can improve the mechanical properties of CPCs, but aggregation and heterogeneous distribution of the fibers are major problems. Instead, short polymeric fillers can be easily dispersed in the cement matrix. In Chapter 7, continuous PLLA fibers with a smooth or porous surface morphology were prepared by electrospinning. PLLA microcylinders were developed, by means of an aminolysis process (Chapter 6) and added to α-TCP or α-TCP/PLGA-based cements. Microcylinders distribution, as well as the morphology, cohesiveness, setting times and mechanical properties, were evaluated. PLLA microcylinders were homogeneously dispersed throughout the cement, while the handling properties were not significantly affected. A decrease in the initial setting times was observed when PLLA was added, while the mechanical properties were comparable to those of the α-TPC or α-TCP/PLGA compositions.

2. Closing remarks and future perspectives

In this thesis, we addressed the limited functionality inherent to synthetic polymeric membranes as applied for GBR procedures. Functionalization
techniques were applied to create new materials with improved mechanical properties or which could promote the osteogenic differentiation of pre-osteoblastic cells. Although it was demonstrated that both pre-spinning and post-spinning functionalization strategies enable the production of i) membranes with improved tensile properties, ii) membranes which can be used as drug delivery vehicles iii) micrometric structures functionalized with reactive amine groups, several challenges still need to be addressed, namely the use of non-toxic solvents and the development of more precise approaches when electrospun materials are used as drug delivery systems.

Strong organic solvents are commonly used to dissolve the synthetic polymers prior to electrospinning. This is a major limitation for the definite translation of electrospinning to industry and the scale-up of this technology. The use of organic solvents on an industrial level presents several concerns: i) most organic solvents are highly flammable and volatile, which results in the need to adopt specific safety measures\[1\] ii) organic solvents are harmful to the environment, and special measures are needed for their storage and elimination\[2\] iii) conventional organic solvents are toxic and not suitable for the dissolution of biomolecules, limiting their use for biomedical applications.\[3\] New electrospinning methodologies, denominated as green electrospinning, which focus on more environmentally friendly approaches show promising results. The term green electrospinning is generally used when the dissolution of the polymers and subsequent electrospinning is performed using non-toxic and environmentally friendly solvents, mainly water or aqueous solutions. Water-soluble polymers as poly(ethylene oxide) (PEO), poly(vinyl alcohol) (PVA) or poly(vinylpyrrolidone) (PVP) have been electrospun by this approach.\[4\] However, water-insoluble polymers require the use of strong toxic organic solvents, as chloroform, dichloromethane or dimethylformamide. The development of suspension electrospinning and colloidal electrospinning is a recent green electrospinning strategy, where the production of electrospun fibers is based on the dispersion of water-insoluble polymers or copolymers in an aqueous medium.\[5, 6\] A second alternative green approach is the electrospinning of thermo-responsive polymers, as poly(N-isopropylacrylamide) (PNIPAM). These polymers are water-soluble below their lower critical solution temperature (LCST), but when electrospun the fibers presented a good stability in aqueous environments.\[7\] Room temperature ionic liquids (RTILs) are also gaining increasing attention due to their high conductivity, low vapor pressure, high decomposition temperature and, the capability to chemically modify and recycle the ions.\[8\] Such properties make them especially attractive to be used as environmentally friendly solvents for electrospinning. Cellulose fibers have already been produced using RTILs\[9\] and efforts are being made to expand the use of ionic liquids for the production of non-cellulosic electrospun fibers.\[10, 11\] The sustained delivery of pharmaceutical compounds and biomolecules from electrospun membranes also remains a challenge. The release of pharmaceutical
compounds or biomolecules incorporated in electrospun fibers by blending or sequential electrospinning is mainly a passive phenomenon, being dependent on the diffusion of the drug or degradation/erosion of the polymeric fibers. On-demand drug delivery systems, where a compound release occurs according to endogenous or exogenous stimuli and on a precise location are considered the next step in the development of drug delivery systems. Temperature, pH or enzyme-cleavable domains can be used to control and direct drug release. Stimuli-based responses can be developed based on the customization and modification of the polymeric backbone or by surface functionalization. Considering the polymeric backbone an interesting approach that should be explored in the future is the development of electrospun fibers based on block-copolymers. Temperature sensitive block-polymers as poly(N-isopropyl acrylamide)\cite{12}, poly(N,N-diethylacrylamide)\cite{13}, and PEG/PLGA\cite{14} have been used in the development of temperature-sensitive nanoparticles. PEG-based block copolymers have been used for the development of pH-sensitive delivery systems.\cite{15} Surface functionalization of electrospun fibers is also promising. Matrix metalloproteinase (MMPs) cleavable domains\cite{16}, thermo-responsive pamidronate\cite{17}, or pH-responsive dextran\cite{18} and chitosan\cite{19} have been used on the surface functionalization of nanoparticles and microparticles and are appealing strategies to be explored for the creation of new on-demand electrospun delivery systems.

In summary, in this thesis electrospinning combined with diverse functionalization techniques were used to develop new biomaterials with improved functional properties for GBR applications and bone tissue engineering. Diverse pre- and post-spinning strategies have led to the development of membranes with improved tensile properties which can potentially be used on GBR applications, and the development of new micrometric structures to be used for drug delivery purposes or as building blocks of new biomaterials. Electrospinning has all the potential to be an important technique in the development of the next generation of biomaterials. For that to occur i) low-cost and environmentally friendly electrospinning methodologies should be explored ii) stimuli-based drug delivery systems should be further investigated.
Summary, closing remarks and future perspectives

References

Chapter 9

Samenvatting, slotopmerkingen en toekomstperspectieven
1. Samenvatting

Electrospinning wordt gebruikt voor een breed scala aan toepassingen, waaronder de ontwikkeling van filtratiesystemen, de productie van substraten voor chemische katalyse, de ontwikkeling van fotoluminescerende materialen, de creatie van nieuwe textielstoffen en voor biomedische toepassingen. Biomedische toepassingen van elektrospinning omvatten de ontwikkeling van antimicrobiële membranen, medicijnafgiftesystemen, nieuwe gentherapiebenaderingen en de ontwikkeling van scaffolds voor weefselregeneratie. Biologisch afbreekbare synthetische polymeren, b.v. poly(lactic acid) (PLA), poly(glycolic acid) (PGA) en poly(ε-caprolactone) (PCL) zijn uitstekende kandidaatmaterialen voor de ontwikkeling van synthetische elektrospinmembranen. Biologisch afbreekbare polymeren hebben het voordeel dat een tweede operatie om het implantaat te verwijderen wordt vermeden. De ideale scaffold, d.w.z. een "slim materiaal" dat niet alleen biocompatibel is, maar ook een actieve rol heeft bij cellulaire proliferatie, cellulaire differentiatie, en weefselregeneratie of genezing is nog steeds niet beschikbaar. Synthetische polymere elektrospinmembranen zijn intrinsiek hydrofoob en biologisch inert, zonder een moleculaire structuur die een gewenste cellulaire respons stimuleert. Deze nadelen hebben geleid tot de noodzaak om nieuwe werkwijzen te ontwikkelen voor de functionalisering van synthetische elektrospinmembranen, waarbij nieuwe biologisch actieve materialen worden gevormd.

Wat is de huidige state-of-the-art met betrekking tot de functionalisering van elektrospinmembranen voor biomedische toepassingen?

Hoofdstuk 2 getiteld "Biofunctionalization of Electrospun Fibres for Tissue Engineering and Regenerative Medicine", was gericht op het evalueren van de meest recente ontwikkelingen in de ontwikkeling, functionalisering en toepassing van elektrospinmembranen voor biomedische doeleinden. Verschillende elektrospinningtechnieken, waaronder elektrospinning met een enkel mondstuk, coaxiaal elektrospinnen en elektrospinnen met emulsie worden besproken, met de nadruk op hun voordelen en beperkingen. Het tweede deel van het hoofdstuk richt zich op post-spinning functionalisatie van elektrospin membranen. De belangrijkste methodologieën die worden gebruikt voor functionalisatie na spinnen worden gepresenteerd en besproken, verdeeld in niet-covalente en covalente modificaties. Een breed scala van technieken wordt beschreven, reikend van fysische adsorptie en laag-voor-laag tot energie-gemedieerde functionalisering en wet-chemie functionalisatietechnieken, zoals oppervlakte-entpolymerisatie. De laatste sectie van dit hoofdstuk is gewijd aan de verschillende toepassingen van elektrospinmembranen voor biomedische doeleinden: ontwikkeling van scaffolds voor cellulaire adhesie, proliferatie en differentiatie; levering van biomoleculen; gentherapie; ontwikkeling van...
Kunnen de mechanische en biofunctionele eigenschappen van PCL-gebaseerde elektrospinmembranen worden verbeterd door de opname van silicium nanodeeltjes?

**Hoofdstuk 3** beschreef de ontwikkeling van een composiet elektrospin membraan voor Guided Bone Regeneration (GBR). De cruciale stap voor GBR-therapie is het inbrengen van een membraan voor steun- en barrièrefuncties. In **Hoofdstuk 3** hebben we het effect bestudeerd van siliciumdioxide nanopartikel (Si-NP) opname in electros spun poly(ε-caprolactone) (PCL) membranen om de mechanische en osteo-geleidende eigenschappen van de membranen te verbeteren. Hiertoe werden Si-NP's eerst gesynthetiseerd en vervolgens gesuspendeerd in PCL-oplossingen die een polair oplosmiddel (2,2,2-trifluorethanol) en water bevatten met de toevoeging van een anionische oppervlakteactieve stof. Nanocomposietmembranen werden vervaardigd uit de oplossingen door middel van een elektrospinningtechniek. Morfologie, structuur en chemische samenstelling en trekeigenschappen van de membranen werden geanalyseerd. Membraanstabiliteit werd bepaald door visueel onderzoek van de membranen na onderdompeling in phosphate buffered saline (PBS). Het effect van de materialen op osteoblastische differentiatie werd geëvalueerd door *in vitro* kweek van de membranen met MC3T3-E1 osteoblastische cellen. De resultaten gaven aan dat Si-NP's met succes werden opgenomen in het inwendige van de PCL-elektrospinvezels tijdens het elektrospinproces. De trekmodulus was significant verhoogd voor samenstelling S50 en de treksterkte nam significant toe voor de samenstellingen S25 en S50. Membranen die Si-NP's bevatten, bleken cytocompatibel te zijn. De verkregen resultaten tonen aan dat de Si-NP's homogeen waren opgenomen in de elektrogesponnen vezels, hetgeen resulteerde in een verbetering van de trekeigenschappen van de bereide materialen.

Kan een thermische behandeling leiden tot een meer aanhoudende afgifte van simvastatine die is opgenomen in PLLA elektrospinmembranen, waardoor ze kunnen worden gebruikt als elektrospinmembranen van GBR?

De opname van osteostimulerende verbindingen kan de biofunctionaliteit van elektrospinmembranen verbeteren, waardoor ze actieve spelers worden in de richting van botregeneratie. Simvastatine heeft aangetoond dat het zowel *in vitro* als *in vivo* osteogene differentiatie bevordert. In de meeste van deze systemen werd simvastatine echter snel afgegeven, wat niet overeenkomt met het tempo van botregeneratie. In **Hoofdstuk 4** ontwikkelden we poly(L-lactic acid) (PLLA) membranen die simvastatine (SV) bevatten met een verhoogde medicijnafgiftesnelheid, compatibel met GBR-toepassingen. Voor
dit doel werd SV gemengd met PLLA en electrospun. De membranen werden onderworpen aan een thermische behandeling om de kristalliniteit van PLLA te verhogen. Morfologische, structurele en chemische eigenschappen van de elektrospinmembranen werden gekarakteriseerd. Het effect van de thermische behandeling op het afgifteprofiel van SV werd geëvalueerd door bijna fysiologische vrijgave-experimenten bij 37 °C. Het osteostimulatoire potentieel werd bepaald door in vitro kweek van de membranen met beenmerg stromale cellen van de rat (rBMSCs). De resultaten bevestigden dat thermische behandeling leidde tot een verhoogde polymeerkristalliniteit en een meer aanhoudende afgifte van SV. In vitro testen vertoonden cellulaire proliferatie in de loop van de tijd voor alle membranen en een significante toename in ALP-activiteit voor de cellen gekweekt met de membranen die SV onderworpen aan thermische behandeling bevatten.

Kan een layer-by-layer (LbL) -strategie, gebaseerd op de depositie van PAA en PLL, worden gebruikt om PLLA elektrospinvezels te functionaliseren, waardoor de integratie van kleine moleculen wordt vergemakkelijkt?

In Hoofdstuk 5 werd post-spinfunctionaliteit van PLLA elektrospinmembranen door een Layer-by-Layer (LbL) strategie onderzocht. Electrospin poly(1-lactic acid) (PLLA) membranen worden veel gebruikt voor biomedische toepassingen. Een belangrijk nadeel voor dergelijke systemen is PLLA zonder biologische functionaliteit, wat leidt tot de noodzaak van het ontwikkelen van functionaliseringsstrategieën die de fysisch-chemische eigenschappen van PLLA veranderen en vandaar de opname van reactieve moleculaire groepen of macromoleculen aan het oppervlak mogelijk maken. Het doel van deze studie was om het vezeloppervlak van elektrospin PLLA-membranen door LbL te functionaliseren, waardoor een systeem wordt gecreëerd dat mogelijk toepassing vindt als een zeer biocompatibel substraat met het vermogen om te functioneren als een reservoir voor kleine moleculen (bijvoorbeeld farmaceutische verbindingen). Voor dit doel werden biocompatible polylektrolyten, poly(1-lysine) (PLL) en poly(acrylic acid) (PAA) gebruikt om PLLA elektrospinmembranen te functionaliseren. Morfologisch en elementair onderzoek van de membranen werd uitgevoerd. LbL-functionalisering werd bereikt, met een evenredige toename in de hoeveelheid poly-elektrolyten afgezet met het aantal onderdempelingsstappen. Verder werden twee modelgeneesmiddelen met een verschillende oppervlaktelading bij fysiologische pH (rhodamine B en clindamycine) opgenomen en hun ladingsrendement en afgifte beoordeeld. Beide geneesmiddelen werden opgenomen, zij het met lage ladingsefficiëntie. Rhodamine B werd vrijgegeven na 8 uur incuberen in phosphate buffered saline (PBS) bij 37 °C, terwijl de afgifte van clindamycine optrad tot 24 uur incubatie. Onze gegevens hebben de haalbaarheid van de aanpak aangetoond, maar er zijn meer studies nodig om de efficiëntie van de
medicijnbelading te verbeteren en de afgifte van geneesmiddelen te beheersen.

Kunnen gefunctionaliseerde microcilinders worden ontwikkeld door een top-down modificatie van elektrospun vezels?

Electrospinning is een veelzijdige techniek, die niet alleen kan worden gebruikt voor het produceren van steigers en membranen, maar ook voor de ontwikkeling van nieuwe structuren en componenten voor composiet biomaterialen. In *Hoofdstuk 6* hebben we een top-down benadering beschreven voor de ontwikkeling van nieuwe gefunctionaliseerde micrometrische structuren op basis van elektrospun PLLA-vezels, die kunnen worden gebruikt voor de ontwikkeling van medicijnafgiftesystemen of als bouwstenen voor nieuwe biomaterialen. Een breed scala aan deeltjes is ontwikkeld voor verschillende toepassingen in medicijnafgifte, weefseltechniek of regeneratieve geneeskunde. In tegenstelling tot traditionele bolvormige deeltjes bezitten niet-sferische (bijvoorbeeld cilindrische) deeltjes verschillende structurele en morfologische voordelen die hen aantrekkelijk maken voor specifieke toepassingen. In *Hoofdstuk 6* werden elektrospinvezels verwerkt tot micro-maat cilinders (d.w.z. microcilinders) met een hoog specifiek oppervlak en met of zonder oppervlaktporositeit. Om deze microcilinders te verkrijgen, werden PLLA-oplossingen onderworpen aan elektrospinning, gevolgd door een op aminolyse gebaseerde chemische splijtingsprocedure. De morfologie, structuur en chemische samenstelling van de microcilinders werden vervolgens gekarakteriseerd. Het specifieke oppervlak en de porositeit van het oppervlak van de microcilinders werden geregeld door de vluchtigheid van de oplosmiddelen en hun lengte was afhankelijk van de duur van de aminolyseractie. Gedurende de aminolyse werden de microcilinders gefunctionaliseerd met aminogroepen, hetgeen mogelijk verdere modificaties mogelijk maakt door enten met verbindingen die gewenste chemische groepen bevatten, b.v. carboxyl, carbonyl of hydroxyl functionele groepen. Bovendien vertoonden de microcilinders in vitro biocompatibele eigenschappen met betrekking tot cellevensvatbaarheid. Deze gegevens toonden aan dat PLLA-microcilinders met een hoog specifiek oppervlak, optionele oppervlaktporositeit, op amine gebaseerde functionele handvatten die additionele functionalisatie verschaffen, en cytocompatibele eigenschappen kandidaat-materialen zijn voor toekomstige biomedische toepassingen.

Heeft de opname van PLLA-microcilinders in calciumfosfaatcementen (CPC’s) een positief effect op hun mechanische eigenschappen?

*Hoofdstuk 7* richt zich op de mogelijke toepassing van de structuren beschreven in *Hoofdstuk 6* als polymere microfillers om de mechanische eigenschappen van calciumfosfaatcementen (CPC’s) te verbeteren. CPC’s zijn uitstekende biomaterialen voor botregeneratie die biocompatibel, resorbeerbaar,
injecteerbaar en osteoconductief zijn. De broosheid van de CPC’s beperkt hun toepassing echter alleen tot toepassingen zonder belasting. De opname van lange polymeervezels kan de mechanische eigenschappen van CPC’s verbeteren, maar aggregatie en heterogene verdeling van de vezels zijn grote problemen. In plaats daarvan kunnen korte polymere vulstoffen gemakkelijk in de cementmatrix worden gedispergeerd. In Hoofdstuk 7 werden continue PLLA-vezels met een gladde of poreuze oppervlaktemorfologie bereid door middel van elektrospinning. PLLA-microcilinders werden ontwikkeld, door middel van een aminolyseproces (hoofdstuk 6) en toegevoegd aan op α-TCP of α-TCP/PLGA gebaseerde cementen. Microcilinders distributie, evenals de morfologie, cohesie, uithardingsstijden en mechanische eigenschappen, werden geëvalueerd. PLLA-microcilinders werden homogeen door het cement gedispergeerd, terwijl de hanteringseigenschappen niet significant werden beïnvloed. Een afname van de initiële instellingstijden werd waargenomen wanneer PLLA werd toegevoegd, terwijl de mechanische eigenschappen vergelijkbaar waren met die van de α-TPC of α-TCP / PLGA-samenstellingen.

2. Slotopmerkingen en toekomstperspectieven

In dit proefschrift hebben we ingegaan op de beperkte functionaliteit inherent aan synthetische polymeermembranen zoals toegepast voor GBR-procedures. Functionalisatietechnieken werden toegepast om nieuwe materialen te creëren met verbeterde mechanische eigenschappen of die de osteogene differentiatie van pre-osteoblastische cellen zouden kunnen bevorderen. Hoewel werd aangetoond dat zowel pre-spinning als post-spinning functionaliseringsstrategieën de productie mogelijk maken van i) membranen met verbeterde trekeigenschappen, ii) membranen die kunnen worden gebruikt als drugsafgiftedragers iii) micrometrische structuren gefunctionaliseerd met reactieve aminegroepen, verschillende uitdagingen moet nog steeds worden aangepakt, namelijk het gebruik van niet-toxische oplosmiddelen en de ontwikkeling van meer precieze benaderingen wanneer elektrospunmaterialen worden gebruikt als medicijnafgifitesystemen.

Sterke organische oplosmiddelen worden gewoonlijk gebruikt om de synthetische Polymeren op te lossen voorafgaand aan elektrospinnen. Dit is een belangrijke beperking voor de definitieve vertaling van elektrospinning naar de industrie en de opschaling van deze technologie. Het gebruik van organische oplosmiddelen op industrieel niveau leveren verschillende bezwaren op: i) de meeste organische oplosmiddelen zijn licht ontvlambaar en vluchtig, wat resulteert in de noodzaak om specifieke veiligheidsmaatregelen te nemen[1] ii) organische oplosmiddelen zijn schadelijk voor het milieu en speciale maatregelen zijn nodig voor hun opslag en eliminatie[2] iii) conventionele organische oplosmiddelen zijn toxisch en niet geschikt voor het oplossen van biomoleculen, waardoor het gebruik ervan voor biomedischetoepassingen wordtbeperkt.[3] Nieuwe elektrospinningmethodieken,
die worden aangeduid als groene elektrospinning, en die zich richten op milieuvriendelijker benaderingen, laten veelbelovende resultaten zien. De term groene elektrospinning wordt over het algemeen gebruikt wanneer het oplossen van de polymeren en daaropvolgende elektrospinning wordt uitgevoerd met behulp van niet-toxische en milieuvriendelijker oplosmiddelen, voornamelijk water of waterige oplossingen. Water-oplosbare polymeren als poly(ethylene oxide) (PEO), poly(vinyl alcohol) (PVA) of poly(vinyl pyrrolidone) (PVP) zijn elektrogesponnen door deze benadering.[4] Wateronoplosbare polymeren vereisen echter het gebruik van sterke toxische organische oplosmiddelen, zoals chloroform, dichloormethaan of dimethylformamide. De ontwikkeling van elektrospinning met suspensie en colloidale elektrospinning is een recente groene elektrospinningstrategie, waarbij elektrospinvezels worden geproduceerd op basis van de dispersie van wateronoplosbare polymeren of copolymeren in een waterig medium.[5, 6] Een tweede alternatieve groene benadering is de elektrospinning van thermoresponsieve polymeren, zoals poly(N-isopropylacrylamide) (PNIPAM). Deze polymeren zijn in water oplosbaar onder hun lagere kritieke oplossingstemperatuur (LCST), maar bij elektrospinning gaven de vezels een goede stabiliteit in waterige omgevingen.[7] Ook ionische vloeistoffen op kamertemperatuur (Room temperature ionic liquids; RTIL’s) krijgen steeds meer aandacht vanwege hun hoge geleidbaarheid, lage dampspanning, hoge ontleidingstemperatuur en de mogelijkheid om de ionen chemisch te modificeren en te recyclen.[8] Dergelijke eigenschappen maken ze bijzonder aantrekkelijk om te worden gebruikt als milieuvriendelijke oplosmiddelen voor elektrospinning. Cellulosevezels zijn al geproduceerd met behulp van RTIL’s[9] en er worden inspanningen geleverd om het gebruik van ionische vloeistoffen voor de productie van niet-celluloseachtige elektrospin vezels uit te breiden.[10, 11]

De aanhoudende levering van farmaceutische verbindingen en biomoleculen uit elektrospinmembranen blijft ook een uitdaging. De afgifte van farmaceutische verbindingen of biomoleculen opgenomen in elektrospinvezels door mengen of coaxiaal elektrospinnen is hoofdzakelijk een passief fenomeen, dat afhankelijk is van de diffusie van het geneesmiddel of afbraak / erosie van de polymeervezels. On-demand medicijnafgiftesystemen, waarbij een afgifte van een verbinding plaatsvindt volgens endogene of exogene stimuli en op een precieze locatie, worden beschouwd als de volgende stap in de ontwikkeling van medicijnafgiftesystemen. Temperatuur, pH of door enzym afbelsbare domeinen kunnen worden gebruikt voor het reguleren en richten van geneesmiddelafgifte. Op stimulansen gebaseerde responsen kunnen worden ontwikkeld op basis van de aanpassing en modificatie van de polymere hoofdketen of door oppervlaktefunctionalisatie. Gezien de polymere hoofdketen is een interessante benadering die in de toekomst moet worden onderzocht de ontwikkeling van elektrospinvezels op basis van blokpolymeren. Temperatuurgevoelige blokpolymeren zoals poly(N-isopropylacrylamide)[12], poly(N,N-diethylacrylamide)[13] en PEG/PLGA[14]
Samenvatting, slotopmerkingen en toekomstperspectieven

Zijn gebruikt bij de ontwikkeling van temperatuurgevoelige nanodeeltjes. Op PEG gebaseerde blokcopolymeren zijn gebruikt voor de ontwikkeling van pH-gevoelige afgiftesystemen. Oppervlaktefunctionalisatie van elektrospinvezels is ook veelbelovend. Matrix-metalloproteinase (MMP’s) knipbare domeinen, thermoresponsief pamidronaat, of pH-responsief dextran en chitosan zijn gebruikt voor de oppervlaktefunctionalisatie van nanodeeltjes en microdeeltjes en zijn aansprekende strategieën om te verkennen voor het creëren van nieuwe on demand demandingsystemen voor elektrospun. Samengevat, in dit proefschrift werd elektrospinning gecombineerd met diverse functionalisatietechnieken gebruikt om nieuwe biomaterialen te ontwikkelen met verbeterde functionele eigenschappen voor GBR-toepassingen en botweefselengineering. Diverse pre- en post-spinning strategieën hebben geleid tot de ontwikkeling van membranen met verbeterde trekeigenschappen die mogelijk kunnen worden toegepast op GBR-toepassingen, en de ontwikkeling van nieuwe micrometrische structuren die kunnen worden gebruikt voor medicijnafgifte of als bouwstenen voor nieuwe biomaterialen. Electrospinning heeft alle potentie om een belangrijke techniek te zijn in de ontwikkeling van de volgende generatie biomaterialen. Om dat te laten gebeuren, moeten i) goedkope en milieuvriendelijke methoden voor elektrospinning worden onderzocht; ii) op stimuli gebaseerde systemen voor toediening van geneesmiddelen moeten verder worden onderzocht.
Referenties

Acknowledgments
List of Publications
Curriculum Vitae
Acknowledgements

The first time I heard about Nijmegen was when I applied to the Department of Biomaterials of Radboud UMC for a research project involving the candidacy to the Ph.D. title. Before that moment Nijmegen was a big mystery and when my family and friends asked me where I was going I simply said: “a Dutch city near Germany”.

The first time I came to Nijmegen was at the end of November to meet with my future supervisors and visit the facilities. It was a true Dutch winter, snowing, cold and dark. I was so nervous that during that short visit of 2 days I did not leave the campus area and not being aware of the Dutch dinner hours I went to have dinner around 21h00…and as you can imagine everything was closing at that time, and my dinner consisted of some chips, chocolate bars and soda, that I bought in a hospital vending machine. The warmth way that I was received by both Dr. Fang Yang and Dr. Jeroen van den Beucken the next day, and our pleasant lunch truly compensated that first impression and I returned to Portugal anxious and enthusiastic to start this new adventure on January 2014.

Nijmegen turned out to be a very dynamic, young, open-minded and friendly city that with time became a second home to me.

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“Your questions are false if you already know the answer.”
José Saramago, Portuguese writer and 1998 Nobel Prize in Literature.
List of publications

Publications related to this thesis:


*These authors contributed equally to this work.

Other publications:


**Book chapters:**
Curriculum Vitae

Antonio Castro was born in 1986 in Luanda, Angola. In 2004 he was enrolled in the Sciences Faculty of the University of Oporto (FCUP) obtaining his bachelor's degree (B. Sc.) in Biochemistry in 2007. That same year he enrolled in the Biochemistry master program of the FCUP. He performed his master's thesis work in a collaboration between the FCUP, the Department of Metallurgical and Materials Engineering of the Engineering Faculty of the University of Oporto (FEUP), and the Dentistry Faculty of the University of Oporto (FMDUP). In 2009, he obtained his master's degree (M. Sc.) with a thesis entitled “Development and characterization of an anorganic bovine-bone derived xenograft”. In 2010 he worked as a researcher in the Department of Bioengineering of the Instituto Superior Técnico in Lisbon (IST), evaluating the capture of Salmonella thyphimurium by ferromagnetic nanoparticles chemically functionalized with polyclonal antibodies, considering the development of a biosensor for the detection of pathogenic bacteria in a liquid medium. From 2011 to 2013 he worked as a researcher in the Department of Materials Engineering and Ceramics of the University of Aveiro (DEMaC-UA), developing new silicon-based materials and coatings by sol-gel procedures for biomedical applications. In 2014, he initiated his Ph.D. candidacy in the Department of Biomaterials at Radboud University Medical Center (Radboud UMC) in collaboration with the Radboud Institute for Molecular Life Sciences (RIMLS), focusing on the production and functionalization of biodegradable polymeric membranes for biomedical applications. The results of his thesis are published in several research articles in international scientific journals, including two publications where he collaborated with the Utrecht Institute for Pharmaceutical Sciences (UIPS).
# PhD Portfolio

**Name:** Antonio Castro  
**Department:** Biomaterials  
**Graduate School:** Radboud Institute for Molecular Life Sciences  
**Ph.D. period:** 01.01.2014-01.01.2018  
**Supervisor:** Prof. John A. Jansen  
**Co-supervisors:** Dr. Fang Yang, Dr. Jeroen J. J. P. van den Beucken

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<td>Laser Assisted Macro and Nano Designing of Ti-6Al-4V Surface for Bio-implant Application- Prof Dr Jyotsna Dutta Majumdar</td>
<td>2014;2015;2016;2017</td>
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<tr>
<td><strong>Symposia &amp; congresses</strong></td>
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<td>Radboud Frontiers</td>
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<td>PhD retreat (incl poster/talk)-poster presentation</td>
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<td>PhD retreat (incl poster/talk)-oral presentation</td>
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<td>NIRM consortium meeting-oral presentation</td>
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<td>NBTE annual meeting- oral presentation</td>
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<tr>
<td>27th European conference on biomaterials 2015-oral presentation</td>
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<td>10th World biomaterials congress 2016- oral presentation</td>
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<td><strong>Teaching activities</strong></td>
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<tr>
<td>Supervision of internships / other</td>
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<td>Master student project: Mechanical reinforcement of calcium-phosphate cement with poly(L-lactic acid) micro-cylinders</td>
<td>2015</td>
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<td>Master student project: Development of a nanocomposite membrane for guided bone regeneration</td>
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<tr>
<td>Supervision Bachelor students: membranen voor de reconstructie van parodontaal weefsel</td>
<td>2017</td>
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<td>Master student project: Fabrication, characterization and biological evaluation of poly-caprolactone/gelatin membranes for guided bone regeneration.</td>
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<td>Supervision exchange student: Development of biodegradable drug-delivery systems</td>
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<td>Supervision exchange student: Development of guided bone regeneration membranes with drug-delivery functionality</td>
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**Total** 32.95