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Ph.D. in PRODUCTS ENGINEERING AND INDUSTRIAL PROCESSES

XXXII CYCLE

"PREPARATION AND CHARACTERIZATION OF SCAFFOLDS FOR TISSUE ENGINEERING"

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TABLE OF CONTENTS

SUMMARY

In this work, it will be explored the use of electrofluidodynamic technologies (EFDTs), namely electrospinning and electrospraying for the development of bioinspired devices for the application in tissue engineering and drug delivery systems. Different strategies were developed via EFDTs by using different biomaterials such as natural proteins and created oriented fibers to improve the cell-material interaction through chemical and morphological signals. The main aims of the work are summarized below:

- Evaluation of the processability of wool keratin to obtain aligned fibers via electrospinning and coating of nanogrooved titanium to provide topographical and biochemical signals to guide growth cells.
- Evaluation of the biocompatibility of bicomponent fibers processed from blended solutions of synthetic polymer (PCL) and natural proteins, gelatin, zein and keratin for their use in tissue engineering applications.
- Design of electrosprayed particles and optimization of integrated systems for drug delivery of antibiotics as Amoxicillin via additive electrospray to evaluate i*n vitro* antibacterial properties for their use in periodontal treatments.

The thesis will be organized in 7 chapters: after a preliminary description of the state of art on the use of natural proteins and electrofluidodynamic technologies for tissue engineering and drug delivery applications, it will described the fabrication, characterization and in vitro validation of different devices obtained by the implementation of tailored experimental setups.

Chapter 1 is a brief overview on the natural proteins that have been studied for the fabrication of different platforms for biomedical applications. Electrofluiodynamic techniques (EFDTs) have been widely used for the fabrication of micro/nanofibers and particles respectively for biomedical applications. In **chapter 2** are described the basic principles, process parameters and setup modifications that have been investigated for the development materials for tissue engineering and molecule release. The aim of this work was to design different devices: aligned fibers, bicomponent fibers, integrated systems and nanoparticles. In **chapter 3**, electrospinning process was optimized to obtain aligned fibers of wool keratin. Once optimized the process, aligned keratin fibers were deposited onto titanium surfaces. The alignment of keratin fibers provided a biochemical and morphological signal to fibroblasts, promoting their growth along fibers. which could be a promissory strategy to improve the interaction of transmucosal implants with soft tissues. The use of bioactive proteins such as keratin, gelatin or zein has been successfully explored to improve the biological interface of scaffolds with cells during tissue regeneration. In **chapter 4** was optimized the fabrication of nanofibers combining gelatin, zein and wool keratin, with polycaprolactone (PCL) in order to design bicomponent fibrous matrices able to provide biochemical and morphological signals to influence cell interactions. Fibers in nanometric size were obtain. Cell interaction and cell proliferation were improved by the presence of proteins, mainly in case of PCL/gelatin and PCL/keratin fibers. Moreover, PCL/keratin fibers were further investigated in **chapter 5**. It is demonstrated that the blending of highly polydisperse keratin with PCL (50:50) improves the stability of the electrospinning process, promoting the formation of nanofibers. Moreover, keratin increases the fiber hydrophilicity thus improving the hMSC adhesion and in vitro proliferation until 14 days. When fibers were in contact with bacterial strains seems to be not specifically inhibited by the contribution of

keratin, so that the integration of further selected compounds (i.e., metal ions) is suggested to more efficiently fight against bacteria resistance. to make them suitable for the regeneration of different interfaces and soft tissues (i.e., skin and cornea). The versatility of EFDTs allowed through electrospraying fabricate nanoparticles of cellulose-graft-polycaprolactone to encapsulate sodium diclofenac, reported in **chapter 6**. Results shown an influence of diclofenac on nanoparticles morphology; however, the encapsulation efficiency was more that 80%. The release profile and cytotoxicity were evaluated. The release was characterized by an initial burst release followed by a sustained release up to 144 hours, influenced by the amphiphilic properties of polymer. During the time release, no cytotoxic effect was detected. In **chapter 7** the integrated use of EFDTs is presented as an alternative to design nanostructured platforms with controlled release. Nanofibers of PCL and loaded nanoparticles of chitosan with amoxicillin were deposited by to form an integrated system. The hydrophobic properties of the PCL network promote a more homogeneous spatial distribution of nanoparticles, improving the antibacterial properties.

The main challenge of tissue engineering is to design scaffolds able to reproduce the natural environment of cells, it means, the native extracellular matrix by including morphological and biochemical cues. From the morphological point of view, natural ECM may be organized randomly or aligned or semi-aligned, meanwhile, the addition of natural polymers is able to improve the biological properties of biomaterials.

CHAPTER 1

PROTEIN BASED SCAFFOLDS FOR IN VITRO INTERACTION AND CELL GUIDANCE

1.1 INTRODUCTION

In the last three decades, the employ of biomaterials in tissue engineering is rapidly evolving to offer a portfolio of innovative devices to support the functionalities of natural tissues. Biomaterials were used as medical devices like pacemakers, biosensors, or implants in the form of sutures, bone plates, joint replacements, ligaments, vascular grafts, heart valves, intraocular lenses, and dental implants [1]. More recently, biomaterials work as synthetic frameworks; namely, scaffolds, matrices, or foams able to guide the mechanisms of tissue regeneration [2–5]. Over the past decade, the growing attention towards the understanding of cell materials interaction has addressed the finding of new materials suitable to accurately reply the local biological microenvironment in order to improve cellular response and more efficiently modeling biological context [6]. Hence, researchers have focused on the innate attitude of natural polymers to guide the cell behavior through biophysical and biochemical cues to mimic the native extracellular matrix (ECM).

From a structural point of view, ECM is composed of protein fibers with diameters in the range of tens to hundreds of nanometers [7]. Main functions of ECM concern the support and preservation of mechanical integrity of tissues and organs, as well as the regulation of cell functions such as proliferation, shape, migration and differentiation, and provide cell-cell and

cell-matrix interactions. Fibrous proteins have an elongated 3D structure, are secreted by cells and can be founded generally as part of the extracellular matrix.

For this purpose, starting from the function of natural fibers of ECM to guide the cell behavior through biophysical and biochemical cues, many researchers are exploring the use of natural proteins to design temporary platforms able to mimic the native ECMz. Fibrous proteins such as collagen, elastin, keratin, laminins, fibronectin and vitronectin with elongated three-dimensional structure (Table 1.1) are primary components that directly interact with cells similarly to the bioactive component of the native ECM. Herein, an overview of recent studies involving structural and functional proteins used for the regeneration of hard and soft tissues was reported [8].

Table 1.1. Summary of proteins for scaffold manufacturing in oral tissue engineering [9].

Protein	Structural properties	Biological properties	References
Collagen and gelatin	Right-handed triple helix, composed of three α-chains (Gly-X-Y)	Good biocompatibility Structural protein	[11,12]
	RGD motifs	Cell adhesion, proliferation and differentiation	
Keratin	RGD and LDV motifs	Cell adhesion	[18,19]
	Content of cysteine	High mechanical strength, inertness, and rigidity	
Silk	Disulfide bonds	Good mechanical properties Biocompatible	[25,26]
Zein	Alcohol-soluble. Rich in glutamic acid, and non-polar amino acids (leucine, proline and alanine)	Drug controlled release Biocompatible	[29,30]

1.2. NATURAL POLYMERS

The increased interest to reply the extracellular environment to promote tissue regeneration has led to develop materials based on natural polymers [8]. Among the most used natural polymers in tissue engineering, the proteins collagen, gelatin, fibrin, elastin, keratin, silk, zein; polysaccharides as chitosan, hyaluronic acid, alginate; and polynucleotides are the most commonly used [9].

1.2.1 Collagen and gelatin

Collagen is the most abundant protein in the human body and the main compound of ECM. Collagen is a triple helix protein, characterized by a repetitive sequence of amino acids, glycine, proline and hydroxyproline [10]. Have been identified more than twenty types of collagen, between them, collagen type I is the most abundant in nature followed by collagen type III [11]. Collagen and its denaturized form, gelatin, have been deeply studied for their use in tissue engineering because of their good biocompatibility and the presence of arginine-glycine-aspartate (RGD) sequences to promote integrin-mediated cell adhesion. Collagen is degraded enzymatically by collagenases such as metalloproteinases. Their degradability can be regulated by diverse methods as physical crosslinking techniques (i.e., ultraviolet (UV) or dehydrothermal (DHT) crosslinking) or chemical modifications (i.e., glutaraldehyde; isocyanates) [12,13].

Gelatin - a fibrous protein extracted from denatured native collagen - shares a similar structure, composition and biological properties of native collagen. According to denaturation hydrolysis process, there are two types of gelatin: type A, it means acid process;

type B, which is the alkali breaking. Alone or in combination with natural or synthetic polymers, gelatin has been widely used for the fabrication of *in vitro* stabile bioactive scaffolds for tissue engineering [14,15]. Gelatin has several advantages for tissue regeneration, as RGD-like sequences to promote cell adhesion, biocompatibility, and because it is a denatured form of collagen, gelatin is less antigenic [16,17].

1.2.2 Keratin

Keratin is a fibrous protein, founded in hair, wool, feathers, nails, and horns of mammals, reptiles and birds. Keratin proteins can be classified into intermediate filament proteins and the matrix proteins. Keratin proteins are classified as α-keratins, known as intermediate filament proteins (IFPs) and β-keratins, both embedded in an amorphous keratin matrix. The α-keratins are in the fiber cortex; are low in sulfur content, with 40–60 kDa of average molecular mass [18]. The matrix proteins are globular, have low weight, and high content of cysteine, glycine and tyrosine residues; surrounding the IFPs and interact with them through intermolecular disulfide bonds [18,19]. The high mechanical strength, inertness and rigidity of α-keratins depend on crosslink between IF-matrix composite. The β-keratins and their function are to protect keratin fibers from physical and chemical damage [20].

Keratin is characterized by the presence of sequences as RGD (Arg-Gly-Asp) and LDV (Leu-Asp-Val) found in several ECM proteins for cell adhesion. Thus, keratin has been proposed as an alternative to collagen to develop biomaterials for tissue regeneration [21]. Besides, several studies have shown that the addition of keratin and adjusting its concentration, the mechanical properties of biomaterials is improved [22,23].

1.2.3 Silk fibroin

Silk fibers extracted from domesticated silkworm *Bombyx mori (B. mori)* are the most commonly used ones for tissue engineering applications. The amino acid composition of silk consists of glycine, alanine, serine [24,25]. For tissue engineering applications, silk fibroin has shown to have better mechanical properties than other natural polymers, good biocompatibility and its degradation products are non-toxic [26].

Silk is composed of a filament core coated with sericin, a hydrophilic protein. Sericin is degummed during silk purification process leaving the core fibers corresponding to silk. In general, flexibility and high tensile strength of silk have made it useful in many fields. On the other hand, sericin was considered to promote hypersensitivity reactions, however subsequent studies have shown that sericin as silk fibrin were immunologically inert when cultured with murine macrophage cells [27]. Sericin can be considered to use as biomaterial since some studies have demonstrated that is no cytotoxic for several cell lines when sericin is added to the culture media [28].

1.2.4 Zein

Zein is a vegetable protein found in the endosperm of corn recently explored for tissue engineering and drug delivery application due to its good biocompatibility [29,30]. Zein is an alcohol-soluble protein, which amino acid composition is particularly rich in glutamic acid, and non-polar amino acids, as leucine, proline, and alanine, but deficient in basic and

acidic amino acids [31]. The amino acid sequence is characterized by hydrophobic and neutral amino acids, and sole polar amino acids. It has been studied for its potential as implant material because its compatibility *in vivo*. Zein scaffolds have shown a proper porous structure and mechanical properties for cell adhesion, migration, proliferation and tissue ingrowth. Due to its composition, zein is an hydrophobic protein, which may contribute to control the material degradation for tissue engineering, and allow longer and sustained release of drugs as a carrier [30,32].

1.3 BIOMEDICAL APPLICATIONS OF NATURAL POLYMERS

The main challenge is to identify natural proteins and manipulate them by recently implemented micro and nanotechnologies to improve their structural and functional stability and more efficiently mimicking the native tissue microenvironments by triggering new functionalities for cells. collagen has been processed in different physical forms including injectable hydrogels, membranes, films, sponges and porous scaffolds, and micro- and nanoparticles depending of the aim applications. Collagen has been widely used to design nerve conduits for the relevant advantages in nerve regeneration due to biodegradability and biocompatibility, as confirmed by the U.S. Food and Drug Administration (FDA) approval for clinical use in peripheral nerve surgery [33,34]. Alternatively, the use of gelatin mixed with synthetic polymers such as polycaprolactone (PCL) for the fabrication of bicomponent electrospun conduits able to support axonal regeneration over longer distances, sustaining nerve regeneration for many months while evoking minimal immune response due to inert release products [35].

Collagen membranes are the gold standard, due to their good biocompatibility, biodegradability, and osteoinductive properties, despite showing some limitations due to their inadequate mechanical properties. As a result, collagen-based scaffolds have been designed for guided bone regeneration in periodontal research blended with other polymers and ceramics to enhance mechanical properties and cellular behavior [36,37]. Alternatively, PCL scaffolds may be also coated either with collagen than HA (Hydroxyapatite), two components of natural bones. In this case, collagen, not only provides the necessary environment for cell attachment, but also helps to increase the poor fracture toughness of HA [38].

Since the advances in the knowledge about extraction, purification, and characterization of keratins, the potential use of keratins as biomaterial has been explored, as alternative to collagen. One reason for the use of keratin in tissue regeneration is to contain cell adhesion sequences, arginine-glycine-aspartate (RGD), and leucine-aspartic acid-valine (LDV) which are found in the extracellular matrix proteins such as fibronectin, and recognized by the integrin family protein of the cells membranes [39,40]. In particular, keratin has been widely studied as a biomaterial for wound healing as films, hydrogel, nanofibers, and three-dimensional blended scaffolds, and also in combination with other materials to get a biocompatible scaffold with antibacterial activity and good mechanical properties for skin repair [41,42]. Keratin, has also been evaluated in dental regeneration especially on pulp-tissue engineering related to their fibrous structure, the intrinsic ability to self-assemble, and adhesive motifs [43]. It may be successfully used to support the self-repairing of soft tissues such as nerves in order to find alternative routes to the use of autografts and surgical intervention. Also, for guided tissue regeneration (GTR), the addition of keratin may be used

to enhance the mechanical properties of GTR scaffolds [44]. To improve the physical and biological properties, gelatin and elastin proteins have been frequently chosen for their biocompatibility, long-term stability and capability to promote fibroblast-attraction [45].

More recently, electrospun zein fibers have been crosslinked with citric acid to enhance their water stability [46,47]. Co-electrospinning of two proteins as gelatin and zein has been also studied, showing that addition of gelatin increases the elastic modulus of the scaffold and cell adhesion, of periodontal ligaments stem cells (PDLSCs); meanwhile, zein confers stability resulting non-degradable at early stage to achieve bone regeneration in the recovery of periodontitis [48].

1.5 CONCLUSIONS

In the last years, biological approaches based on the use of biomolecules inspired by tissue engineering principles are tracing new routes for the restoration/regeneration of natural tissues. Several studies have confirmed that proteins from natural sources may be candidate as gold standard biomaterials, due to their bioactive properties that provide better interactions with cells, thus enhancing the final performance in biological systems. In particular, advances in biotechnology and synthetic biology currently allow designing protein-based materials derived from living organisms with specific chemical, mechanical, or structural properties that were once limited to the domains of inorganic and organic chemistry. In this perspective, the incorporation of proteins to biomaterials and their blending with other natural or synthetic polymers are attractive approaches to follow in the development of biomaterials. Moreover,

their process by EFDTs offers an innovative resource to design scaffolds with improved biochemical and topographical signals to allow the regeneration of tissues.

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CHAPTER 2

ELECTROFLUIDODYNAMIC TECHNOLOGY: PROCESS OPTIMIZATION

2.1 INTRODUCTION

Electrical assisted spinning or electrofluidodynamic techniques (EFDTs) are highly flexible and low-cost processes to generate biomaterials by using electrostatic forces. Electrospinning and electrospraying are EFDTs to generate fibers or particles, respectively, of a wide variety of materials in micro and nanometric sizes by adjusting process parameters [1,2] (Figure 2.1).

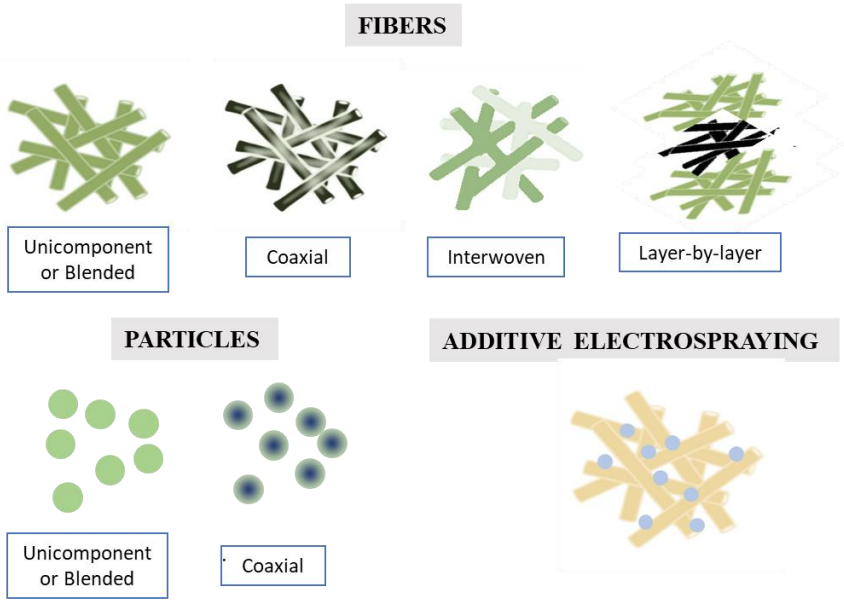

Figure 2.1 Different structures that can be obtained by EFDTs [2].

Electrospinning is used for the fabrication of fibers, generally of polymeric materials, that can be used in different fields, as filtration, sensor technology, biomedical devices, tissue engineering and drug delivery [3,4]. For biomedical applications, electrospun fibers offer an

interconnected structure with pores to facilitate cell migration, the high surface area for oxygen permeability and abroad of spatial arrangements to mimic the native extracellular matrix (ECM) of tissues and enhance the regeneration process [5]. In the other wise, electrospraying is used for the fabrication of particles that can be used in microelectronic devices, food industry, imaging, and molecule/drug delivery systems [1,6,7].

2.2 BASIC PRINCIPLES OF EFDTs

The principle of EFDTs is based on the stretching of a polymer solution by the application of high voltage. The basic setup of electrofluidodynamic technology consists of a syringe with a steel needle connected to a syringe pump, a high-power supply and the grounded collector (Figure 2.2).

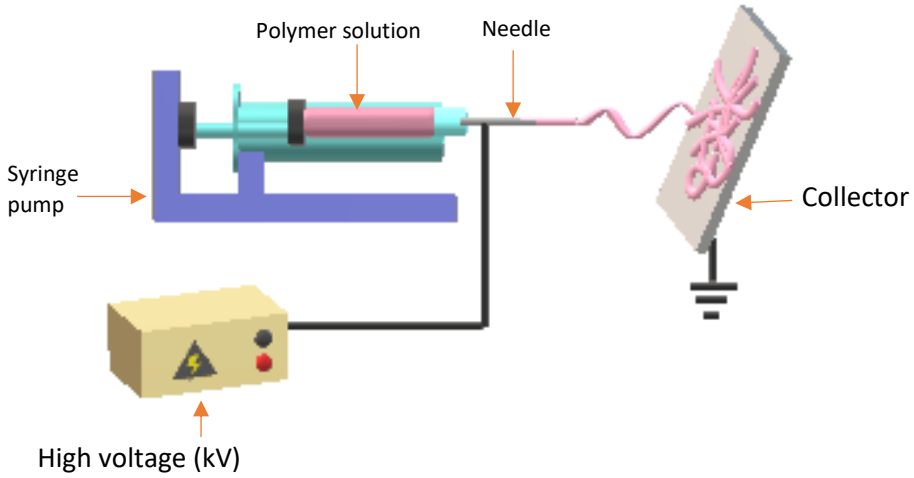

Figure 2.1. Electrofluidodynamic basic setup.

The process starts when a polymer solution is delivered in a constant flow rate by the syringe pump to form a drop at the tip of the needle. Then, the polymer solution interacts with the high voltage through the metallic needle, causing a polymer jet ejection from the needle to

the grounded collector [8,9]. The physical principle is that the interaction between polymer solution and the high applied voltage overcomes the surface tension forces of the solution through repulsive electrical forces generated when the electrical field reaches to a critical value. With the increased electric field, the droplet deforms to a conical shape, called Taylor cone, at the tip of the needle and then a stable jet is ejected in direction to the collector. The distance between the needle tip to the collector has to be adjusted to allow the evaporation of the solvent during the jet ejection.

EFDTs can be affected by different parameters that are classified into three main groups: solution properties, process parameters and environmental parameters (Table 2.1). The appropriate combination of all these factors allows the formation of fibers or particles, without defects, with controllable size, shape and arrangement [10].

Table 2.1 Electrofluidodynamic parameters

Solution properties	*Molecular weigh* *Concentration* *Viscosity* *Surface tension* *Solvent volatility* *Dielectric constant and conductivity*
Process parameters	*Applied voltage* *Tip-to-collector distance* *Flow rate* *Collector*
Environmental parameters	*Temperature* *Humidity*

2.3 SETTING OF MATERIALS AND PROCESSING PARAMETERS

2.3.1 Solution properties

Biodegradable polymers have been studied as a promissory approach to design biomaterials for biomedical applications. For the development of devices via EFDTs, solution properties must be considered for the optimization of the process, it means polymer molecular weight, concentration, viscosity, elasticity, conductivity and surface tension.

a) Polymer molecular weight, concentration and viscosity

The molecular weight of polymers influences the viscosity of solutions, affecting the morphology of fibers [11]. Thus, the polymer concentration should be considered since is proportional to the viscosity of the solution that may influence the morphology and diameter of fibers [12,13].

The molecular weight of polymers indicates the intermolecular entanglement, thus high molecular weights are preferred because when polymer solution is stretched during the process, prevent the jet breaking, maintaining a continuous and stable jet to form fibers. In case of lower molecular weights, the viscosity decreases resulting in beads formation [14].

In order to increase the viscosity of low molecular weight polymers in solution, it is necessary to increase the polymer concentration resulting in a similar effect as high molecular weight polymers. By increasing polymer concentration the diameter of fibers is also increased [15]. When viscosity has increased the shape of beads changes from spherical to spindle-like and

finally results on the formation of smooth fibers [14]. However, when the viscosity is higher, is not able to pump out the solution or dries at the tip of the needle before the jet formation, due to the voltage is insufficient to stretch the polymer solution [16,17].

b) Surface tension

The process of electrospinning is based on the stretching of a polymer solution through the application of an electric field to charge the solution and overcome its surface tension. In general, is considered that surface tension affects fiber morphology [18,19]. Low surface tension is attractive for the formation of fibers without beads at low voltages, since the liquid surface opposes less resistance to the external force of the electric field.

c) Solvent volatility

The importance of solvent is related to different properties of polymer solution for the electrospinning process. The selection of solvent depends of the type of polymer, the boiling point of solvent and the dielectric constant. The volatility of solvents is an important parameter to allow the fiber deposition, since the solvent should evaporate during the path between the needle tip to the collector [20]. Solvents with low boiling point have a high evaporation rate, thus the electrospinning process can be interrupted because the solution dries and blocks the needle tip. The contrary case, solvents with high boiling point are less volatile resulting in the formation of beads or formation of films instead of fibers. In this regard the evaporation rate of solvents can be controlled by changing the distance between

the tip and the collector and controlling environmental parameters as temperature and humidity for the obtention of smooth fibers [21]. Solvent volatility can be used for the formation of porous fibers by the phase separation before deposition of fibers onto the collector or using a system of solvents, it means the use of solvents with different boiling points [22–24].

d) Dielectric properties and conductivity

In electrospinning the properties of solvents and their interaction with polymers are important for the obtention of fibers. One of the major properties of solvents is their dielectric constant, a measure of their polarity [25]. In general, solvents with high dielectric constant are preferred to avoid beads formation. Several studies have considered that higher dielectric constant of solvents generates thinner fibers, due to the higher net charge density which allows the jet elongation under the electrical field [17,26].

The conductivity of solutions allows the jet stretching thus, it is a solution property that influences the morphology and fiber diameter. It has been studied that solutions with low conductivity are not appropriate for the electrospinning process because of the absence of surface charge. However, it is possible to provide a better conductivity by adding salts to solutions as NaCl, LiCl, and $MgCl_2$ [19,27]. On the other hand, when conductivity is increased, high quality fibers are obtained, and the fiber diameter can decrease with higher conductivity. Moreover, the high solution conductivity enables the use of lower applied voltage. However higher conductivity prevents the formation of Taylor cone because the electrostatic force along the surface of the fluid is decreased.

2.3.2 Process parameters

The main process parameters include applied voltage, tip-to-collector distance and flow rate. There are variables as the design of the nozzle, the type of collector for the obtention of different arrangements or the composition of fibers or particles.

a) Applied voltage

The applied voltage is a basic parameter of EFDTs for the formation of Taylor cone under the electrostatic forces. The high voltage applied to the droplet at the needle tip creates a strong electrostatic field, with increasing the electrostatic field intensity, the charges on the surface repel each other and produce shear stress. The repulsive forces act in the opposite direction to the surface tension, leading to the formation of Taylor cone. Once it reached a critical voltage, the balance of repulsive forces is broken and the jet is ejected from the Taylor cone [28].

In general, it is considered that increasing applied voltage promotes the formation of thinner fibers because the stretching of polymers is more extensive and promotes the faster evaporation of the solvent. [3,12]. However, this effect is related to other parameters as solution properties and flow rate. The increase of high voltage accelerates the jet ejection, in which a fixed flow rate can generate an unstable and small Taylor cone [29]. There are studies we In case of polymer solutions with low viscosity, the increased voltage may promote the

formation of secondary and smaller jets [16]. Some studies suggest that the voltage effect on fibers can transit from round to flat/ ribbon fibers [30].

b) Tip-to-collector distance

The distance between the needle tip and the collector is the path that jet will travel giving enough time to the solvent to evaporate, resulting in the deposition of dry fibers. It has been studied that decreasing distance does not allow the correct evaporation of the solvent, thus may generate merged fibers, with less porous structure, beads or form a film [31,32]. Distance may have an inversely proportional effect to the electric field strength, it means when distance decreases, jet behavior is similar to increasing applied voltage, thus an increased field strength that can cause high instability and formation of beads [10,33]. On the other hand, good solvent evaporation is allowed with longer distance, and favors the decrease of fiber diameters or beyond the critical value the dimeter may increase due to the weak field strength [34,35].

c) Flow rate

The maintenance of the Taylor cone depends on the applied voltage and the constant amount of polymer solution available for the electrospinning process. The flow rate depends on the properties of the polymer solution, more specifically of solvent properties, it means that solutions with highly volatile solvent should be pump at higher rates, thus, when higher flow rates are applied, the electric field should be higher for the jet stretching [36]. By increasing

the flow rate, the diameter of fibers is increased [32]. However, in case flow rates over the limit, beads with bigger diameters can be formed.

2.3.3 Ambient parameters

Environmental factors as humidity and temperature influence diameter and fiber morphology. The effects environmental factors are related to the polymer solution constituents, more specifically with viscosity and solvent evaporation.

a) Temperature

The environmental temperature and relative humidity depend on polymer solution components and influence the morphology of fibers. At higher temperatures, the solvent evaporation is faster, producing thicker fibers. The viscosity is also influenced by the effect of temperature, thus higher temperatures reduce the viscosity of the solution, with formation of thinner fibers [37].

b) Humidity

There are studies that correlate humidity with the fiber diameter depending on the polymer used. Different polymers and blends have been studied, resulting in decreased dimeters with the increase of humidity, however, it is considered that more than 70% of relative humidity, beaded fibers can be obtained or no-uniformed structures [38]. In some cases, when humidity

is low, volatile solvents evaporate rapidly, which causes the clogging of the needle, stopping the electrospinning process [39]. Relative humidity influences the morphology of fibers, by a competition between the dynamics of phase separation and the rate of solvent evaporation [40].

2.4 ELECTROSPRAYING

Among the EFDTs, electrospraying is a versatile technique based on the interaction of a polymer solution with high voltage as in case of electrospinning that offers great versatility to fabricate particles for the development of drug delivery carriers, diagnostic or theranosthics applications [9]. The principle of electrospraying is based on the theory of charged droplets. Briefly, once the high voltage interacts with the polymer solution, the electric charge generated on the droplet causes an electrostatic force inside the droplet to finally form the Taylor cone [7]. Once the surface tension is overcome, micro or nano-scale droplets are ejected to the collector. As in the case of electrospinning parameters related with the process, solution and environment influence the morphology, size and surface of particles, but also offers a great versatility to fabricate particles for the development of drug delivery carriers, diagnostic or theranostics applications [9,41].

The flow rate influences the morphology and size of particles. This effect is related to the Coulomb fission during solvent evaporation. High flow rates promote the formation of polydisperse droplets, meanwhile low flow rates promote the formation of a more strict distribution of particles size [42,43]. Regarding the use of solvents in electrospraying, those with low vapor pressure and high boiling point are preferred for electrospraying process to

promote the formation of particles with smaller size and smoother surface; for particles with textured, porous and hollow surfaces, solvents with high vapor pressure and low boiling point are selected. Distance is another parameter which is also related solvent evaporation, with a similar effect to that describe for electrospinning, short distances generate wet particles with broad size distribution.

2.5 SETUP MODIFICATIONS

The increased use of EFDTs has addressed to develop several approaches to design multicomponent biomaterials to include selected molecular and topographical signals able to improve the bioactive response of scaffolds for tissue engineering or improve the release of therapeutic agents [44–46]. For this propose, the basic setup of EFDTs can be modified to obtain different structures (Figure 2.3).

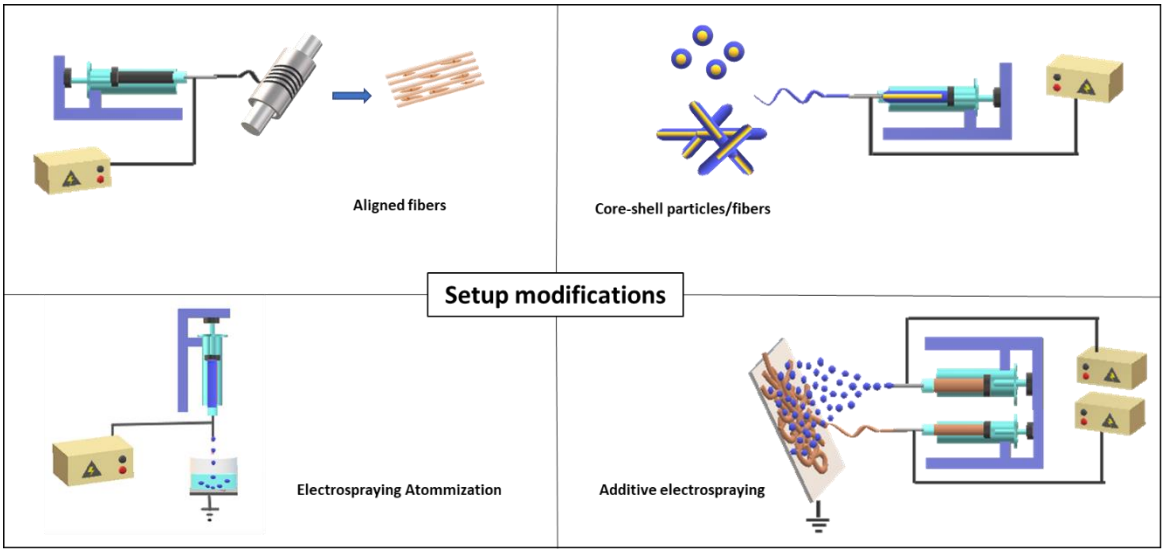

Figure 2.2. Setup modification for the fabrication of different materials by EFDTs.

To control the orientation of fibers, specifically for their alignment, a rotting mandrel at high speed is being used as a collector for the deposition of fibers along it [44,45]. Depending of the solution the speed rotation may vary from hundreds to thousands of revolutions per minute (rpm) [47]. Highly aligned fibers have been obtained by using two conducting collectors placed in parallel with a gap between them to create an electric field profile to force the charged fibers to span the gap [48].

Single and blended polymers have been used for the incorporation of molecules as drugs, proteins and cells for the development of devices for tissue engineering or drug delivery systems. Therefore, several studies have developed core-shell fibers or particles to improve the release by using a coaxial nozzle to pump two different polymers solutions, where usually the core contains the active molecule [49–51].

In recent years, electrospraying is widely used to design carriers for biomedical applications. There are different electrospraying modes, one is the basic electrospraying of a polymeric solution, for the deposition of charged droplets on a grounded collector with solvent evaporation, resulting in the formation of single or agglomerated particles [52]. A recent approach is based on the deposition of charged droplets into a bath with chemical or ion crosslinking agent solution, to bind the polymer in the droplet with high efficiency of encapsulation [53,54].

Post-treatments of electrospun fibers have been investigated in order to functionalize the fiber surfaces. However, these processes can alter the morphology of fibers, and an unpredictable release profile. In this context, additive electrospraying has resulted in an innovative and

simple strategy on the coupling fibers and particles processed by electrospinning and electrospraying respectively [55,56]. For this propose, there are two different setups to integrate nanoparticles to fibers, the sequential process, which consists on two-step technique, resulting in a coating of electrospun fibers; meanwhile the simultaneous process is a one-step, where fibers and particles are deposited at the same time, assuring a more uniform distribution of particles onto the fibers [57,58].

2.6 APPLICATIONS EFDTs-PROCESSED MATERIALS IN TISSUE ENGINEERING AND DRUG DELIVERY

The development of biomaterials for tissue engineering and drug delivery is focus on the use of biodegradable and biocompatible materials to restore the damage tissues and control the release of molecules during the time.

2.6.1 Tissue engineering

Tissue engineering is focusing on the development of biomaterials to mimic the extracellular matrix (ECM) of native tissues. ECM is a hierarchical three-dimensional fiber network of proteins and polysaccharides surrounding cells, that stimulate cells through interactions cell-ECM, provides support, and stores growth factors and signaling molecules [59,60]. In recent years, fiber scaffolds in micro- or nano- scale fabricated by electrospinning have been widely studied since it is possible to mimic the architecture of ECM, besides have a microporous structure and high surface-area-to-volume ratio to facilitate the adhesion, proliferation and

differentiation of cells [61,62]. For this propose, biodegradable materials have been studied as a promising approach to design biomaterials for tissue engineering. Natural and synthetic polymers have been extensively used as biomaterials due to their biodegradability and biocompatibility for different applications, i.e., wound healing, bone, nerve, tendon, muscle, cartilage and cardiac tissue engineering [62–66].

Biocompatible synthetic polymers are promissory materials with tunable properties as microstructure, degradation rate, chemical composition, and mechanical properties. The most commonly used synthetic degradable polymers for tissue engineering and drug delivery are mainly aliphatic polyesters including poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(caprolactone) (PCL) and their copolymers [67–70]. The increased interest to replay the extracellular environment to promote tissue regeneration has led to develop materials based on natural polymers. Between the most used natural polymers in tissue engineering are the proteins collagen, gelatin, fibrin, elastin, keratin, silk, zein; polysaccharides as chitosan, hyaluronic acid, alginate; and polynucleotides [71,72].

Several approaches using electrospinning have been studied to enhance the cell adhesion, proliferation and osteogenic differentiation of cells [73,74]. For instance collagen fibers have been widely used because in nature is responsible to guide the mineralization process [75]. Meanwhile, PCL electrospun fibers have shown to improve the stability of biomaterials able to provide a long-time support for cells to allow the synthesis of new ECM and its mineralization [76]. Moreover, blended solutions of synthetic and natural polymers have been evaluated to improve the stability and biocompatibility of biomaterials, as well the addition of inorganic materials for mineralized tissues. PCL fibers are characterized by its hydrophobic properties, thus, blending PCL solution with natural proteins as collagen or

gelatin has resulted in the improvement of wettability and better interaction between cells and electrospun fibers able to promote osteogenesis for bone tissue regeneration [77,78].

The flexibility of EFDTs and the understanding of the hierarchical structure of bone has lead to incorporate inorganic materials for mineralized tissues. Electrospun scaffolds of polycaprolactone (PCL), collagen I and hydroxyapatite (HA) have been shown to support the adhesion, integrin-related signaling and proliferation of mesenchymal stem cells (MSCs), suggesting these matrices serve for bone regeneration [79]. In the same line, electrospun fibers of gelatin modified with calcium phosphate and PCL to facilitate cell interaction and mineralization [80]. Electrospun fibers of silk fibroin for bone tissue engineering have been used due to the presence of arginine-glycine-aspartate (RGD) sequences specific for cell adhesion and used for the incorporation of HA nanoparticles [81].

Proteins from vegetal sources have gain attention in their use for biomedical applications. Zein is the main component of endosperm corn that has been processed by electrospinning with controllable morphology and diameter of fibers, by modifying process parameter [82,83]. Zein has been used in combination with polymers as cellulose acetate and gelatin to design electrospun fibers for tissue engineering applications [84,85]. For cardiac tissue engineering electrospun fibers of poly(glycerol sebacate) and zein have been explored, results shown improved mechanical and physicochemical properties by the addition of zein [86]. Modified zein with poly(L-lysine) has been processed to form nanfibrous scaffolds with an efficient adhesion, proliferation and differentiation suitable for neural stem cells [87]. As well, polysaccharides have been considered as biomaterials. Chitosan is the most used for tissue engineering and as drug carrier for different molecules [88]. Chitosan fibers have been prepared by electrospinning and treated to enhance the formation of HA [89]. Results have

shown that cell proliferation increased and enhanced cell differentiation, due to the fiber structure and composition.

The increased interest on the use of renewable resources, the extraction of proteins from waste as keratin [90]. Recently the use of wool or hair keratin to design biomaterials has increased. For instance, wool and hair keratin fibers have been evaluated for their use in tissue engineering. Results showed that keratin improves the cell adhesion due to the presence of RGD-like and LDV sequences recognized in native ECM [91,92].

The arrangement of fibers is an important factor for muscle, tendons, periodontal ligament and nerve regeneration; thus, the use of rotator collector has been implemented as a modification of the basic setup of EFDTs [93,94]. For instance, cells seeded onto PCL/gelatin and PCL/elastin aligned electrospun fibers shown a preferential direction in their morphology and the extent elongation of neurite along the fibers [45,95].

EFDTs have been also used for modification of surfaces as titanium alloys to improve the interface between cells and material devices. Titanium surfaces have been coated with natural proteins as keratin to improve the cell adhesion of fibroblast for dental implant applications by electrospinning process [96]. Moreover, aligned keratin electrospun fibers deposited onto these Ti surfaces allow the alignment of fibroblast along the fibers, similar to periodontal ligament arrangement in nature [44].

2.6.2 Drug delivery systems

The research on biomaterials has been focused on the development of drug delivery systems to avoid the first-pass metabolism when substances are orally administered. For the regeneration of damaged tissues, the release of growth factors in the injured zone has been evaluated. For this propose EFDTs have offer a good alternative to encapsulate active molecules as antibacterial agents, growth factors, proteins or peptides and cells to enhance the wound healing and regeneration of tissues [97,98]. Moreover, application on molecular delivery systems of EFDTs have an attractive feature as its high loading capacity, high encapsulation efficiency, ease of operation and cost-effectiveness, high surface-to-volume ratio, which can accelerate the solubility of the drug in the aqueous solution and improve the efficiency of the drug, and the possibility to use biodegradable synthetic or natural polymers which can modulate and protect the molecules or drug maintaining the bioactivity of the material [99].

Numerous studies have reported the development of nanofiber spun mats for molecular delivery applications with reported successfully activity in the biomedical field. Antibacterial agents have been the most common drug molecules encapsulated, using different polymers such as PLA, PLGA and PCL for its biodegradability and also used for controlling the release pattern of the drug. The development of these carriers by EFDTs allows to choose the polymeric or composite system for the preservation of the therapeutic effect, for example blending electrospinning method where drug encapsulation is achieved through electrospinning in a single step, because drugs are dissolved or dispersed in the polymeric solution [100]. Multilayer devices prepared by electrospinning of PCL and poly(ethylene-co-

vinyl acetate) have shown to have good encapsulation efficiency increasing the sustained realese time from 6 to 15 days [101].

Co-axial electrospinning or electrospraying have shown that molecules encapsulated in the core of fibers or particles have a more controllable release [50,102]. The treatment of pain in chronical diseases as rheumatoid arthritis, has led to the development of new molecular delivery systems, as capsules or particles at micro and sub-micrometric size scale, to be used as carriers for controlled drug and molecular release. For instance, special synthesis using electrohydrodynamic atomization an electro-dropping technology was able to develop a mono-component device made of cellulose acetate based on the use of coaxial needles to design core/shell architectures to confine anti-inflammatory drugs (ketoprofen lysine) as microcarriers with mono- (MC) or bi-phasic (BC) composition as more efficiently for oral delivery applications [103]. The treatment of chronic skin wounds requires the prevention of infections in exposed tissues, thus have been developed strategies as collagen combined with PCL to encapsulate drugs as gentamicin sulfate, to be release locally [46].

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CHAPTER 3

DESIGN OF ALIGNED KERATIN FIBERS FOR SURFACE MODIFICATION OF TITANIUM PLUGS FOR TRANSMUCOSAL IMPLANT APPLICATIONS

3.1 INTRODUCTION

Medical devices made from inert materials as titanium are widely used as substitute of structures as joint replacement protheses, femoral stems, fracture plates, spinal cages, and dental implants [1]. However, the cell response can be influenced by chemical and topographic signals, thus, the research in biomaterials has been focused on surface modification of these devices to improve the cell attachment and promote tissue regeneration [2]. Moreover, electrospinning allows to create a topography in order to mimic the extracellular matrix (ECM) of tissues as nerve, muscle, tendons, and periodontal ligament [3–8]. In this context, several techniques have been used to create substrates with physical topographies (i.e. pits, groves, steps, oriented fibers) [9]. Electrospinning has been presented as useful technology to generate aligned micro/nano fibers, by using a rotating collector instead a plate collector. The parameter process mentioned in the first chapter (i.e., process parameters, solution properties and environmental conditions), and the rotatory speed of collector should be optimized for the obtention of aligned fibers.

In particular, transmucosal implants body has contact with bone, meanwhile the collar has contact with soft tissues. However, at a long time, there are different risk factors that can cause periimplantitis, between them, the lack of sealing between the soft tissue and dental implant that cause the epithelium surrounding implant grows apically exposing the implant

and allowing the formation of biofilm similar to periodontitis diseases [10–13]. Thus, different strategies have been developed to modify the surface of titanium implants to promote the protein adsorption and promote the cell growth [14]. Titanium surface modification has been explored by different technologies based on electrochemical and physical techniques. Moreover, the biological functionalization of titanium surfaces with natural polymers by electrospinning technique has been reported to improve the interaction of fibroblast *in vitro* [15]. Randomly oriented keratin fibers have been deposited onto nanogrooved Ti surfaces [16]. Results showed a predominant biological signal induced by the presence of random keratin fibers with lose of guidance by the aligned patter of nanogrooves, thus fibroblast growth randomly. Keratin is a natural protein founded in hair, feathers, wool, nails, and horns, able to improve cell proliferation [17,18]. Wool keratin can be recovered from wastes, thus support a green and sustainable resource to use in biomedical applications due to its good biocompatibility and biodegradability, and it supports the adhesion and proliferation of cells [19,20]. Hence, the proposed work is aimed to optimize the electrospinning process and investigate the possibility to obtain submicrometric wool keratin fibers aligned to the nanogrooves of a Ti-substrate in order to overcome the main issues related to the randomly oriented fibers, to stimulate topographically and biochemically fibroblasts to improve the adhesion and maximize their orientation.

The design of materials for tissue engineering is based on mimic the structure of the extracellular matrix (ECM) of biological systems. The complex architecture of tissues such as nerve, smooth muscle, tendons, and periodontal ligament has led to manufacture materials with specific topographies to stimulate the cellular behavior.

3.2 MATERIALS AND METHODS

3.2.1 Optimization of aligned keratin fibers.

Keratin obtained by a green approach, extracted from discarded wool by sulfitolysis with sodium metabisulfite, as previously described [16,21] was used for the electrospinning process. Wool keratin was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Sigma-Aldrich, Italy) by stirring at room temperature for 24 h until obtaining a clear solution (10% (wt/v).

3.2.2 Deposition of aligned keratin fibers onto nanogrooved Ti-substrate.

Commercially pure titanium foils (Titanium foil 0.025 mm, 99.94%, Alfa Aesar) of 10 x 10 mm were used as substrates for keratin fiber deposition. The shape of the substrates was selected according to the requirements of the rotating collector necessary for the deposition of aligned submicrometric-fibers. The morphology and roughness of these commercial titanium foils were compared with the one previously obtained onto titanium disks [16].

Electrospun fibers were processed by using a commercial electrospinning system (NANON 01, MECC, Japan) equipped with a rotating collector and 18 Ga needle as similarly reported elsewhere [9]. Aligned submicrometric-fibers were fabricated by setting optimal parameters as follows: flow rate of 0.5 ml/h, applied voltage 25 kV, spinneret/collector distance 150 mm, collector rotating rate 2000 rpm. Noteworthy, the process was performed at room temperature and controlled humidity degree (35–40%) to assure optimal environmental conditions for keratin fibers formation. Two different times of deposition (15 and 45 min) were considered.

3.2.3 Morphology characterization

Morphology of the fibers was investigated by Field Emission Scanning Electron Microscopy (FESEM; Quanta 200 FEI, the Netherland) under low vacuum conditions. The average diameter of the fibers was measured from selected SEM images (n = 10) using open source image analysis software (ImageJ 1.50i, National Institutes of Health, USA) whereas fiber alignment via FFT analysis and Orientation plugin (i.e., conversion from spatial to the frequency domain).

The coated samples were subjected to thermal treatment (2h at 180°C) in order to stabilize the fibers as reported previously [4,10]. This process produces at the same time sterile samples suitable for biological characterizations. In this case, morphology and spatial distribution of submicrometric keratin fibers onto titanium substrates were further investigated after sputter coating with a thin Pd-Au layer (ca. 18 nm).

3.3 RESULTS AND DISCUSSION

Biomaterials surface modification represents a very attractive tool to modulate host tissue response. Natural polymers as fiber or structural proteins have been proposed for the modification of materials as transmucosal implants to improve the interface interaction with natural surrounding tissues through biochemical signals to mimic the native environment of fibroblasts in periodontal ligament [16]. As first attempt the optimization of the process as tip-to-collector distance (9, 12 and 15 cm) and rotation speed (RS, 500 to 2000 rpm) of

collector were evaluated at a set voltage and flow rate for the obtention of aligned keratin fibers (Figure 3.1). By increasing the distance, it is possible to observe some aligned fibers, thus 12 and 15 cm of distance were considered to evaluate the rotating rate at 500, 1000 and 2000 rpm. Fibers start to orient themselves along tangential direction, with more efficient fiber alignment in the case of higher rotating rates. These results are in accordance with other works, which demonstrate that increasing the rotation speed allows the formation of bead-free fibers aligned along the movement of rotatory collector. preferential direction of fibers [22,23]. In order to control the effect of rotation onto solvent evaporation, the higher needle/collector distance (15 cm) showed a more efficient control of fiber deposition.

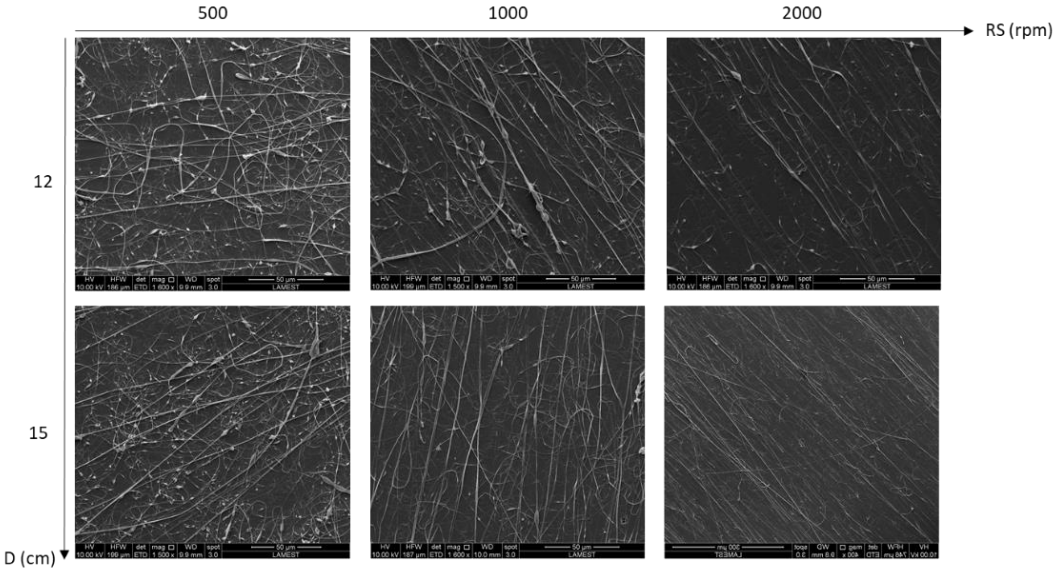

Figure 3.1. Optimization process for wool keratin aligned fibers.

Once optimized the process, nanogrooved Ti substrates were used to electrospun wool keratin fibers, considering two different times of deposition (15 and 45 min). The average diameter of short and long-time deposition fibers measured from SEM images were 0.614 ± 0.2 μm and 1.02 ± 0.44 μm, respectively (Figure 3.2). In terms of fiber orientation, fiber alignment

was analyzed via FFT analysis. Results showed a no significant difference in the alignment of fibers as a function of deposition time. Meanwhile the color map represents the coherency of fibers in the same direction. A slight right shift in the fibers size distribution may be recognized for longer deposition times, due to the growing contribution in the presence of thicker layers which reduces the stretching force applied to the keratin solution during the fiber formation process, as has been denoted in other studies [24].

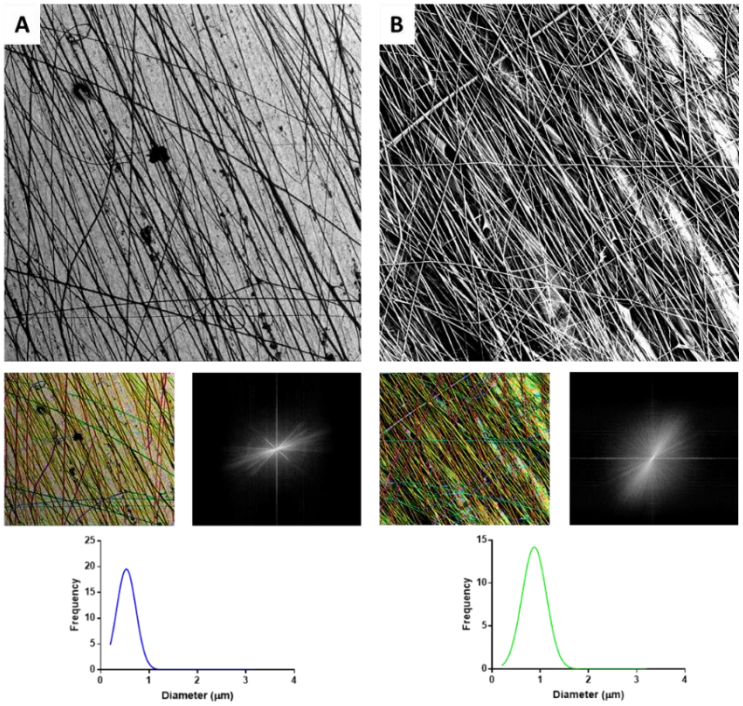

Figure 3.2. Aligned wool keratin fibers onto Ti surfaces after 15 (A) and 45 (B) minutes of deposition time .

Noteworthy, the formation of fibrous layers with different thickness may be relevant to influence mechanism of cell interactions *in vitro*. In the case of longer deposition time, the morphological signals of titanium surfaces can be hidden and not provide the correct stimuli for cells interaction due to the thicker layer of fibers (Figure 3.3). However, when keratin

fibers are thinner, cells can be stimulated by biochemical and topographical cues, which can improve the fibroblast response.

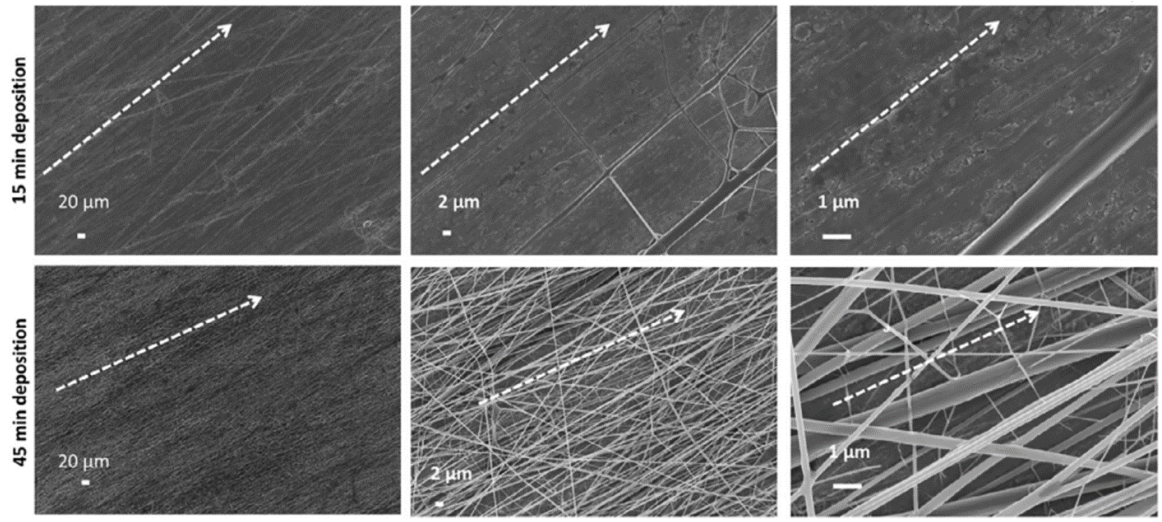

Figure 3.3. SEM images denote the keratin aligned fibers along same direction than nanogroove patterns onto Ti surfaces.

In collaboration with the Department of Applied Science and Technology, Politecnico di Torino, Torino, in Italy biological characterization until 48 h was performed (Figure 3.4).

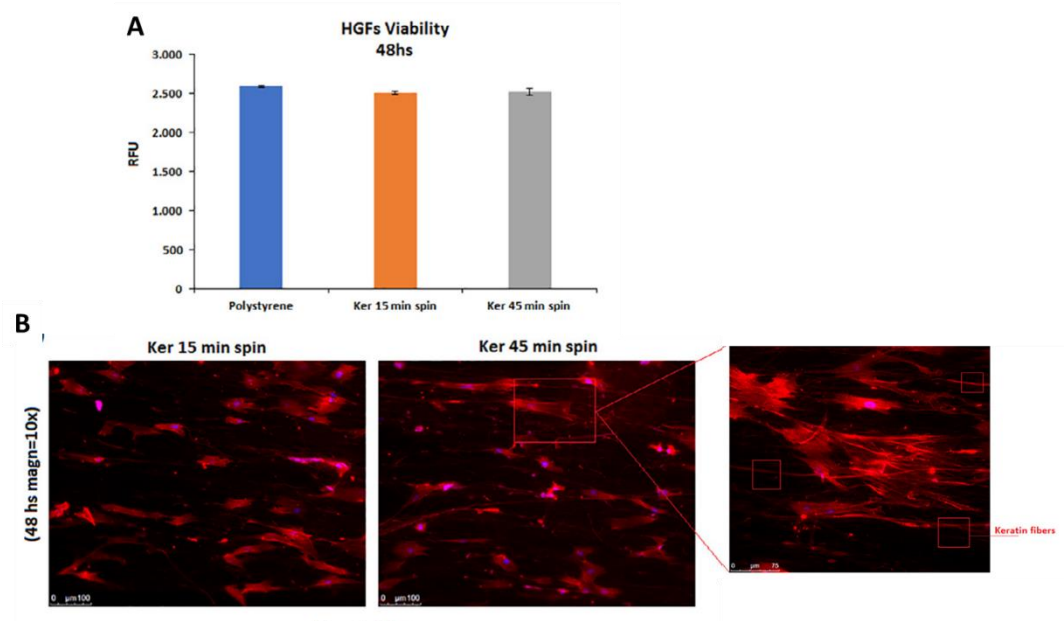

Figure 3.4 Fibroblasts culture onto keratin coated Ti surface. A) Cell viability 48 h, (B) and fluorescence observation of cells alignment.

Their results have shown a cell viability comparable to control in the presence of keratin nanofibers, independently of time deposition. Moreover, confocal images revealed that cells were able to sense the topography of fibers and growth along keratin fibers.

3.4 CONCLUSIONS

Keratin obtained by discarded wool by a green approach was used for the preparation of aligned fibers. The electrospinning technique with the use of rotatory collector allows to align the submicrometric keratin fibers onto nanogrooved Ti surfaces to provide topographical and biochemical signals to support ‘‘along the same spatial direction” the biological stimulation of fibroblasts. Thus, keratin aligned fibers has been deposited onto cylinders of nanogroove Ti, emulating the collar of transdermal implants.

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CHAPTER 4

DESIGN OF PROTEIN ADDED NANOFIBERS FOR TISSUE ENGINEERING

4.1 INTRODUCTION

Tissue engineering is a multidisciplinary field that involves the use of technologies to develop biomaterials to promote tissue regeneration. The interaction between scaffolds, cells and biochemical signals is based on mimic the extracellular matrix (ECM) of native tissues. The ECM has different functions as support for cells, store of growth factors, stimulate cellular differentiation, maintain the homeostasis of tissues and response in case of tissue injury [1]. The ECM is organized in a 3D network of fibrous proteins and polysaccharides assembled in a unique topography which is able to provide biochemical and biophysical signal to cells.

The interest to mimic ECM of tissues has open the use of natural polymer-based materials to enhance the cellular response during the regeneration process [2]. Between the most used natural polymers in tissue engineering are the proteins collagen, gelatin, fibrin, elastin, keratin, silk, zein; polysaccharides as chitosan, hyaluronic acid, alginate; and polynucleotides [3].

The main component of ECM is collagen and is the most abundant protein in the human body with more than twenty types, being type I the most abundant followed by collagen type III [4]. Collagen is a triple helix protein, characterized by a repetitive sequence of amino acids (X-Y-Glycine, where X and Y commonly correspond to proline and hydroxyproline [5]. The triple helix consists of three strands coiled around each other stabilized by glycine residue.

These triple helices can form a quaternary structure depending of collagen type. The complicated structure of collagen makes difficult to extract and isolate the protein. Collagen has poor immunogenic properties compared with other proteins; however it has antigenic determinants, in the non-helical telopeptide region and in the sequence of the helical region of α-chains [6,7]. Gelatin is the denatured form of collagen, derived from the partial hydrolysis of collagen and depending on the extraction and manufacturing method there are type A, acidic treatment, and type B, alkaline treatment [8]. Gelatin has several advantages for tissue regeneration, as biocompatibility, biodegradability and contains Arg-Gly-Asp (RGD)-like sequences to promote cell adhesion [9].

Keratin is a fibrous protein, founded in hair, wool, feathers, nails, and horns of mammals, reptiles and birds. Keratin proteins can be classified in intermediate filament proteins and the matrix proteins. The characteristic secondary structure of intermediate filaments is α-helix, known also as α-keratins, characterized by low sulfur content. The matrix proteins are globular, are high sulfur content and are surrounding the intermediate filament proteins interacting through disulfide bonds [10]. The presence of cell binding motifs, as RGD and LDV (Leu-Asp-Val), the ability to self-assembly make it a good natural polymer for the design of biomaterials for tissue regeneration [11].

Zein is a vegetable protein found in the endosperm of corn that has been explored for tissue engineering and drug delivery application due to its good biocompatibility [12,13]. The amino acid sequence is characterized by non-polar, uncharged residues as glutamine, leucine, proline, and alanine [14]. Due to its composition, zein is an hydrophobic protein, which may prevent cells attachment, however, it has been widely proposed for its use in drug delivery systems to allow a controllable and sustained release [13,15].

Despite the good cell biocompatibility and cell affinity of natural polymers, there are some disadvantages as instability in water, and low mechanical strength. There are different strategies to modified natural-based materials as chemical or physical crosslinking. For instance, chemical crosslinking can add some cytotoxicity to biomaterials or change the final morphology of scaffolds [16]. Thus, blending natural and synthetic polymers has resulted in improved stability and biochemical signal of biomaterials [17,18]. The most commonly used synthetic degradable polymers for tissue engineering and drug delivery are mainly aliphatic polyesters including poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(caprolactone) (PCL) and their copolymers. PCL is a semi-crystalline polymer that can be easily processed and dissolved with organic solvents. PCL has slow degradation rate through hydrolysis of ester linkages, so, has been studied for tissues with longer regeneration process and for sustained drug release [19–21]. Because its hydrophobic properties, PCL has been blended with other polymers, mainly natural polymers as collagen, gelatin, keratin, elastin, silk among others to enhance the hydrophilicity and cell interaction [18,22–24].

As has been mentioned in the previous chapter, electrospinning allows the formation of 2D/3D fibrous matrices to mimic the ECM of cells. In this chapter, blended polymers of PCL with gelatin, zein and keratin were processed via electrospinning and characterized and evaluated their ability to improve proliferation of mesenchymal stem cells *in vitro*.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of bicomponent fibers

Gelatin (type A from porcine skin, Sigma-Aldrich, USA), zein (Sigma-Aldrich, USA), keratin (provided by ISMAC/CNR) and PCL (Mw: 65 kDa, Sigma-Aldrich, Italy) were dissolved individually in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (Sigma-Aldrich, Italy) by stirring at RH by 24 h to get 10% (w/v) solution of each one. Then, PCL was mixed in a ratio of 50:50 with gelatin (PCL/gelatin), zein (PCL/zein), and keratin (PCL/keratin) to obtain the blended solution.

Electrospun fibers were produced via electrospinning by commercialized equipment (NANON 01; MECC, Japan). The solutions were placed in 5 mL syringe connected to a stainless-steel needle. The parameters for the deposition of fibers were optimized as indicated in table 4.1.

Table 4.1. Electrospinning parameters used for the obtantion of bicomponent fibers.

Polymer solution	Voltge Applied (kV)	Flow rate (ml/h)	Tip-to-collector distance (mm)
PCL	22	0.1	120
PCL/Gelatin	15	0.1	130
PCL/Zein	12	0.1	130
PCL/Keratin	25	0.1	120

4.2.2 Morphology characterization

To evaluate the morphological features of PCL/gelatin, PCL/zein and PCL/keratin fibers, scanning electron microscopy (SEM; Quanta FEG 200 FEI, The Netherlands) was used under high vacuum conditions. Previous observation, samples were coated with a Pd-Aunanolayer, using a sputter coating (Emitech K550, Italy) to improve the conductivityThe average diameter of fibers was measured on selected SEM images, while data were elaborated by using an open-source image analysis software (ImageJ 1.50i; National Institutes of Health). Results are expressed as mean ± standard deviation (SD).

4.2.3 Attenuated total reflection Fourier transform infrared spectroscopy analyses

Chemical composition of fibers was investigated by means of Fourier transform infrared spectroscopy coupled with attenuated total reflectance technique (ATR-FTIR, Perkin Elmer Spectrum 100 FTIR spectrophotometer). The spectra were acquired in the spectral region between 4000 and 400 cm^{-1}. The analysis was performed using the Origin software (OriginPro 8 SR0; OriginLab Corporation).

4.2.4 hMSCs Cell Culture

Biological assays were perform using human mesenchymal stem cells (hMSCs; Sigma-Aldrich, Italy, SCC034). hMSCs were cultured in Eagle's alpha minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum, antibiotic solution (100 μg/ml

streptomycin and 100 U/mL) and 2 m*M* L-glutamine, incubated at 37 °C in humidified atmosphere with 5% CO^2 and 95% air.

4.2.5 Biological tests

Previous biological assays, nanofibrous scaffolds were cut into a disc shape and then sterilized with UV light for 1 hour. Biological assays were performed using hMSC at 5×10^3 onto nanofibers scaffolds of PCL/gelatin, PCL/zein and PCL/keratin in Eagle's alpha minimum essential medium supplemented with 10% of fetal bovine serum, antibiotic solution (streptomycin 100 μg/mL and penicillin 100 U/mL) and 2 m*M L*-glutamine.

For cell proliferation, the cell counting kit-8 (CCK-8, Dojindo) was used to analyze the proliferation of hMSCs. Briefly, at 1, 3, 7 and 14 days in culture, the culture media was changed by fresh media and 10 %(v/v) of CCK-8 reagent was added. After 4 h of incubation, the medium was removed and placed into a 96-well plate reader. The masurament of absorbance was recorded at 450 nm in spectrophotometer (Wallac Victor3 1420, PerkinElmer, Boston, MA).

4.2.6 Cell morphology

The morphology of cells onto nanofiber scaffolds of PCL, PCL/gelatin, PCL/zein and PCL/keratin was evaluated by FESEM after 24 hours. The hMSCs were cultured onto fibers scaffolds and fixed with 4% paraformaldehyde. Then, the samples were washed with PBS

and dehydrated with increasing concentrations of alcohol (25–50–75–90–100%, 5 min each) and air-dried.

For cell morphology via fluorescence microscopy, before seeding onto nanofiber scaffolds, culture cells were incubated with CellTracker Deep Red (Thermo Fisher scientific) in phenol red-free medium at 37°C for 30 min. Subsequently, the cell culture was washed with PBS and incubated for 1 h in complete medium. After recovery, cells were trypsinzed, and incubated for 24 h onto nanofiber scaffolds and evaluated for cell–material interaction.

4.3 RESULTS AND DISCUSSION

The development of biomaterials for tissue engineering is focus on develop instructive materials to provide biochemical and morphological cues to guide the cellular response. For this propose, different technologies have been explored for the fabrication of fibers in micro and nano scale to mimic the fiber structure of ECM. Electrospinning is a versatile technique to process natural and synthetic polymers that can be used for tissue engineering applications [25]. Synthetic polymers as PCL are biocompatible, with good mechanical properties, however, are hydrophobic, which may influence negatively the cell adhesion [26,27]. Meanwhile, natural polymers have good biocompatibility and provide biochemical signals to cells, however, they have poor stability [28]. In this work, different blended solutions were processed for the obtention of bicomponent fibers.

By controlling electrospinning parameters, randomly nanofibers of PCL/gelatin, PCL/zein and PCL/keratin were obtained. As a control, fibers of PCL solution were electrospun. Random fibers were obtained under optimized conditions. Fibers from the PCL solution showed the presence of some defects along fibers, and diameter average of 0.171 ± 0.036 μm (Figure 4.1 A). These characteristics are related with the high permittivity of HFIP used in the solution, that ensured stronger polar interactions among the polymer chains mediated by solvent molecules, but increased instability phenomena at lower flow rates [29,30]. In contrast, fibers from blended solutions of PCL/gelatin, PCL/zein and PCL/keratin showed defect free nanofibers characterized by diameters of 0.564 ± 0.102 μm, 0.153 ± 0.027 μm, 0.124 ± 0.036 μm, respectively (Figure 4.1 B, C and D).

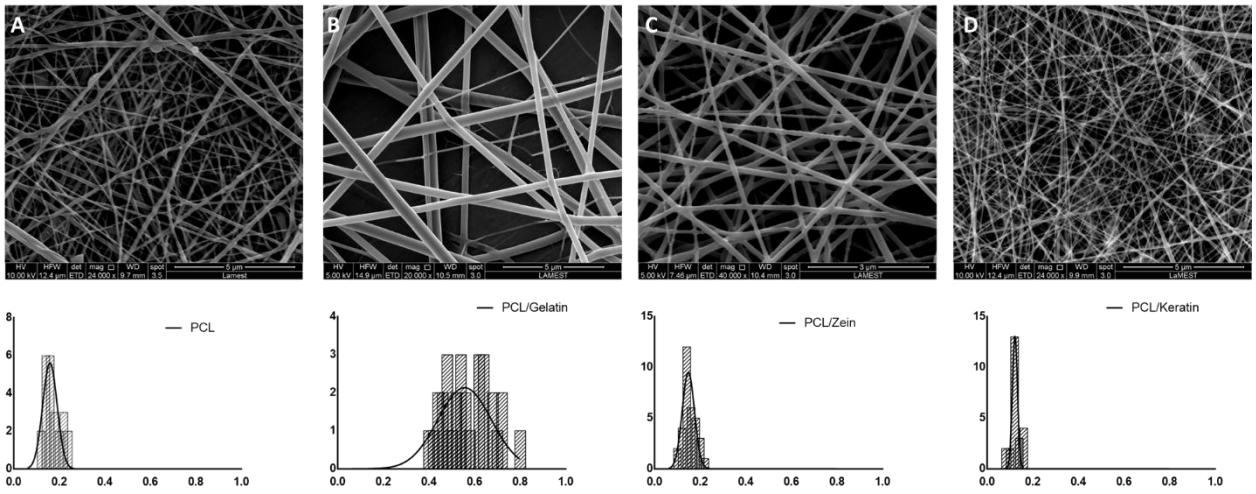

Figure 4.1. Scanning microscopy of PCL (A), PCL/gelatin (B), PCL/zein (C), and PCL/keratin (D) fibers with diameter distribution.

Once optimized the morphology of fibers, to verify the incorporation of the natural polymers into the fibers, ATR-FTIR spectroscopy analyses were performed. To characterize the spectra of natural polymers, electrospun fibers from the pure solution of gelatin, zein and keratin were obtained. All three materials exhibit similar bands as expected due to their protein nature (Figure 4.2). The characteristic bands corresponding to amide I, amide II and amide III are present at 1650 cm^{-1} (C=O stretching), 1540 cm^{-1} (N-H bending associated with C-N stretching), and 1240 cm^{-1} (C-N and N-H groups of bound amide), respectively; and amide A at 3286 cm^{-1} [31–35].

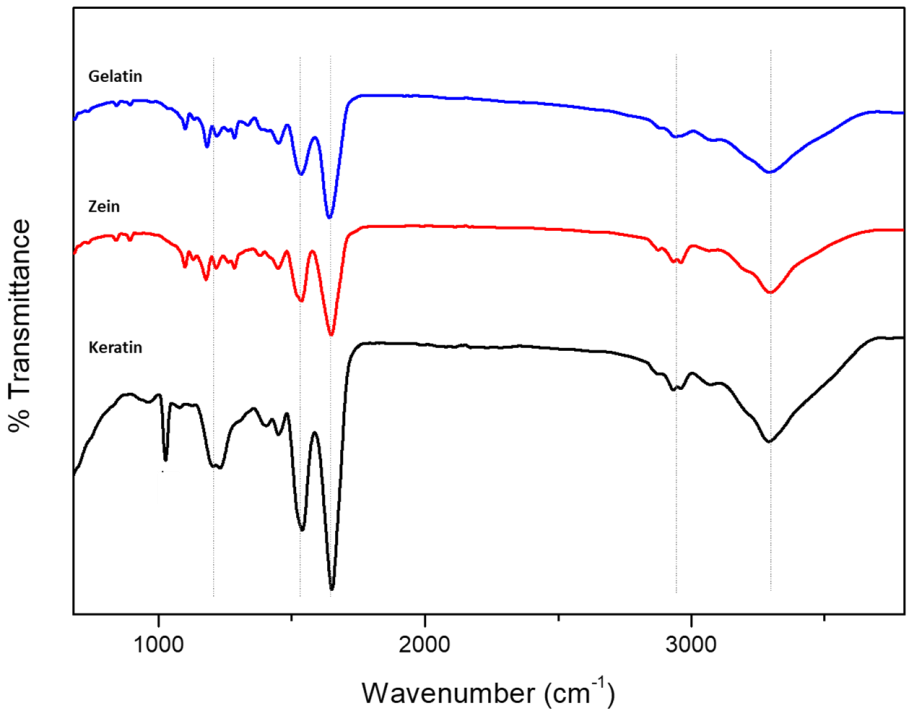

Figure4.2. ATR-FTIR of electrospun fibers of gelatin, zein and keratin.

Meanwhile, figure 4.3 shows the spectra of electrospun fibers from pure PCL solution. The characteristic spectrum of PCL are present at 2949 cm^{-1} (asymmetric CH_2 stretching), 2865 cm^{-1} (symmetric CH_2 stretching), 1727 cm^{-1} (carbonyl stretching), 1293 cm^{-1} (C-O and C-C stretching), 1240 cm^{-1} (asymmetric C-O-C stretching) and 1170 cm^{-1} (symmetric C-O-C stretching) [36]. The ATR-FTIR of bicomponent fibers showed the characteristic bands of PCL and those corresponding to amide I (1650 cm^{-1}), amide II (1530 cm^{-1}) distinctive of proteins, thus confirming the presence of gelatin, zein and keratin into the fibers, respectively.

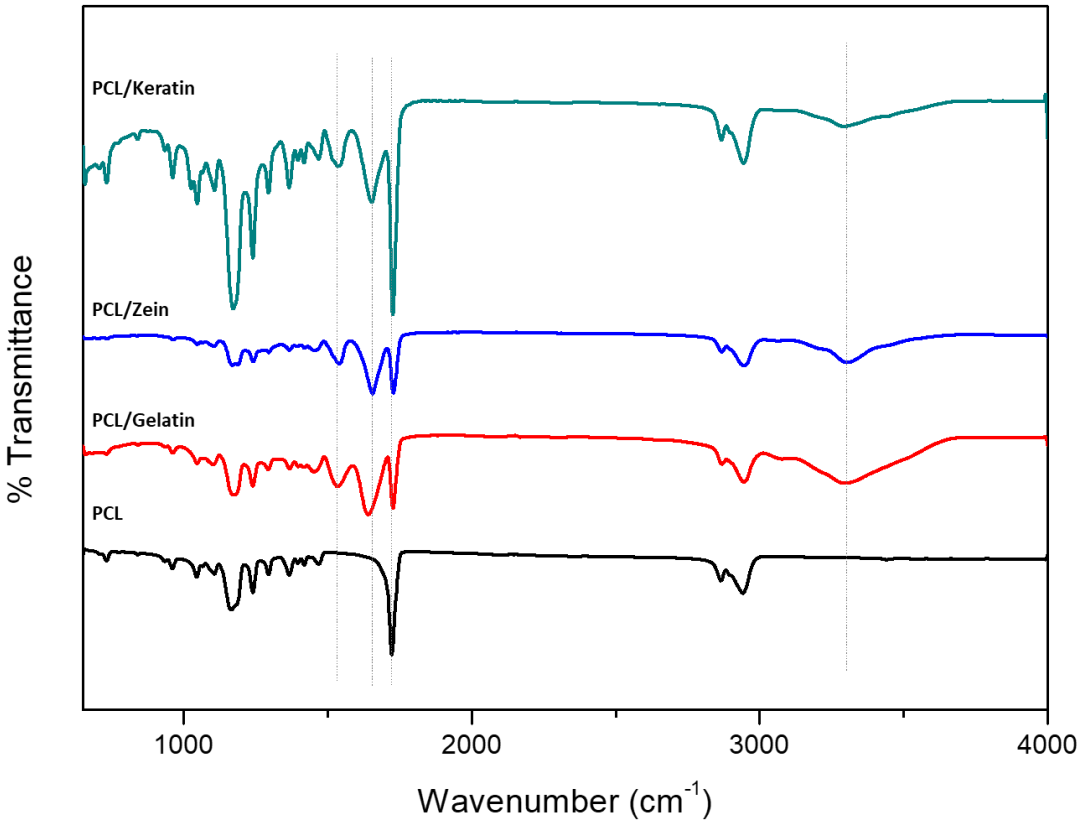

Figure 4.3. ATR-FTIR spectra of PCL fibers and bicomponent fibers.

Cell morphology of hMSCs seeded onto scaffolds was observed by SEM (Figure 4.4 A-C). After 24 hours in culture, hMSCs distributed onto the bicomponent fibers and formed clusters, and it is possible to distinguish elongated structures along fibers. Moreover, after 3 days in culture, confocal images showed the cell interaction with bicomponent fibers (Figure 4.4 A'-C'). hMSCs in contact with bicomponent fibers of PCL/gelatin and PCL/keratin appeared to have projection of their cytoplasm which correspond to lamellipodia and cell density appear to be greater. For bicomponent fibers of PCL/zein, cells grew isolated onto the scaffolds, with rounded morphology and shorter cytoplasmatic projections, as can be distinguish in the confocal images.

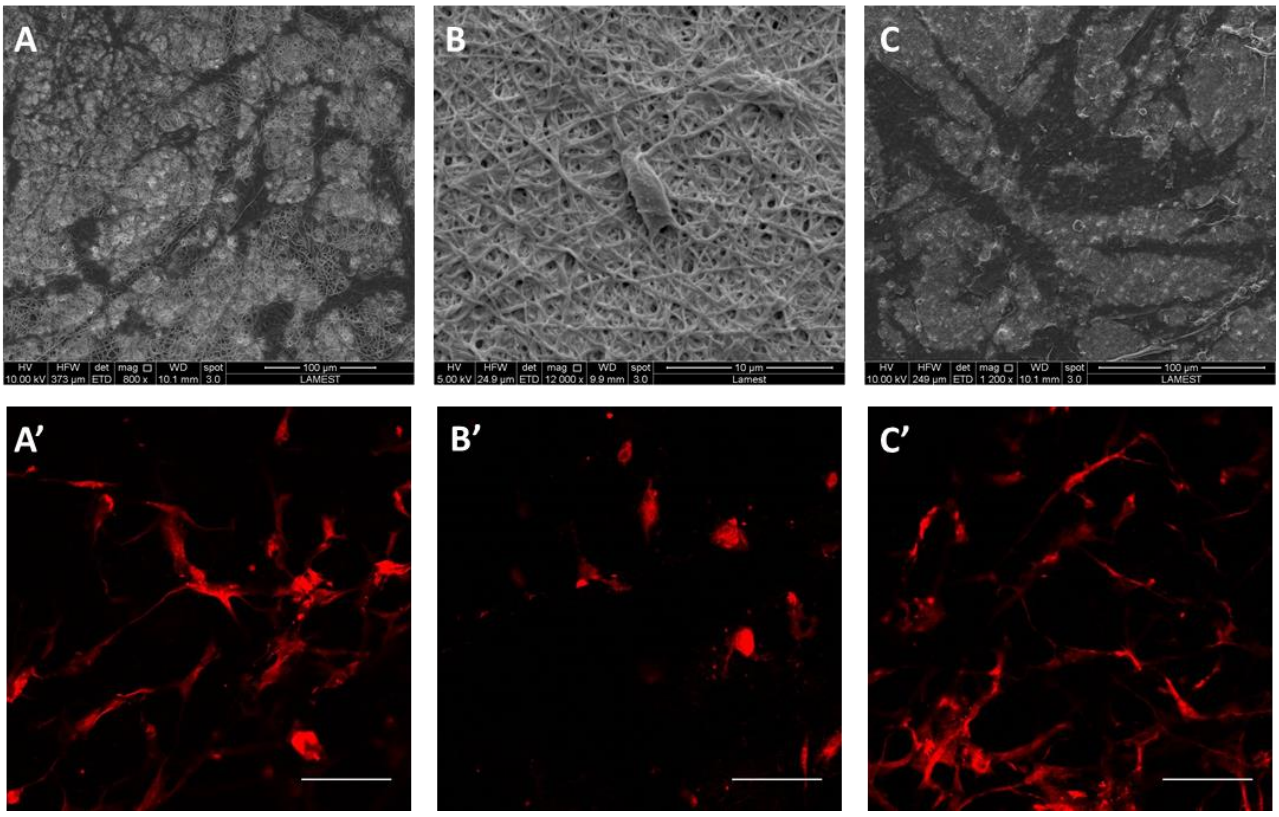

Figure 4.4. SEM images of hMSCs after 24 h in culture onto bicomponent scaffolds of PCL/gelatin (A), PCL/zein (B), and PCL/keratin (C). Confocal images of hMSCs cultured after 3 days onto bicomponent scaffolds A'- C', respectively.

PCL is a biocompatible synthetic polymer, however, is characterized by its high hydrophobicity, thus the adhesion of cells can be affected. Thus, to improve the biological response, natural proteins as gelatin, zein and keratin were added to improve the biological response. To analyze cell biocompatibility and the influence of different component of scaffolds, CCK-8 assay was performed (Figure 4.5). The amount of the formazan dye, generated by the activities of dehydrogenases in cells, is directly proportional to the number of living cells. According to the assay, the cell density increases onto all bicomponent fibers with the time. From the first day it is possible to observe a higher response of cells seeded onto bicomponent fibers with gelatin and keratin compared to PCL alone ($p<0.05$).

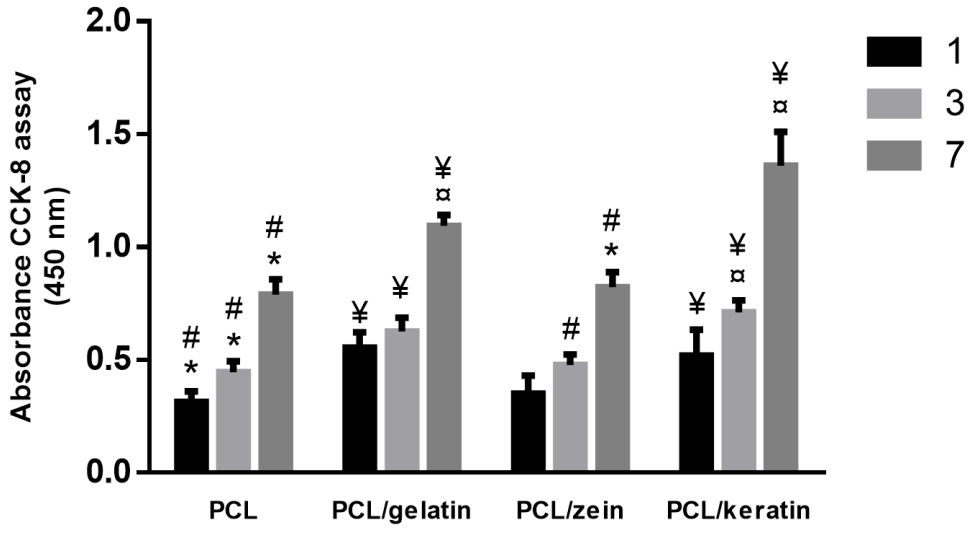

*Figure 4.5. ¥ denotes statistical significance PCL fibers; * denotes statistical significance difference compared to PCL/gelatin fibers; ¤ denotes statistical significance compared to PCL/zein fibers; # denotes statistical significance difference compared to PCL/keratin fibers. (p<0.05)*

Gelatin is considered an excellent alternative of collagen-based materials since it is nonimmunogenic, moreover, gelatin, differently to collagen does not show any denaturation

phenomena due to the interaction with the applied electric field during electrospinning process [37]. Keratin and gelatin are proteins that can influence similarly the cell proliferation, due to their content of RGD-like and LDV-like adhesion motifs that improve the interface between cells and materials [38,39]. Therefore, cell viability was enhanced in presence of gelatin and keratin proteins compared to bicomponent fibers with zein. Despite the good biocompatibility of zein, cell behavior is similar to PCL fibers, which can be related to the less hydrophilic property of zein compared with other natural proteins [40]. Thus, there is a higher response of cells in bicomponent fibers of PCL/gelatin and PCL/keratin where both morphological and chemical signals influenced on cell behavior.

4.4 CONCLUSIONS

A broad variety of biomaterials have been explored for their use in biomedical applications, between them polymers have gained attention because their biodegradability and biocompatibility. Electrospinning has been widely used to produce scaffolds because it allows to process different biomaterials, which is an advantage to promote tissue regeneration through biochemical and morphological signals. In this work, bicomponent fibers of PCL blended with gelatin, zein and keratin, respectively were fabricated via electrospinning. Blended fibers with zein protein have improved biocompatibility, but similar to PCL fibers, which can be related to the hydrophobic nature. Meanwhile. was demonstrated that cells have similar behavior in presence of gelatin or keratin into the fibers. Thus, the use of blended natural and synthetic polymers is a large field that has been further investigated, for their use in wound healing and tissue engineering.

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CHAPTER 5

HIGHLY POLYDISPERSE KERATIN ADDED NANOFIBERS: INSTRUCTIVE SCAFFOLDS OR ANTIBACTERIAL PLATFORMS?

5.1 INTRODUCTION

Tissue engineering research has been focusing on the design of scaffolds able to reproduce the natural extracellular matrix (ECM) in order to enhance the healing and regeneration process [1]. In particular, the use of collagen for the fabrication of nanofibers via electrospinning technique has been long time considered an effective strategy to design fiber scaffolds with different, morphology, structural arrangement, surface roughness, and porosity, by an accurate control of process parameters and solution properties [2,3]. More recently, alternatives to collagen have been explored for the development of biomaterials as gelatin, silk, keratin, chitosan or zein [4–7].

In this regard, keratin is an abundant protein founded in wool, hair, fur, hooves, and feather that can be used for biomedical applications due to its biodegradable and biocompatible properties [8,9]. Several studies have demonstrate that keratin is able to promote wound healing by stimulating human keratinocytes migration [10,11]. In particular, wool keratin can be extracted from wastes from the textile industry, thus represent a way to use renewable sources [12]. Moreover, the presence of cell binding motifs, such as leucine–aspartic acid–valine (LDV) and arginine–glycine–aspartic acid (RGD) binding residues, contributes to support cellular adhesion and proliferation [13,14]. Keratin extracted from wool can be classified in two groups: intermediate filament proteins (IFPs) known as low-sulfur keratin;

and matrix proteins, which are high-sulfur keratins. There are different chemical extraction methods for wool keratin as alkali hydrolysis, sulfitolysis, reduction, oxidation, and extraction using ionic liquids [15]. In this work, wool keratin extracted by sulfiitolysis was used, characterized by two-high molecular mass bands (60-45 kDa) and low molecular mass bands (28-6 kDa) which represent the low-sulfur keratin of the microfibrils and the high-sulfur keratin of the matrix, respectively, reported in previous works [16].

Despite the good biological properties, keratin-based materials have poor stability and structural integrity due to the high swelling under aqueous environments. Therefore, the combination of keratin with synthetic polymers has been proposed to improve the *in vitro* stability. In the last years, the use of bicomponent fibers combining synthetic polymers with natural polymers as collagen and gelatin [17–20], silk [21], elastin [22,23], and chitosan [24] has been just largely explored. In the previous chapter different proteins were used in combination with PCL, resulting in the improvement of cell proliferation at 7 days. Herein, bicomponent fibers of PCL and highly polydisperse wool keratin nanofibers were optimized and evaluated the *in vitro* stability and biocompatibility at longer time.

5.2 MATERIALS AND METHODS

5.2.1 Preparation of fibers via electrospinning process.

PCL and keratin solutions were prepared individually, and then mixed to create PCL/keratin a solution. PCL (Mw: 65 kDa, Sigma-Aldrich, Italy) and keratin powder (gently provided by ISMAC/CNR) were dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (Sigma-Aldrich) by stirring at RH by 24 h to get 10% (w/v) solution of each one. Different PCL/keratin

solutions with two different mass ratios, 50:50 and 30:70 were prepared. Moreover, solutions with 100:0 (only PCL) and 0:100 (only keratin) mass ratio were used to prepare the positive and negative controls.

Electrospun fibers were produced via electrospinning by commercialized equipment (NANON 01; MECC, Japan). The solutions were placed in a 5 mL syringe connected to a stainless-steel needle. The flow rate of the syringe pump was set at 0.1 mL/h. To optimize the final morphology of PCL/keratin fibers, different parameters—that is, applied voltage ranging from 15 to 25 kV, tip-to-collector distance from 90 to 120 mm—were used. For the PCL fibers, an applied voltage of 22 kV and a tip-to-collector distance equal to 120 mm were used. Finally, samples were properly cut in the form of circular disks (6 mm as diameter) for the biological studies.

5.2.2 Morphology characterization

To evaluate the morphology of PCL/keratin fibers, scanning electron microscopy (SEM; Quanta FEG 200 FEI, The Netherlands) was used under high vacuum conditions. Previous observation, samples were coated with a Pd-Aunanolayer, using a sputter coating (Emitech K550, Italy) to improve the conductivity. The average diameter of fibers was measured on selected SEM images, while data were elaborated by using an open-source image analysis software (ImageJ 1.50i; National Institutes of Health). Results are expressed as mean ± standard deviation (SD).

5.2.3 Attenuated total reflection Fourier transform infrared spectroscopy analyses

Chemical composition was investigated by means of Fourier transform infrared spectroscopy coupled with attenuated total reflectance technique (ATR-FTIR, Perkin Elmer Spectrum 100 FTIR spectrophotometer). The spectra were acquired in the spectral region between 4000 and 400 cm^{-1}. The analysis was performed using the Origin software (OriginPro 8 SR0; OriginLab Corporation).

5.2.4 Wettability via contact angle measurements

The wettability of keratin and PCL/keratin electrospun fiber scaffolds was evaluated by contact angle (CA) measurement. The CA of scaffolds was measured at time zero by a single bidistilled water droplet (5 μL), by the support of a high frame camera. In this case, these strips were cut—length 50 mm, width 10 mm—to perform 10 consecutive measurements on the same specimen.

5.2.5 Degradation of bicomponent nanofibers

To evaluate the structural stability of nanofibers in an aqueous environment, PCL/keratin fibers were cut into squares and incubated in PBS solution up to 14 days. The buffer was changed every 3 days. The bicomponent nanofibers were taken out from the solution and dried under a hood at room temperature. Then PCL/keratin nanofibers were processed for scanning microscopy to examined changes in fiber morphology. To observe chemical changes, TR-FTIR was performed under the above-mentioned conditions.

5.2.6 hMSCs Cell Culture

To examine the cell/material interaction, nanofibrous scaffolds were cut and sterilized with 75% (v/v) ethanol solution for 20 min, washed three times with sterile phosphate buffered saline (PBS) and air-dried. Human mesenchymal stem cells (hMSCs; Sigma-Aldrich, Italy, SCC034). hMSCs were cultured in Eagle's alpha minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum, antibiotic solution (100 μg/ml streptomycin and 100 U/mL) and 2 m*M* L-glutamine, incubated at 37 °C in humidified atmosphere with 5% CO^2 and 95% air.

5.2.7 In vitro assays

Biological assays were performed using hMSC at 5×10^4 onto nanofibers scaffolds of PCL and PCL/keratin in α-MEM at the same conditions mentioned above. For cell adhesion, crystal violet assay was performed at 4 and 24 h of culture in triplicate. Briefly, after the time period, nanofiber scaffold were rinsed three times with PBS to remove non-adherent cells, then adherent cells were fixed with 4% paraformaldehyde and incubated with 0.1% crystal violet solution for 15 min, rinsed with distilled water and then the dye was extracted with 0.1% of sodium dodecyl sulfate (SDS) and absorbance was recorded at 545 nm. Conventional polystyrene 24-well culture plates were used as a control.

For cell proliferation, alamarBlue (AB) assay (Invitrogen) was performed at 3, 7, and 14 days of culture in triplicate. Briefly, after each time of culture, the culture media was changed by

fresh media and AB was added. Fresh medium without cells was also incubated with AB to serve as blank. After 4 h of incubation, the medium was removed and placed into a plate reader. Absorbance was recorded at 570 nm, using 600 nm as a reference wavelength.

5.2.8 Cell morphology

The morphology of cells onto nanofiber scaffolds of PCL and PCL/keratin was evaluated by SEM. The MSC cultures onto fibers scaffolds were fixed 20 min at RH with 4% paraformaldehyde. Then, the samples were washed with PBS and dehydrated with increasing concentrations of alcohol (25–50–75–90–100%, 5 min each) and air-dried.

For cell morphology via fluorescence microscopy, before seeding onto nanofiber scaffolds, cells were incubated with CellTracker Green 5-chloromethylfluorescein diacetate in phenol red-free medium at 37°C for 30 min. Subsequently, the cell culture was washed with PBS and incubated for 1 h in complete medium. After recovery, cells were trypsinized and counted to the desired cell concentration and incubated for 24 h onto nanofiber scaffolds and evaluated for cell–material interaction.

5.2.9 Antibacterial tests

[3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT; Sigma-Aldrich) assays against *Escherichia coli* (*E. coli*, ATCC 33780) and *Staphylococcus aureus* (*S. aureus*, ATCC 25923) was assessed. The growth of pure culture of the *E. coli* and *S. aureus* strains were harvested from agar plate. For qualitative analysis, the disk diffusion method was used.

Briefly, *E. coli* and *S. aureus* pure strains were spread onto the agar plate, with previously circular cut samples of PCL, PCL/keratin, and chlorhexidine disk as a control. For viability, after pure culture of bacterial strains, were resuspended in trypticase soy broth (TSB, BD Bioxon) supplemented with 0.3 μg/mL menadione and 5 μg/mL hemin (Sigma-Aldrich), and adjusted to optical density = 1 at 600 nm. The PCL and PCL/keratin mat samples were placed individually in 24-well plates incubated at RT with 100 μL of TSB for 20 min and then inoculated with 300 μL of bacterial solutions stock (1 × 106 cells/mL) added to each well in a total volume of 1 mL and incubated for 24 h (35°C, natural-light illumination) under aerobic conditions. All experiments were run in triplicate. After culture time, to evaluate biofilm bacterial growth inhibition, bacteria-incubated mats were rinsed with TSB, placed in clean culture-well plates and incubated with MTT:TSB solution (1:10) for 4 h at 35°C. Bacteria-metabolized formazan crystals were solubilized in 2-propanol:dimethyl sulfoxide (1:1) and solution absorbance was read at 570 nm (FilterMax F5). In order to observe the bacterial adhesion on the PCL and PCL/keratin mat sample surfaces, another set of samples was prepared for SEM following standard procedures. Briefly, after incubation and washing, all the tested surfaces were fixed in 2% glutaraldehyde 24 h at RH, then washed three times with PBS and dehydrated through a series of graded ethanol solutions with concentrations as follows: 20, 40, 60, 80, and 100%. Samples were vacuum dried overnight, sputtered with an Au thin film, and observed by SEM at 10 kV.

5.2.10 Statistical analyses

One-way analysis of variance followed by Tukey's post hoc was assessed for the biological assays. Data were presented as mean ± standard error. $p < 0.05$ was considered as statistically significant.

5.3 RESULTS AND DISCUSSION

In the last decade, the use of natural polymers has provided an important tool for the design of biomaterials. Moreover, EFDTs versatility allows the fabrication of biomaterials with different components. Despite, electrospun fibers are promising structures since they are able to mimic the basic fibers morphology of native ECM [25]. Hence, bicomponent fibers combining natural and synthetic polymers have offered a great alternative to develop stable and instructive materials to promote tissue healing and regeneration. In this work, was proposed the combination of polycaprolactone, which has been widely characterized as

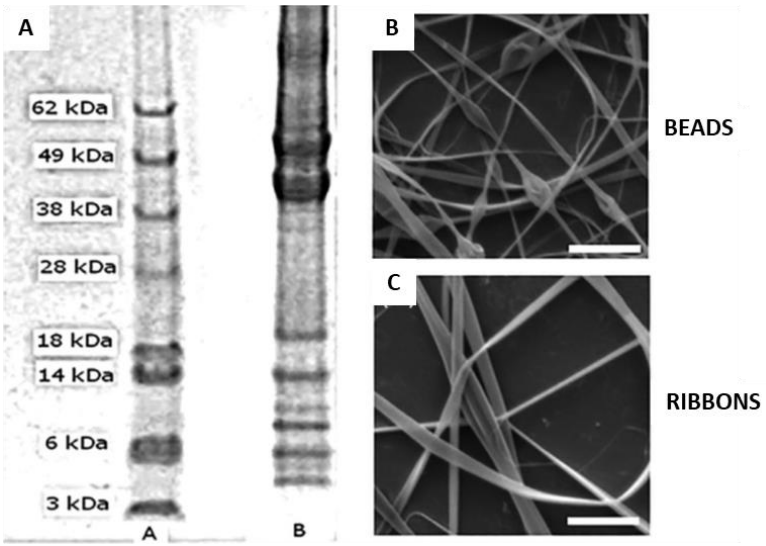

Figure 5.1. A) SDS-PAGE: standard (line A) and wool keratin (line B). B and C) Different morphologies of wool derived keratin nanofibers (scale bar 3 µm); SEM images of beaded and ribbons-like fibers.

biocompatible material, with keratin, a structural protein, to fabricate nanofibers scaffolds via electrospinning.

Keratin can be extracted from hair, wool and feathers for biomedical applications. In this work, keratin from wool fibers extracted by sulfitolysis was used, which is characterized by the presence of high and low molecular weight bands, as mentioned in chapter 2. As has been discussed in the first chapter, the molecular weight can influence the morphological features of nanofibers. In case of electrospun fibers of keratin solution, it is possible to recognize two different morphologies, beaded fibers, and fibers with a gradual transition from rounded to ribbon-like fibers, that can be related to the polydispersity of protein molecular weights (Figure 5.1), as has been reported for other proteins [26].

Taking into consideration the high solubility of the protein and to improve the fiber morphology, keratin was processed with PCL in HFIP. To optimize the process a PCL:keratin ratio of 50:50 was used, and PCL/keratin nanofibers were prepared by changing the applied voltage and tip-to-collector distance. SEM images showed that increasing the voltage there is a reduction of beads density along fibers, as reported in previous studies [27]. Noteworthy, the mechanism of beaded fibers formation is regulated by voltage-to-gap ratio as has been reported in previous studies [28]. Hence, it was also possible to recognize a slight increase of beads density as the tip-to-collector distance decreased from 120 to 90 mm (Figure 5.2). In summary, less beaded fibers were obtained by using 25 kV as applied voltage, with the collector placed at 120 mm from the needle tip.

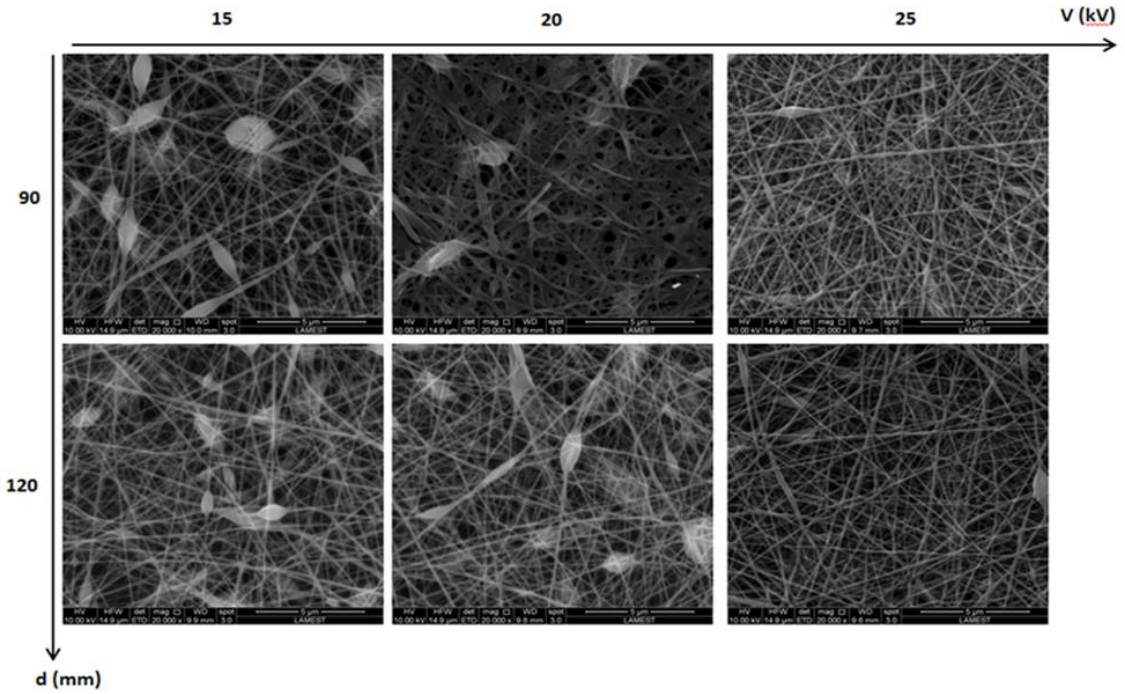

Figure 5.2. Optimization of process parameters for the fabrication of 50/50 PCL/keratin fibers: effect of voltage and electrode distance on fiber morphology and beads formation.

Once optimized the process parameters, different PCL/keratin mass ratio were used (100:0, 50:50, 30:70 and 0:100). In all cases, was possible to obtain random nanofibers with uniform spatial distribution, however the diameters and morphology of fibers were influence by the content of keratin (Figure 5.3). In particular, keratin fibers (0:100) showed a ribbon-like shape, meanwhile in the case of 50:50 ratio, the presence of PCL prevented the formation of such structures. The high polarity of keratin molecules strictly packed to PCL chains tended to dampen the contribution of the solvent evaporation, so generating thicker fibers in comparison with PCL alone, in agreement with similar studies performed on PCL/gelatin blends [29,30]. In case of solutions with higher content of keratin (30:70), it is possible to recognize two sort of fibers, with characteristics of PCL fibers (100:0) and keratin fibers

(0:100), which can be due to the higher content of keratin not blended homogeneously with PCL.

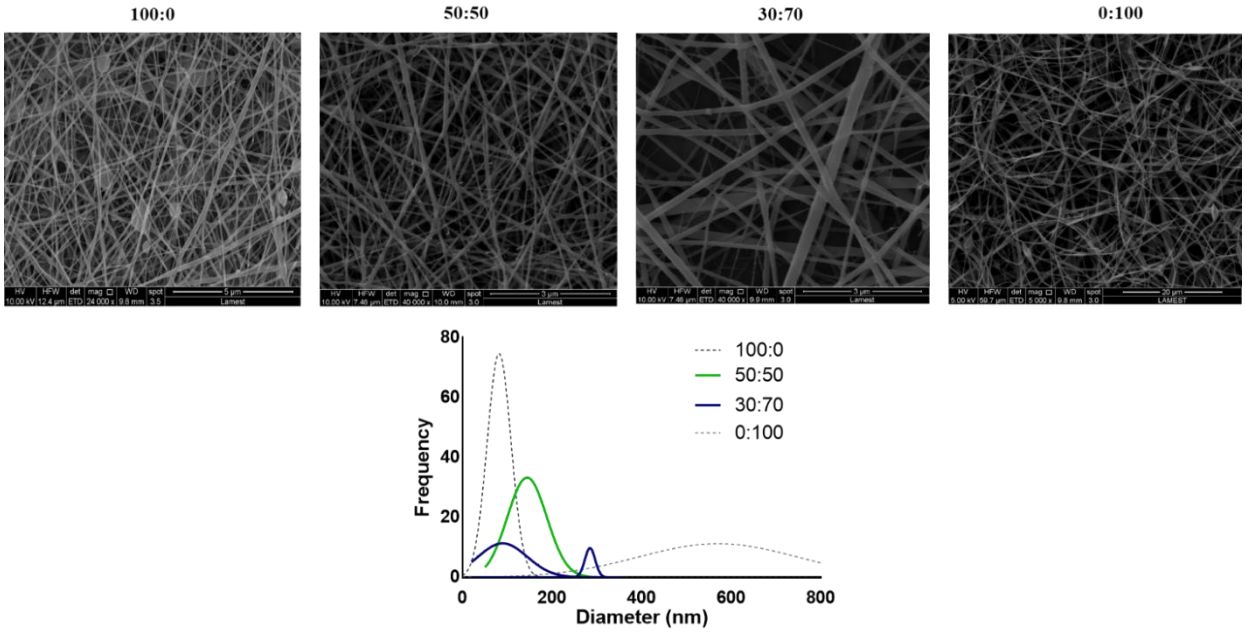

Figure 5.3. Effect of keratin on fiber morphology. SEM images of PCL/keratin fibers with different relative mass ratios and comparative distribution of fiber diameters via image analysis.

The average diameter of PCL (100:0) and keratin (0:100) was 81.7 ± 26.7 and 572.76 ± 18.16 nm, respectively as reported in previous works [30,31]. In the case of 30:70 PCL/keratin, there are fibers with 96.5 ± 44.5 nm and 265.9 ± 45.1 nm, corresponding to those of PCL and keratin fibers, respectively. PCL/keratin with a 50:50 ratio allowed better control of morphology, with an average diameter of 144.1 ± 43.9 nm with homogeneous distribution, proving the good miscibility of PCL and keratin solutions, differently to fibers 30:70 fibers where there is a tendency to separate fibers. Therefore, the blend composition plays an important role in determining the diameter distribution of fibers.

Hence, fibers of 50:50 PCL/keratin were selected for further chemical, physical and biological characterization. To recognize the incorporation of keratin into the fibers ATR-FTIR spectroscopy was performed to PCL, keratin and bicomponent fibers (figure 5.4). The characteristic spectra of keratin include amide I (1650 cm^{-1}), Amide II (1540 cm^{-1}), amide III (1230 cm^{-1}), which are mainly represented by C=O stretching, N-H bending coupled with C-N stretching and, C-N stretching, of the amide groups respectively; the peak at 1025 cm^{-1} was representative of S-O stretching vibration [32,33]. In PCL/keratin fibers, amide I and II were detected, thus confirming the presence of keratin into the fibers. Moreover, amide III, was covered by the region of C-O-C stretching corresponding to a large and characteristic peak of PCL (1722 cm^{-1}) for the carbonyl group (C-O) [19,34]. The local interaction between PCL and keratin can be detected with a moderate shift of the amide II peak in comparison with keratin spectra, that can be related to the interactions among carbonyl groups and amides weak binding via hydrogen bonds [34,35]. Moreover, the peculiar position of amide bands at 1650 and 1540 cm^{-1} in amides I and II, respectively are related to the secondary conformation of proteins confirmed the presence of keratin into α-helix/random coil secondary structure of the protein usually promote by electrospinning process, with potential benefits for *in vitro* cell response [36,37].

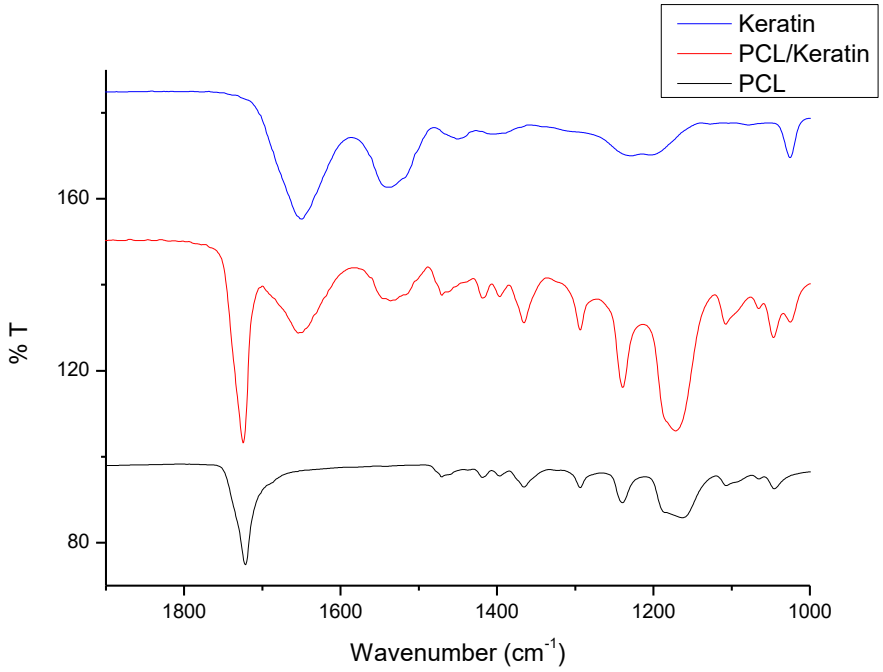

Figure 5.4. ATR-FTIR characterization of PCL, keratin and bicomponent fibers of PCL/keratin.

The main limitation of synthetic polymers as PCL in tissue engineering is related to their innate hydrophobic nature [38]. Thus, a comparative analysis of wettability via CA measurements was performed onto bicomponent fibers of PCL/keratin and fibers of PCL (Figure 5.5). The use of bioactive proteins such as keratin can be extremely important to improve the interface among cells and synthetic polymers [39]. In particular, PCL/keratin fibers showed a smaller CA equal to 51.2 ± 8.4° respect to PCL fibers with CA of 103.0 ± 7.8° as a consequence of the hydrophilic signals exerted by the protein, that can positively influence *in vitro* adsorption of proteins, as recent studies that confirm a preferential protein adsorption on moderate hydrophilic surfaces, characterized by 55° CA [40].

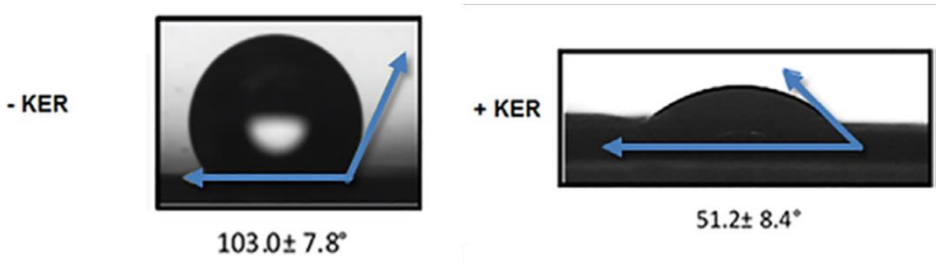

Figure 5.5. Ccomparative analysis of CAs for wettability measurements.

Hence, the interaction of PCL/keratin fibers with stem cells and different bacteria has been investigated in order to evaluate the contribution of keratin as cell or bacteria promotive signal. Cell adhesion of hMSCs onto PCL and PCL/keratin fibers were analyzed by crystal violet at the time intervals previewed. The adhesion in presence of keratin increased after 4 and 24 hours in comparison with PCL fibers ($p<0.05$) (Figure 5.6, A). This result was also confirmed by the fluorescence images that showed cytoplasmatic extension of cells, and more elongated morphology of hMSCs when they are in contact with PCL/keratin nanofibers (Figure 5.6, B). The presence of keratin into the fibers improve the interaction between the cells and fibers, similarly to other natural polymers [2,41,42]. Besides, wool keratin is mainly characterized by arginine, glycine, aspartic acid, valine, and leucine, all included into the native proteins of the ECM. In this case, their organization in specific sequences (i.e., LDV, RGD) basically influences cells adhesion and spreading, just after 24 h, through an integrin-mediated mechanism, where keratin acts as ligand of α4β3 integrin to induce intracellular signaling pathways [43,44].

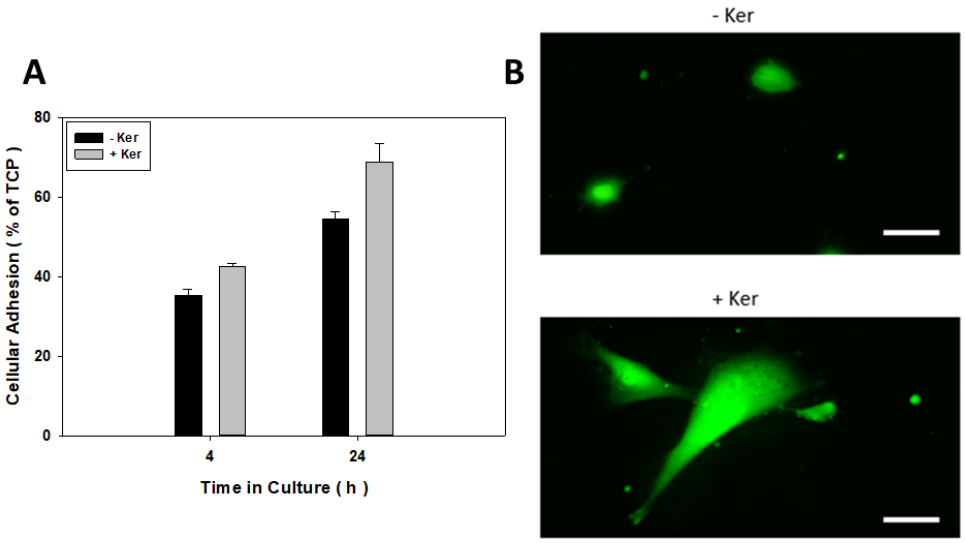

Figure 5.6. In vitro hMSCs response onto PCL and PCL/keratin nanofibers: A) cell adhesion via crystal violet assay and B) cell morphology by confocal images.

Blending natural polymers as wool keratin with synthetic polymers represent an advantage to anchor the protein into the fibers without the use of physical or chemical treatments conventionally used to improve the *in vitro* chemical stability of proteins. In this work, PCL/keratin fibers were incubated in water to evaluate the morphology and chemical stability of fibers. Results confirmed that after 7 days and 14 days in PBS solution, there is a tendency of keratin to be swollen onto the fiber surface, without any drastic alteration of the fibrous network, thus, can support adhesion of cells (Figure 5.7). In this context, the presence of polymers with slow degradation rates improve the chemical and morphological stability of fibers. The ATR-FTIR spectra of fibers after PBS incubation also showed the characteristic bands of amide groups, which means PCL assured the retention of keratin into the fibers. This result was in agreement with similar experimental evidences on different similar

systems [19,45]. That confirmed the capability of PCL fibers to release wool keratin more slowly than other ones with faster in vitro degradation (i.e., PLLA) [46].

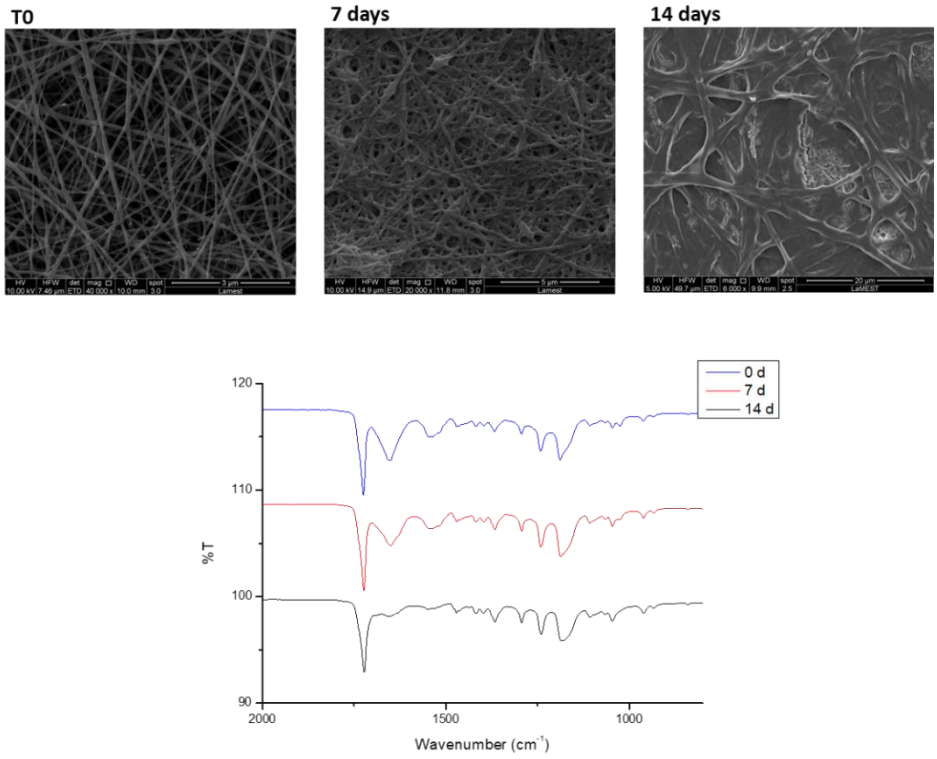

Figure 5.7. SEM and ATR-FTIR characterization of fibers after 7 and 14 days of PBS incubation.

The viability of hMSCs was further enhanced by the presence of keratin into the fibers. SEM images of cells after 3 days in culture into the fibers elongated shape, corroborating that cells preferentially adhered onto bicomponent fibers (Figure 5.8). After a few days in culture, keratin signal onto the fibers enhanced the cell proliferation until 14 days, meanwhile in the case of PCL fibers there is an arrest of metabolic activity of hMSCs.

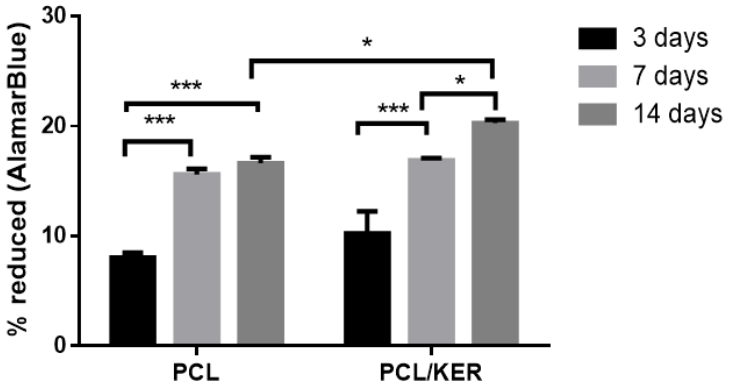

Figure 5.8. In vitro hMSC response onto PCL and PCL/keratin nanofibers.

The process of healing or regeneration can be affected due to the biofilm formation on biomaterials. Recently, the antibacterial property of keratin from feather was evaluated [47]. In this work, the *in vitro* response of two different bacteria population, *E. coli* and *S. aureus*, respectively, was tested onto 50:50 PCL/keratin and PCL nanofibers. Disk diffusion method showed that independently upon the presence of wool keratin, no antibacterial response can be properly attributed to wool keratin (Figure 5.9) on the contrary of positive control (chlorhexidine).

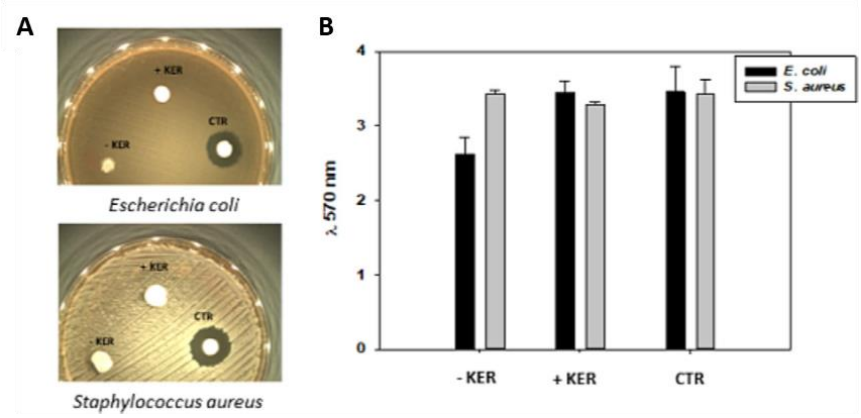

Figure 5.9. Effect of keratin on in vitro early bacterial response. A Disk diffusion method to evaluate E. coli and S. aureus growth in the presence of keratin mats (CTR in chlorhexidine inhibits growth cell. B) Bacterial viability by MTT, similar to culture plate control (without any treatment).

This effect could be related to the secondary structure of the protein, which in case of wool keratin is characterized by high content of α-helix. A comparative study of keratin from different sources (i.e., hair, feather and wool) demonstrated that keratin with β-sheet and random form as feather keratin have more antimicrobial activity [48]. However, wool keratin can be functionalized with antimicrobial agents [49].

5.4 CONCLUSIONS

In this work, the electrospinning process was optimized for the fabrication of bicomponent fibers of PCL and keratin and characterized for their potential use as cell instructive scaffolds for tissue engineering. It is demonstrated that wool keratin blended with PCL into nanometric fibers offers the opportunity to fabricate nanostructured scaffolds suitable for *in vitro* studies. hMSC well adhere and proliferate due to chemical and morphological properties of fibers obtained by electrospinning process and the presence of wool keratin. In the future perspective, the inclusion of further components (i.e., metal ions) could be easily implemented to improve the antibacterial response of keratin rich nanofibers, opening the mind toward new interesting insights for the design of functional interfaces for soft tissue regeneration (i.e., cornea, skin).

Published data

- **I. Cruz-Maya**, V. Guarino, A. Almaguer-Flores, M.A. Alvarez-Perez, A. Varesano, C. Vineis, Highly polydisperse keratin rich nanofibers: Scaffold design and in vitro

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CHAPTER 6

DESIGN OF ELECTROSPRAYED NANOPARTICLES AS VECTOR TO RELEASE BIOACTIVE MOLECULES

6.1 INTRODUCTION

Drug delivery systems are mainly designed to control the molecule release respect to the time, in a specific location. In recent years, electrospraying, an EFDTs able to produce submicrometric droplets, has been used for molecule encapsulation [1,2]. From a technical point of view, it is a rapid single-step technique to prepare capsules or particles to work as carriers of drugs, proteins, enzymes, growth factors and cells [2,3].

The electrospraying setup has the same components as electrospinning, a high voltage supply, a syringe with steel needle a syringe pump and a grounded collector [4]. The electrospraying process is based on the application of an electric field to a liquid droplet which is able to deform the interface of the droplet. This generates an electrostatic force of the droplet, to finally form the Taylor cone [5,6]. When the electrostatic force overcomes the surface tension of the droplet and there is an excess charge, dissipated and smaller charged droplets are ejected from the needle tip to the collector, with production micro or submicrometric particles. Thus, the principle of electrospraying corresponds to the theory of charged droplets [6]. Once droplets are ejected solvent evaporation is produced, which results in solid polymeric particles. To control the size, and obtain reproducible morphologies, electrospraying parameters have been controlling, similar to those mentioned in chapter 1 for

electrospinning, as solution properties, electrospraying parameters and environmental conditions [5,7–9].

The use of polymeric particles is extensively used to encapsulate bioactive molecules, because allow the modification of the components and structure to control the release of active molecules. Synthetic-based, protein-based and polysaccharide-based nanoparticles have been widely processed via electrospraying to encapsulate active molecules [10]. In this chapter we will focus on the use of polysaccharides, specifically the use of chitosan and cellulose-based materials processed by electrospraying for therapeutic treatments.

6.2 POLYSACCHARIDES FOR NANOPARTICLES

Polysaccharides are natural polymers that can be extracted from plant, alga, animal and microbial sources[11]. This group of polymers is composed by monosaccharides linked by O-glyosidic linkages. Biomaterials of chitosan, hyaluronic acid, alginate, and cellulose have shown good biocompatibility, biodegradability and low toxicity [12]. These group of natural polymers are preferred for their use as drug delivery carriers due to the high hydrophilicity, mucoadhesive properties, and can be chemically modified [13–15].

6.2.1 Cellulose-graft-PCL electrosprayed nanoparticles

Recently, the use of amphiphilic polysaccharides for biomedical applications has increased due to the balance between the hydrophobic and hydrophilic fragments. The recent synthetized amphiphilic polysaccharides present a heterogeneous hydrophobic and

hydrophilic chain segments which provide molecule protection and interaction through specific sugar moieties. Cellulose and its derivates are widely used for sustained drug delivery systems due to their properties as low cost, good biodegradability and biocompatibility. Many types of cellulose-based materials have been used, however, recently cellulose graft copolymers are emerging as amphiphilic materials suitable to fabricate smart nanoparticles for drug delivery applications [16,17].

More recently, the use of self-assembled micelles based on amphiphilic cellulose derivatives has forcefully emerged for the fabrication of delivery carriers able to more efficiently encapsulate not only highly water-soluble molecules but also pharmaceutical active ingredients with poor water solubility [18,19]. Cellulose-graft-polycaprolactone (cell-graft-PCL) copolymer has been synthetized by a procedure reported previously [19]. That amphiphilic polymers with a suitable hydrophilic/hydrophobic balance can form a micellar structure by self-assembly process when exposed to a selective solvent concentration value. In case of amphiphilic copolymers, a solvent able to dissolve fastly one portion, also tends to induce the precipitation of the other one, thus promoting the assembly of the copolymer chains to form micellar aggregates. Micellar structures tend to be formed spontaneously above a critical micelle concentration (CMC) when forming a bilayer structure that internalize the hydrophobic portion of the amphiphile, leaving the hydrophilic segment exposed to the inner and outer aqueous environments [20,21].

In the last years, electrospraying allowed the production of agglomeration-free uniform small size particles at micro- and nanometric size scale with controlled sizes , thus promoting the development of innovative drug delivery systems [22]. In our group, electrospraying has been evaluated as innovative manufacturing strategies for the fabrication of amphiphilic

nanoparticles made of cell-graft-PCL, to overcome the limitation of processes, based on evaporation mechanisms.

Different morphologies have been presented, due to the influence of different process conditions [23]. For example, the presence of DS influences on the shape of nanoparticles (Figure 6.1 A). This was directly related to the capability of molecules to influence polar interactions with grafted chains of polymer in solution, during the application of electrostatic forces. In case of electrospinning, the morphology of particles is generally determined by the competition between chains entanglement formation and Coulomb fission at the level of the single droplet. As the solvent evaporates from the droplet, an increase of polymer concentration occurs, with the consequent formation of new entanglements able to stabilize the droplet, thus preventing further subdivision and preserving the spherical shape [24]. Thus, the intercalation between hydrophobic PCL branches grafted along the cellulose backbone induced an alteration of the entanglement density at the equilibrium, promoting the deformation of particles into irregular shapes (concave surfaces), differently to cell-graft-

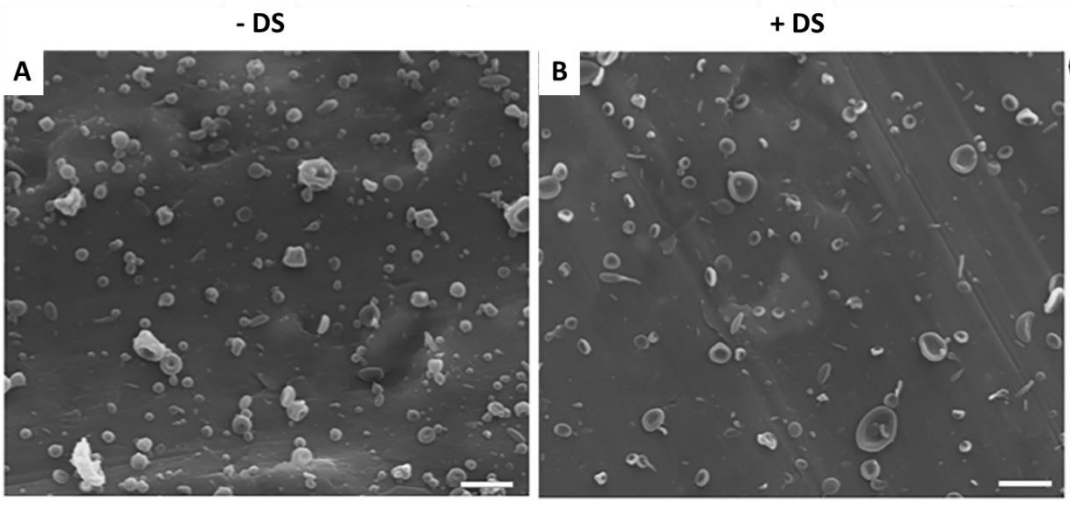

Figure 6.4 Electrospraying process to fabricate cell-g-PCL and DS loaded cell-g-PCL nanoparticles: effect of hydrophilic properties of drug on particle shape (scale bar 1 μm). SEM images of cell-g-PCL electrosprayed (A) unloaded and (B) DS loaded nanoparticles were reported

PCL without drugs, where the elasticity of the entangled network, was able to recover the spherical shape (Figure 6.1 B).

The morphology of particles was also affected by tip-to-collector distance, and flow rate (Figure 6.2 A and B respectively). As increased offspring droplets tended to freeze their shape at the time of fission, before to reach the collector, thus forming non-spherical particles with different shapes Meanwhile, as the flow rate increase promoted the formation of

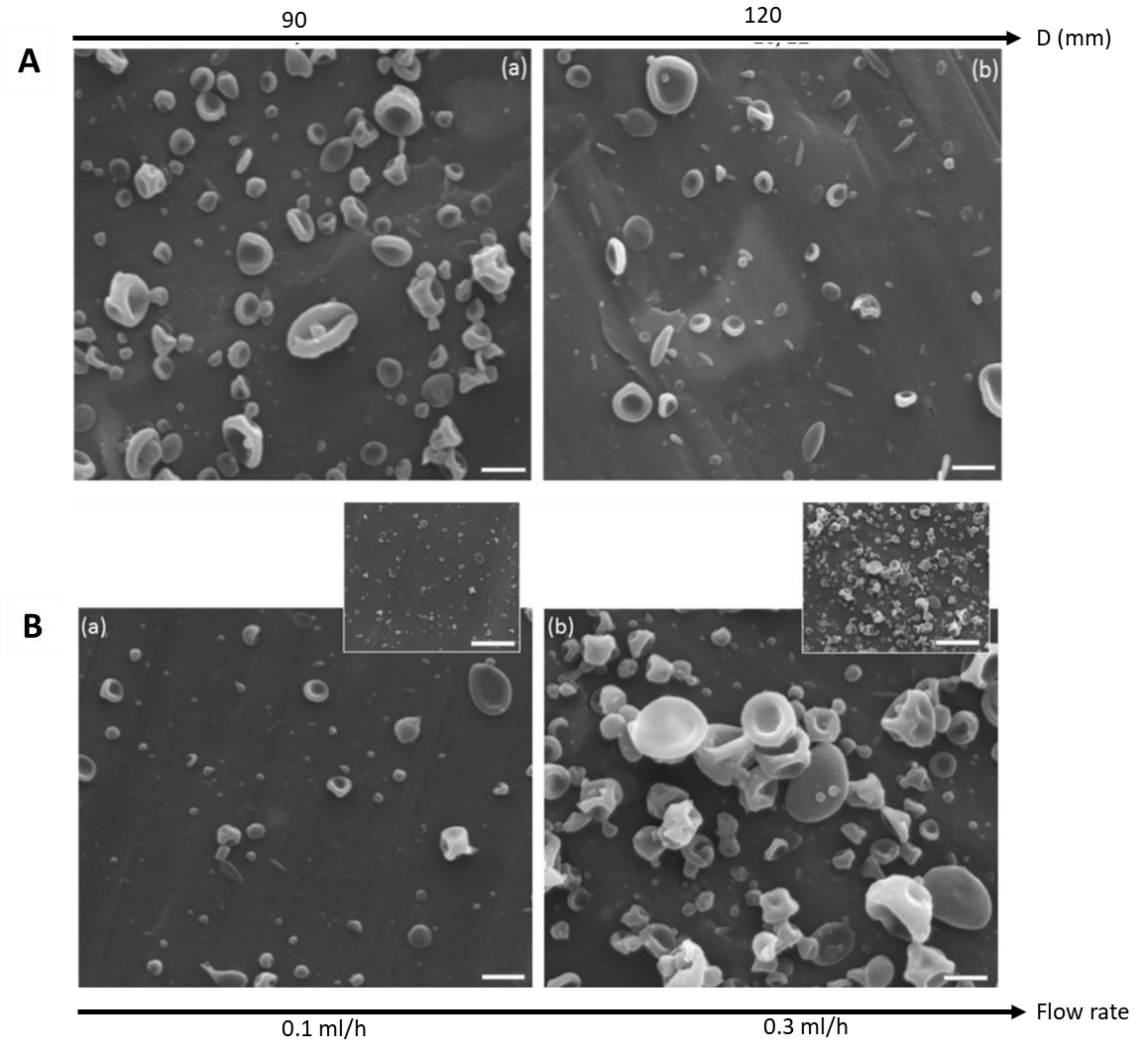

Figure 6.5 .A) Effect of distance on particle shape: SEM images of DS loaded cell-g-PCL nanoparticles (scale bar 500 nm) obtained for (a) 90 mm and (b) 120 mm. B) Effect of flow rate on nanoparticle size and density: SEM images of DS loaded cell-g-PCL nanoparticles (scale bar of 500 nm). In the square, SEM images at low magnification (scale bar 4 µm).

different populations of particles with different size and shape because of the growing instability phenomena in agreement with previous studies [25].

Moreover, the presence of DS was corroborated by ATR-FTIR of nanoparticles (Figure 6.3). As expected, the spectrum of DS loaded nanoparticles showed the characteristic bands at 745 cm^{-1} (C-CL stretching), 1574 cm^{-1} (C=O stretching) and 1607 cm^{-1} (C=C stretching), that can be typically recognized in the DS spectrum [31].

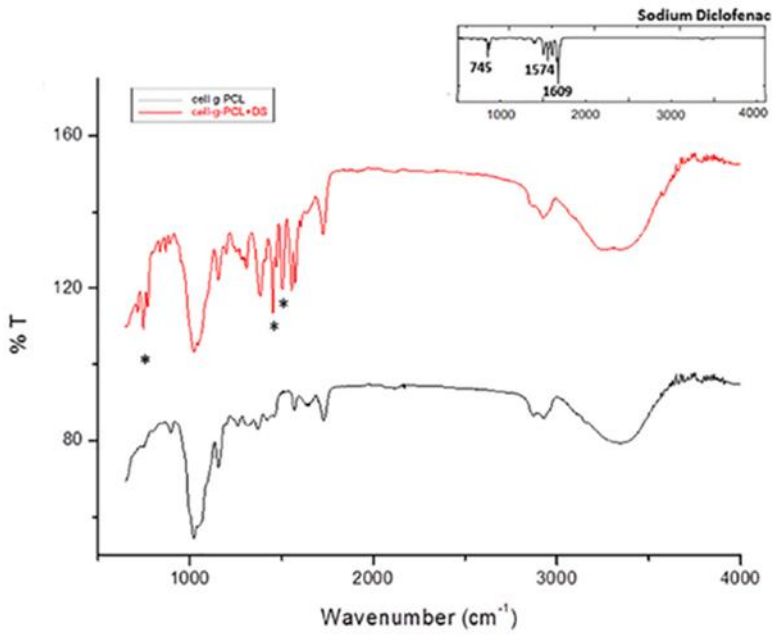

Figure 6.6. ATR-FTIR of cell-graft-particles to confirm the presence of DS onto the elecrtrosprayed nanoparticles.

Electrospraying allowed to efficiently encapsulate DS hydrophilic molecules, thus forming nanocarriers with peculiar shape and morphology and sustained molecular release until 6 days after their administration, with no cytotoxic effect *in vitro* to mesenchymal stem cells (Figure 6.4).

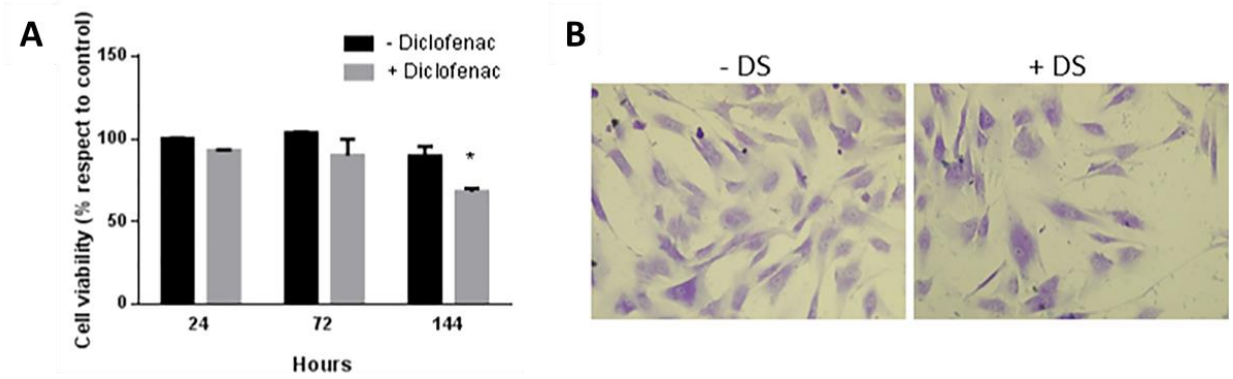

Figure 6.7. A) Quantitative analysis of cytotoxicity (CCK-8 assay). B) Qualitative analysis of hMSCs morphology after exposure to DS released from electrosprayed nanoparticles by optical microscopy (Toluidine blue stain).

In this perspective, a more accurate control of the particle shape and surface properties could be performed by modifying the electrospraying process and more modifying the basic setup, i.e. using sheath working fluids through a coaxial needle system able to influence the molecule/matrix interactions thus concurring to extend the release time for a more efficient sustained release.

6.2.2 Chitosan nanoparticles

Chitosan is derived from the *N*-deacetylation of chitin, found in the exoskeleton of crustaceans. Chitosan has been widely used for oral treatments due to its properties as bioadhesivity, biodegradability, biocompatibility, and antimicrobial activity [26]. Chitosan properties have led to develop different biomaterials as films, hydrogels, and fibers for tissue engineering [27]. Chitosan is a cationic polysaccharide because of its primary amino groups

responsible of the sustained release of molecules, thus has been used widely for local molecule delivery systems, as for periodontal applications [28,29].

In our group, electrosprayed nanoparticles have been fabricated for delivery of antibiotics, as amoxicillin [30]. It is well-known that fiber and/or particle morphology depends upon a large number of factors, including intrinsic solution properties, processing and environmental parameters as singularly discussed in several previous works [1]. In this case,solution properties and main process parameters (i.e. voltage, flow rate, distance) have been preliminarily screened to identify the best parameters to obtain round-shaped particles with submicrometric sizes.

Regarding to solution properties, result shown that morphology of fibers is influenced by mixing of solvents with different boiling points. Chitosan solution was prepared with different ratio of acetic acid and water (9:1 and 8:2). When acetic acid content is high, rounded nanoparticles with well-defined shapes are formed with lower tendency to form clusters; contrariwise, the increase of water content raises up the boiling point of the solution, increasing the tendency to form nanoparticle agglomerates (Figure 6.5).

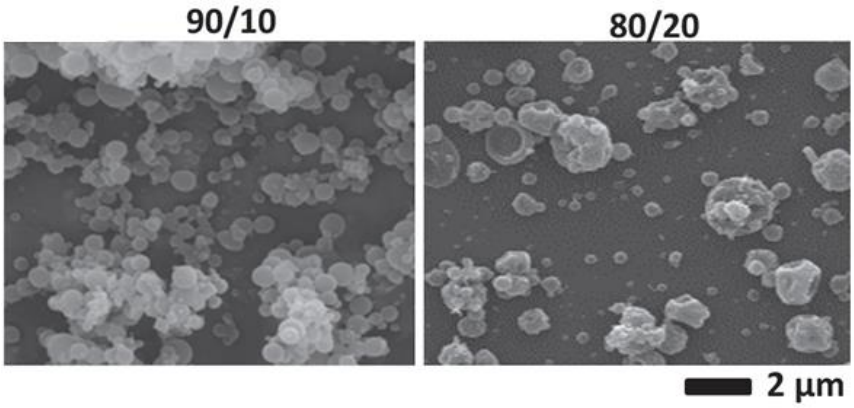

Figure 6.5. SEM images of the effect of solvent system on chitosan electrosprayed nanoparticles morphology

The polymer concentration is another parameter that can affect the morphology of particles. In case of chitosan particles, two different concentrations have been processed. TEM images showed that increasing the concentration, thus the viscosity of polymer solution, the polymer solution tends to generate local forces among polymer chains, directly ascribable to the peculiar viscoelastic behavior of the polymer, which interferes with the conventional forces occurring among polar groups during nanoparticle formation, thus promoting the formation of nano-needles and/or nanoparticles with different shape factors, and also a broad population of nanoparticles (Figure 6.6 A).

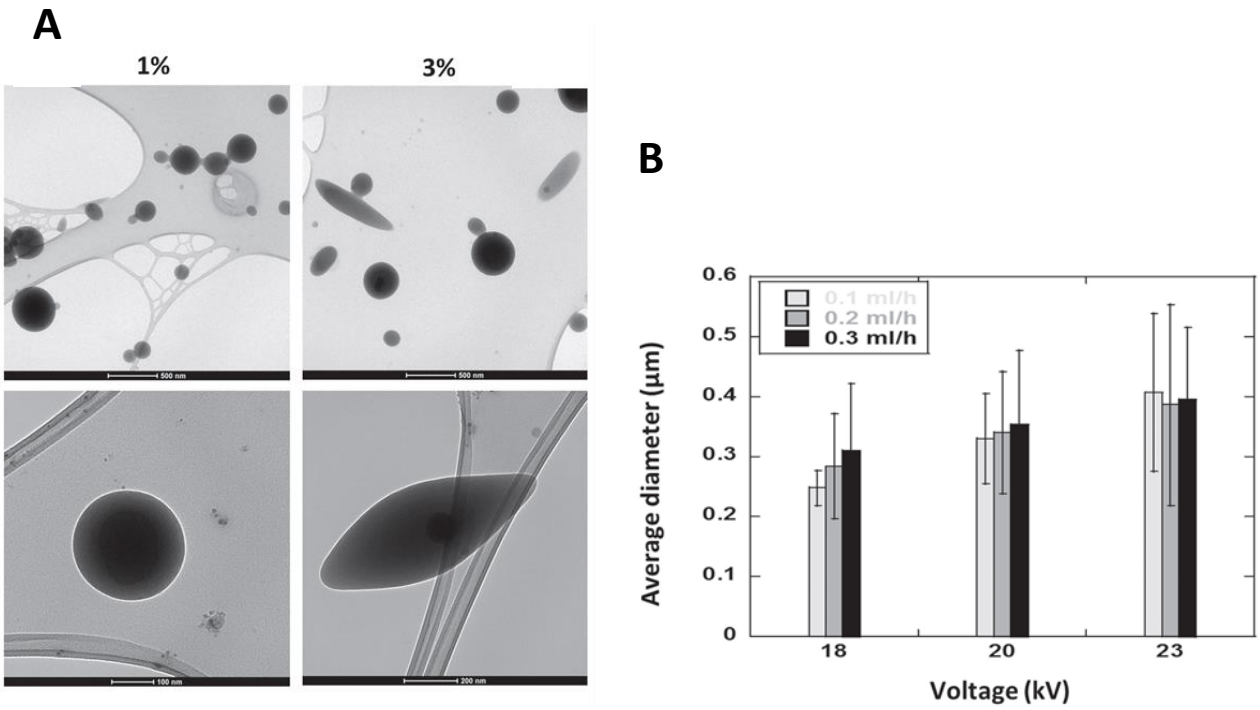

Figure 6.6. A) Effect of solution concentration on shape factor of nanoparticles. TEM analysis on chitosan electrosprayed nanoparticles. B) Effect of voltage and flow rates on average diameter of nanoparticles

Process parameters may also influence nanoparticle sizes, when voltage increases from 18 kV to 23 kV, average nanoparticle sizes vary from 0.25±0.03 to 0.61±0.13 μm; while nonsignificant variation is detected as a function of flow rate changes from 0.1 to 0.3 ml/h at constant voltage (Figure 6.6 B). Thus, the generation of chitosan loaded particles is possible once the electrospraying process is optimized, however, it must tke into consideration the influence of active molecules to solution properties.

6.3 POTENTIAL APPLICATIONS OF ELECTROSPRAYED NANOPARTICLES

Electrospraying has emerged as a similar technique as the electrospinning for the production of micro or nanostructures. Controlled morphology and monodispersity of particles can be obtained with electrospraying, with high encapsulation efficiencies and without unfavorable denaturation of bioactive molecules throughout the process [31].

There is a growing the fabrication of molecule release systems chemically stable in the highly acidic environments, but able to rapidly dissolve in the presence of neutral (or slightly basic) environmental conditions [32]. For instance, CA has been recently used to design nanoscale molecule depots in the form of core-shell fibers able to release ferulic acid (FA)[33] Cellulose based carriers have been designed to achieve a sustained release of KL for several hours after the in vitro administration [34]. The obtained results demonstrate that CA-based microspheres hold strong potential to be used as carriers for a delayed oral administration of anti-inflammatory molecules.

Nanoparticles do not only have potential as molecule delivery carriers, but they also offer non-invasive routes of administration such as oral, nasal and ocular routes. Recently, research on chitosan-based nanoparticles for non-parenteral drug delivery is based on the understanding of chitosan properties and methods of chemical or physical modification, which are applied to the optimization of nanoparticle molecule loading and release features [35]. For instance, nanoparticles were prepared from chitosan/acetic acid solution in one-step by the electrospray technique [36]. Once optimized the solution and process parameters, the average diameter of the chitosan particles was reduced to 124 nm, suggesting that electrospray is promising in producing solid micro- and nanoparticles for oral and pulmonary drug delivery systems.

The increased bacterial resistance has led search the administration of antibiotics, avoid the systemic administration and deliver locally the substances. In this regard several studies using loaded chitosan nanoparticles have been explored. Ampicillin-loaded microparticles and nanoparticles have been designed by optimizing the electrospraying process were characterized for sustained nasal release and gastrointestinal tract delivery [37]. Similarly the encapsulation of tetracycline into chitosan nanoparticles processed by electrospraying have confirmed the control of morphology particles able to release the encapsulated antibiotic [38]. Nanoparticles have been explored as delivery systems of anticancer molecules. Electrospraying process has shown to improve the encapsulation efficiency with a prolongated release of doxorubicin loaded chitosan nanoparticles [39]. Recently the use of hydrogels for cell or molecule encapsulation, innovative molecule delivery systems have been designed by changing the basic setup of electrospraying, based on electrohydrodynamic atomization. This modification is based on the deposition of charged droplets in a

crosslinking agent solution, i.e., calcium chloride ($CaCl_2$) for alginate particles, prior to the solution overcharging, by the perturbation and cutting of polymer jet until the formation of microsized particles [40].

6.4 CONCLUSIONS

Chitosan nanoparticles generated by electrospraying techniques can be efficiently loaded with molecules such as antibiotics and are therefore potentially useful as molecule delivery vehicles to release therapeutic agents by targeted and/or sustained delivery. Thus, the implementation of process parameters and novel modification to the basic setup makes electrospraying a suitable technique to fabricate controllable devices to use in tissue engineering and drug delivery.

Published data

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- V. Guarino, **I. Cruz-Maya**, R. Altobelli, W.K. Abdul Khodir, L. Ambrosio, M.A. Alvarez Pèrez, A.A. Flores, Electrospun polycaprolactone nanofibres decorated by drug loaded chitosan nano-reservoirs for antibacterial treatments, Nanotechnology. 28 (2017) 505103. doi:10.1088/1361-6528/aa9542

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CHAPTER 7

ADDITIVE ELECTROSPRAYING FOR FABRICATION OF INTEGRATED SYSTEMS WITH TISSUE ENGINEERING AND DRUG DELIVERY APPLICATIONS

7.1 INTRODUCTION

Nanotechnology offers a wide range of tools to design materials with specific and highly controlled structure and topography. In recent years, electrofluidodynamic technologies (EFDTs) have been used to fabricate micro or nano size particles/fibers for drug delivery systems [1,2]. The electrospun fibers are used for tissue engineering and drug delivery due to their characterized by high porosity and high surface to volume ratio. Moreover, the further incorporation of different active molecules, such as drugs, natural active compounds or metal ions may be used to control bacterial adhesion and subsequent biofilm formation onto micro and nanostructured surfaces [3–5]. Particularly, studies have shown bacterial contamination after periodontal treatments, which alters the attachment of fibroblast leading to membrane exposure [6,7]. Thus, have been proposed different strategies to incorporate antibiotics to void the initial colonization and improve the regeneration of periodontal tissues [8,9]. Between the current strategies to incorporate active agents into nanofibers are physical blending, molecular confinement by emulsion, core/shell encapsulation, and post-treatments for selective chemical of physical binding [10–13]. However, these techniques still show several limitations related to extended initial release bursts and relatively high encapsulation levels. To date, additive electrospraying (AE) has been emerging as a versatile technique to

simply and more efficiently functionalize electrospun fibers for molecular release [14,15]. This technology offers an improvement respect to the current strategies to inhibit the bacterial adhesion by modifying the single topography and adding antibacterial agents [16]. By using AE, the morphological signals exerted by nanofibers can be complemented by the presence of nanoparticles that additionally can be loaded with antibiotics that eventually are released to avoid microorganism populations.

In this work, it is proposed to fabricate antibacterial platforms by merging electrospinning and additive manufacturing criteria, to assemble drug-loaded nanoparticles and nanofibers into a unique device, with major outcomes in terms of resource costs and product feasibility. Recently, other works have just introduced a similar technological approach to fabricate hierarchically structured composite systems with multilevel porous structure with pore sizes of few micrometers for biomedical applications [17]. Contrariwise, here is described a novel method to fabricate nanostructured platforms by decorating polycaprolactone (PCL) nanofibers with amoxicillin (AMX-DHT) loaded chitosan (CS) nano-reservoirs able to release locally antibacterial drugs. In particular, morphology and antibacterial properties will be carefully investigated in order to optimize additive electrospraying (AE) process conditions and identify the most appropriate experimental setup to be used for a sustained release of the drug.

7.2 MATERIALS AND METHODS

7.2.1 Fabrication of integrated system of PCL fibers with loaded-chitosan nanoparticles.

For fibers, PCL (Mw 65 kDa, Sigma–Aldrich, Italy) pellets were dissolved in methylene chloride/methanol (1:1 v/v) to form a clear and homogenous solution (10% wt/v). For nanoparticles, CS powder was dissolved in 90% (v/v) acetic acid solution to form 2% (wt/v) CHI solution. AMX (1% and 3%w/w respect to the polymer) was added in 2% (w/v) CHI solution and stirred until complete dissolution of the drug.

The integrated systems were obtained via simultaneous and sequential deposition by using two separate nozzles in the same spinneret, using a commercial electrospinning equipment (NANON 01, MECC, Japan). The electrospraying parameters for AMX-loaded chitosan solution were 16 kV as voltage, 0.2 ml/h as flow rate. Meanwhile, electrospinning parameters for PCL fibers were 16 kV, 0.5 ml/h as flow rate. The tip-to-collector distance was 10 cm. In the case of simultaneous electrospinning, the spinneret was translationally moved (rate of 1 mm/s, along 50 mm width) to improve drug distribution homogeneity.

7.2.2 Morphological properties

The morphology of PCL electrospun fibers and CS nanoparticles was investigated by scanning electron microscope (Quanta 200 FEI, The Netherlands) working at low voltage electron emission (2 kV). Average diameters were estimated using Image J software (version

1.37) on 20 randomly selected images at the same magnification (scale bar 2 μm) onto a population of about 15/20 nanoparticles.

7.2.3. Drug release and antibacterial properties

The AMX release from drug-loaded CS/PCL integrated platforms was estimated by ultraviolet (UV) spectroscopy (Cary 100 Varian, Italy) at λmax of 280 nm. Prior to UV measurements, samples were centrifuged at 5000 g for 30 min and a linear calibration curve (CC) for different drugs was generated. Encapsulation efficiency and drug loading were calculated [22]. Drug absorbance was recorded in terms of concentration according to CC, while the released drug relative amount was calculated as cumulative release (%) versus time (hours).

The inhibitory activity was evaluated against *Escherichia coli* (*E. coli*, ATCC 11775), *Staphylococcus aureus* (*S. aureus*, ATCC 25923), and *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*, ATCC 43718). *E. coli* and *S. aureus* were grown on enriched TSA (Trypticase Soy agar, Bioxon, Becton Dickinson) supplemented with hemine (5ml) and vitamin K (0.3 μg/ml) at 37 °C for 24 h under aerobic conditions. *A. actinomycetemcomitans* was grown in enriched HK media (agar base for Mycoplasma, Becton Dickinson) with BHI (brain heart infusion agar, Becton Dickinson), also supplemented with 5% of defibrinated lamb's blood (Microlab) at 37 °C for 7 days, under anaerobic conditions (80% N, 10% CO^2 and 10% N_2). Pure bacteria cultures were transferred on agar plates covering circular specimens—5 mm as diameter. After the

incubation time, bacterial inhibition zone size—i.e. halo diameter increase—was measured in comparison with those of tetracycline (TCH) loaded samples, used as the positive control.

7.3 RESULTS AND DISCUSSION

Drug loaded CS particles and PCL fibers have been electrosprayed and electrospun respectively to fabricate an innovative drug delivery platform with antibacterial properties. In order to optimize the biological response, fibers and nanoparticles with and without drugs have been investigated from morphological point of view. It is well-known that fiber and/or particle morphology depends upon a large number of factors, including intrinsic solution properties, processing and environmental parameters as singularly discussed in several previous works [18,19]. In this case, solution properties and main process parameters (i.e. voltage, flow rate, distance) have been preliminarily screened to identify the best parameters to obtain thin fibers and rounded particles with submicrometric sizes (Figure 7.1). The use of co-solvent system to process PCL allowed the formation of fibers in nanometric scale and reduced the formation of beaded fibers also minimizing fluid dynamic instability phenomena of the polymer jet during the process [20].

As for drug-loaded nanoparticles, the addition of AMX the particle size, as confirmed by the reduction of the average diameter from 0.350 ± 0.18 μm to 0.22 ± 0.11 μm and a stricter distribution of particle sizes. Other studies have proposed CS directly as an antimicrobial agent to fight different bacteria populations [9]. Hence, it has been verified, at the first stage, that CS in the form of nanoparticles does not present any antimicrobial effect which is mainly ascribable to the contribution of the drug (Figure 7.1 C). As expected, the presence of drugs also improves the antibacterial properties of the nanoparticles, generating a more extended inhibition zone with respect to CS nanoparticles.

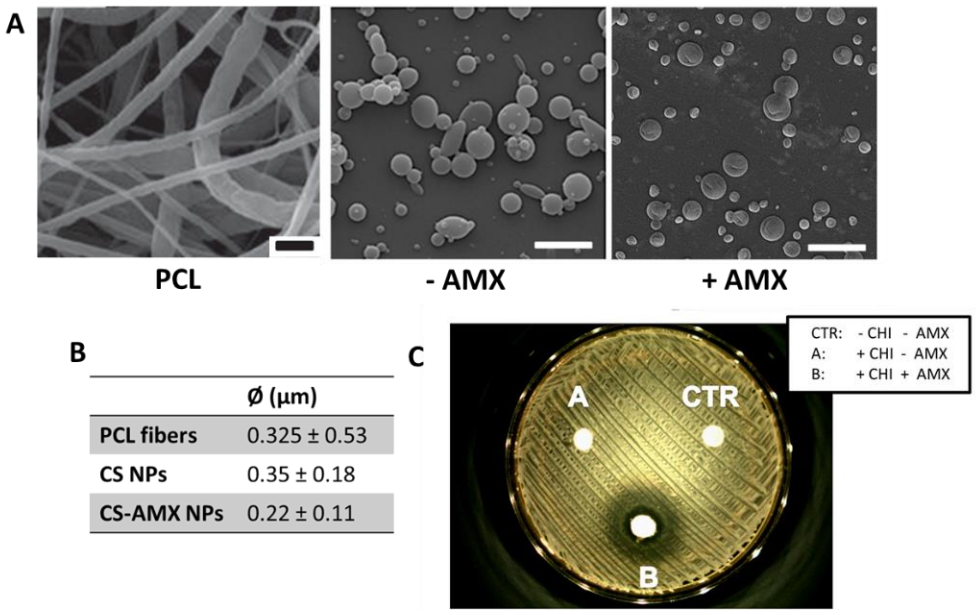

Figure 7.1. SEM imges of PCL fibers and CS nanoparticles with and without AMX. B) Fibers and particles average diameters. C) Antibacterial effect of CS-AMX nanoparticles [29].

The use of different processing modes was evaluated since may significantly influence the mechanism of drug release (Figure 7.2 A). The sequential deposition mode, AMX was released rapidly, presenting a pronounced initial burst release (over 90%) followed by a sustained release during the next two hours, mainly ascribable to the dissolution mechanism of chitosan particles, exposed along the scaffold surface (figure 5.2 B). In this case, hydrophilic chitosan nanoparticles may release antibiotic molecules for bacteria interactions [21].

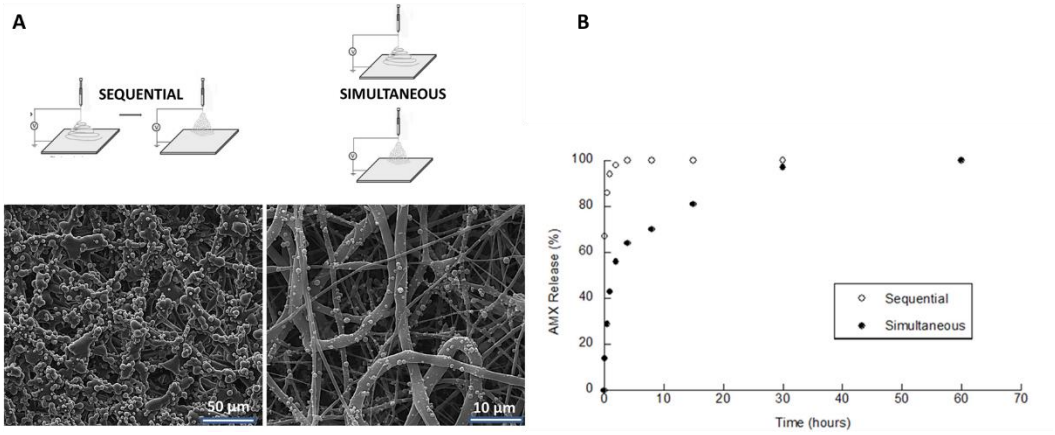

Figure 7.2. A) Sequential and simultaneous deposition modes, with SEM images. B) Reales curve of amoxicillin influenced by the deposition mode [29].

Contrariwise, in the case of simultaneous process, AMX was gradually released, with a more reduced burst effect (not over 50%) followed by a more extended sustained release (Figure 7.2 B). Previous studies have just discussed how PCL fibers can influence drug retention in terms of sustained release and antimicrobial properties [22]. This can be related to the effect on the fluid permeability through the hydrophobic PCL network, able to slow down the dissolution of nanoparticles mainly embedded into the deepest regions. Hence, the release

mechanism of drug loaded CS/PCL integrated platforms fabricated by simultaneous deposition mode have been used for antibacterial tests.

The hydrophobic properties of the PCL network promote a more homogeneous spatial distribution of nanoparticles, improving the activity against bacteria by a more efficient drug confinement. Therefore, the response of three different population of bacteria, *S. aureus*, *E. coli* and *A. actinomycetemcomitans*, in contact with Tetracycline and Amoxicillin loaded-CS nanoparticles decorated PCL nanofibers has been investigated (Figure 7.3). In some reports, micro/nanostructured platforms directly including TCH or AMX have shown a reduction of bacterial growth, preventing bacterial penetration [23]. In particular, a clear halo indicating the inhibition of bacterial growth may be recognized only in the case of drug loaded platforms, as a consequence of the initial burst release into the agar. The burst effect is suitable in tissue regeneration therapies, giving the opportunity to reduce the local contamination of the implant site just during the first minutes after treatment [8,24]. However, some differences as a function of the peculiar drug kinetic release through the fibrous network may be recognized.

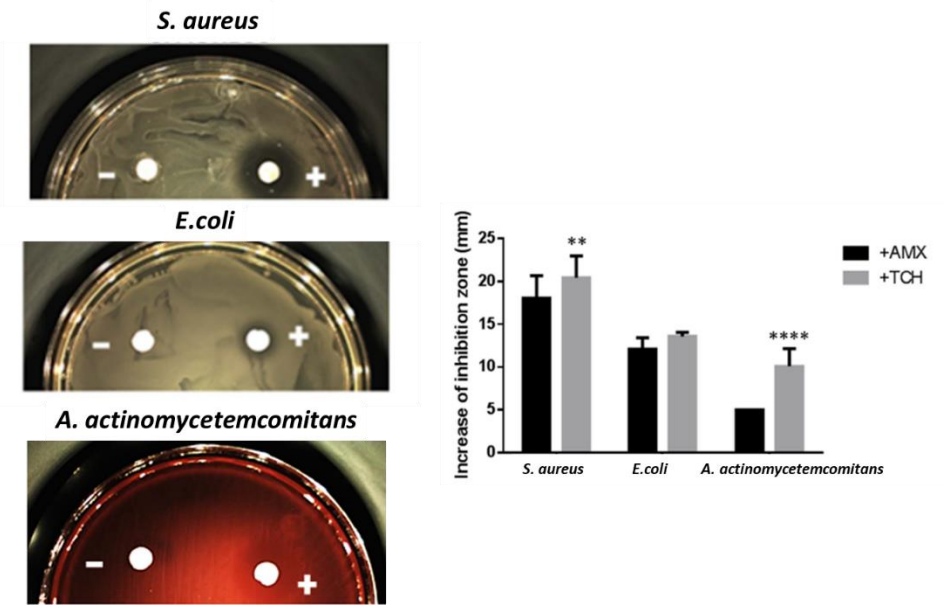

Figure 7.3. Antibacterial effect of amoxicillin released from integrated systems [29].

In the case of AMX and TCH being released from CS/PCL integrated platforms show the highest effect against *S. aureus*, as confirmed by an inhibition halo diameter equal to 18.05±2.63 mm and 20.45±2.57 mm, respectively. Contrariwise, a lower growth of *E. coli* has been detected (less clear inhibition halo). In the case of AMX loaded samples, halo size increases 12.10±1.33 mm being lower than those of TCH loaded ones 13.59±0.48 mm. This result is in agreement with those of previously investigated AMX loaded electrospun fibers for biomedical applications [25,26]. Noteworthy, *A. actinomycetemcomitans* is more resistant to AMX than TCH, as confirmed by the strong difference in size increase of inhibition zone, equal to 10.10±2.04 mm for TCH, and for AMX is close to zero, overlapped with the membrane. These data underline an improvement of antibacterial response with respect to conventional electrospun fibers loaded with antibiotics [26–28].

7.4 CONCLUSIONS

This work confirmed the effective advantage of using EFDTs, particularly, additive electrospraying technology to amplify the effectiveness of nanoparticles with its zero-dimensional nature as molecular and drug release systems. The optimization of EFDTs allowed the design of tailor-made nanostructured platforms containing nano-shuttles able to release antimicrobial agents in a timely way. From this perspective, the capability to guide drug release and, ultimately, in vitro antimicrobial response by a customized design via EFDTs of platforms, could open new innovative routes for multiple drug release, as more effective therapies to overcome the systemic administration in the presence of periodontal diseases.

Published data

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