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Development of Wound Dressings Based on Cyanobacterial Extracellular Heteropolysaccharides

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"I would rather have questions that can't be answered than answers that can't be questioned."

Richard Feynman

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Resumo

A esperança média de vida dos humanos tem vindo a aumentar desde o século XX devido a avanços na medicina, no entanto um dos problemas associados com o aumento da longevidade são as doenças crónicas, como as feridas crónicas por exemplo. Estas feridas são caraterizadas por uma má regeneração e um prolongado estado inflamatório, sendo muito suscetíveis a infeções bacterianas, o que pode atrasar ou parar por completo a regeneração da ferida. Os tratamentos disponíveis atualmente promovem um ligeiro aumento da regeneração da ferida, no entanto são incapazes de prevenir eficientemente o surgimento de infeções e a dor no paciente ou uma fácil manutenção do tecido ferido. Assim, há uma necessidade de desenvolver produtos inovadores que sejam capazes de promover a regeneração natural de uma ferida crónica, evitando infeções e desconforto ao paciente, e que sejam facilmente aplicáveis pelos profissionais de saúde, contribuindo para uma boa relação custo-benefício.

As cianobactérias são procariotas fotossintéticos capazes de produzir substâncias poliméricas extracelulares (EPS), que podem permanecer associadas à parece celular ou serem libertadas para o meio extracelular (RPS), o que facilita o seu isolamento. Usualmente, estes polímeros são heteropolissacarídeos complexos, com uma carga geral aniónica e com capacidade de apresentar diversas conformações estruturais, o que resulta em propriedades únicas, tais como a prevenção da aderência bacteriana ou efeitos anti-inflamatórios.

O objetivo deste trabalho foi utilizar RPS previamente conhecidos e que são produzidos por duas estirpes de cianobactérias do mesmo género - *Cyanothece* sp. CCY 0110 (CCY 0110) e a *Cyanothece* sp. VI 22 (VI 22) - para o desenvolvimento de soluções poliméricas que possam ser aplicadas em feridas crónicas, nomeadamente ajustando-se à sua topologia, promovendo uma melhor regeneração da ferida e prevenindo infeções bacterianas. Neste âmbito, as cianobactérias foram cultivadas para a extração dos RPS, que depois foram dissolvidos em meio de cultura celular em várias concentrações para estudar os seus efeitos de citotoxicidade e proliferação celular em células humanas, usando os ensaios de redução do tetrazólio (MTT), bromodesoxiuridina (BrdU) e de migração. Os resultados obtidos revelaram as duas concentrações ótimas de RPS para os testes de viabilidade *in vivo* em ratos Wistar, nos quais foram efetuadas lesões dorsais onde soluções poliméricas foram aplicadas a cada dois dias durante uma semana. Em suma, os resultados de citotoxicidade e proliferação celular sugerem que a solução de RPS da CCY 0110 não é citotóxico ou anti proliferativo para concentrações de polímero inferiores a 0.50% (w/v), enquanto que a solução de RPS da estirpe VI 22 possui as mesmas caraterísticas para concentrações de polímero inferiores a 0.10% (w/v). Estes resultados foram confirmados pelos testes *in vivo*, no entanto, a regeneração da ferida não foi promovida significativamente pelos RPS, em comparação com as feridas sem soluções poliméricas. Este trabalho revela o potencial do polímero produzido pela cianobactéria *Cyanothece* sp. CCY 0110, que pode ser melhorado através de estratégias de funcionalização, de forma a promover o processo de regeneração da ferida, ao possibilitar a criação de um substituto para os tratamentos disponíveis para feridas crónicas, com as vantagens de ser antiaderente para bactérias, barato, não causar dor para o paciente, de fácil manipulação e preparação, e de origem natural.

Abstract

Life expectancy of humans has been increasing since 20th century due to breakthroughs in medicine, however one major problem related to the increased longevity are chronic diseases, such as chronic wounds. These wounds are characterized by a poor regeneration and a prolonged inflammatory state, being highly susceptible to bacterial infections, which can delay or even halt the wound healing. The currently available treatments promote a slight increase on wound regeneration, however are incapable of efficiently preventing bacterial infections and patient pain or allowing an easy wounded tissue maintenance. Therefore, there is a necessity to develop innovative products that can promote the natural wound healing of a chronic wound, avoiding infection and patient discomfort, and being easily applied by health practitioners, contributing to a good cost-benefit relation.

Cyanobacteria are photosynthetic prokaryotesthat are capable of producing extracellular polymeric substances (EPS), which can remain attached to the cell surface or be released to the extracellular medium (RPS-released polysaccharides), facilitating its isolation. Usually, these biopolymers are complex heteropolysaccharides, with an overall anionic charge and capable of presenting various structural conformations, which can result in unique properties, such as preventing bacterial adhesion or anti-inflammatory effects.

The main objective of this work was to utilize previously known RPS produced by two cyanobacterial strains of the same genus - *Cyanothece* sp. CCY 0110 (CCY 0110) and *Cyanothece* sp. VI 22 (VI 22)- to develop polymeric solutions that can be applied on chronic wounds, namely by adjusting to their topology, promoting an enhanced wound regeneration and preventing bacterial infections. For this purpose, the cyanobacteria were grown to extract the RPS, which then were dissolved in cellular culture medium at various concentrations to study its cytotoxicity and cellular proliferation effects in human cells, using the tetrazolium reduction assay (MTT), bromodeoxyuridine assay (BrdU) and the cell culture wound closure assay. The results obtained revealed the two optimal RPS concentrations for the *in vivo* viability assays using Wistar rats, where dorsal wounds were performed, and polymeric solutions were applied each two days for one week. In summary, cytotoxicity and cell proliferation results suggest that the CCY 0110 RPS solution is not cytotoxic or anti-proliferative for polymer concentrations below 0.50% (w/v), whereas VI 22 RPS solution has the same characteristics for polymer concentrations below 0.10% (w/v). These results were confirmed by *in vivo* assays; however, the wound regeneration was not significantly promoted by the RPS, in comparison to wounds without polymeric solutions. This work reveals the potential of the polymer produced by the cyanobacterium *Cyanothece* sp. CCY 0110, which can be further enhanced by functionalization strategies, in order to promote the wound healing process, by allowing the creation of a substitute for available chronic wound treatments with the advantages of being anti-adhesive for bacteria, not expensive, painless to the patient, easily manipulated and prepared, and of natural origin.

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Abbreviation and Symbols

Abbreviation List

ASN	Artificial sea water
BrdU	Bromodeoxyuridine Assay
CCY 0110	*Cyanothece* sp. CCY 0110
CCY	Culture Collection of Yerseke
CPS	Capsular Polysaccharides
DW	Dry Weight
ECM	Extracellular Matrix
EPS	Extracellular Polymeric Substances
EU	European Union
FGF	Fibroblast Growth Factor
HA	Hyaluronic acid
MCP-1	Monocyte Chemoattractant Protein 1
MMP	Matrix Metallo-Protease
MTS	Tetrazolium Reduction Assay
OD	Optical Density
PDGF	Platelet-Derived Growth Factor
RPS	Released Polysaccharides
RT	Room Temperature
TGF-β	Transforming Growth Factor Beta
UK	United Kingdom
USA	United States of America
VEGF	Vascular Endothelial Growth Factor
VI 22	*Cyanothece* sp. VI 22

Symbol List

$	Dollar
%	Percentage
(w/v)	Weight by volume
€	Euro
˚C	Degree Celsius
mm	Millimeters
nm	Nanometers

Chapter 1

Introduction

1.1 - Motivation and Aim

Wound dressings are usually the go-to treatment for chronic wounds with several types of wound dressings being available for different patient needs, from the typical gauze to advanced tissue engineered skin substitutes and hydrogels. These wound dressings were developed to protect the wound site from infection and to promote wound healing. However, wound infections are recurrent in most chronic wound patients since employed wound dressings fail to prevent bacterial infection, compromising the patient treatment and health, and representing an increased economic cost to the national health system.

The aim of this work was to develop a polymeric solution based on released polysaccharides (RPS) from cyanobacteria *Cyanothece* sp. CCY 0110 (CCY 0110) and *Cyanothece* sp. VI 22 (VI 22), to cover chronic wounds by adjusting to their unique topology, promoting the wound healing process and preventing bacterial adhesion. This strategy was employed due to the need for an efficient wound dressing that is of natural origin, cheap and easily applied to the patient, minimizing their pain during wound management. This work is divided in three major goals: (i) growth of VI 22 culture and the extraction of RPS, (ii) cytotoxicity and cell proliferation assessment of both RPS solutions using human cells and (iii) *in vivo* confirmation of cytotoxic and wound healing effects of both RPS solutions. For this, cyanobacterial cultures were grown in 1 L bioreactors and their RPS was extracted and purified accordingly to optimized protocols. Then, various concentrations of the polymeric solutions were obtained by dissolving the RPS from each cyanobacterial strain in cell culture mediums, accordingly to the human cell line utilized in the cell viability, proliferation and injury assays. From these results, the best concentrations were identified and utilized for *in vivo* cytotoxicity and wound healing tests to understand the effects of each RPS solution in an *in vivo* context.

1.2 - Structure of the Dissertation

In this dissertation, a thorough literature study regarding chronic wounds, wound healing, wound dressings and cyanobacteria was performed to understand the diverse concepts and assess the actual state of art of chronic wounds treatments and cyanobacteria possible uses.

In chapter 2, chronic wound definition, problems and proposed solutions are explored.

In chapter 3, material and methods are described followed by the results obtained by these methods in chapter 4.

Results are discussed in chapter 5 based on current literature and the conclusions and future work are addressed in chapter 6.

Chapter 2

Literature Review

2.1 Chronic Wounds and Diabetes

Populations across the world are ageing due to the generalized improvements in society and medicine leading to an enhanced life quality, however, this leads to an increased incidence of chronic diseases (Järbrink et al., 2017). Chronic wounds are described as wounds that have failed to follow a normal wound healing pathway in an orderly and timely manner leading to the full regeneration or repair of anatomical and physiological tissues, in a time period inferior to 3 months (Lazarus et al., 1994). This problem has a significant burden on patients and on medical system, as estimations indicate an annual cost of US$20 billion for chronic wound care in the United States of America (USA), although only 2% of the population is affected by the disease (Frykberg & Banks, 2015). On the other hand, costs associated with chronic wound treatments in the United Kingdom (UK) are lower than the USA, despite the incidence of the disease in the UK being approximately 3% (Posnett & Franks, 2008). Nonetheless, chronic wounds impose a significant financial burden in society as the patient experiences pain, reduced mobility, social isolation, emotional and physical distress, which can result in reducing productivity and inability to work, impacting the country economy (Walshe, 1995).

Chronic wounds can be classified as leg ulcers, diabetic foot ulcers and pressure ulcers depending on their characteristics (Mustoe et al., 2006). Leg ulcers are subdivided into arterial and/or venous ulcers, depending on their underlying cause that is correlated to the damage of superficial and/or deep venous/arterial systems of the leg, which decreases blood flow to certain regions of the leg, ultimately leading to ulceration (Grey, 2006). Diabetic foot ulcers are caused by diabetes that can cause arterial damage and neuropathy in the wound site, aggravating the patient condition. Approximately 15% of diabetic individuals will be affected by a foot ulcer in their lifetime (Reiber et al., 1998). Pressure ulcers are more frequent on old and frail individuals that are immobile or have some sort of spinal cord injury. This type of

chronic wounds is the result of persistent direct pressure and shear forces constantly applied to the same local, and impaired skin conditions (Grey & Enoch, 2006).

An example of the three different types of chronic wounds that an individual can develop, depending on different comorbidities, is presented below (fig. 1). It is noteworthy that some foot digits are necrotic due to the ischemic condition of the chronic wound (fig. 1a), caused by a damaged venous or arterial system. Diabetic foot ulcers that are infected have a yellowish color at the wound site and surrounding tissues (fig. 1b) (Nunan et al., 2014). In fact, many patients with diabetic foot ulcers are prone to the development of bacterial infection, which can spread to the deeper fascia or even bones, hindering the wound healing process (Jeffcoate & Harding, 2003). Pressure wounds are only visible on the plantar surface of this individual (fig. 1c), as it is a zone subjected to constant pressure every time that the individual walks.

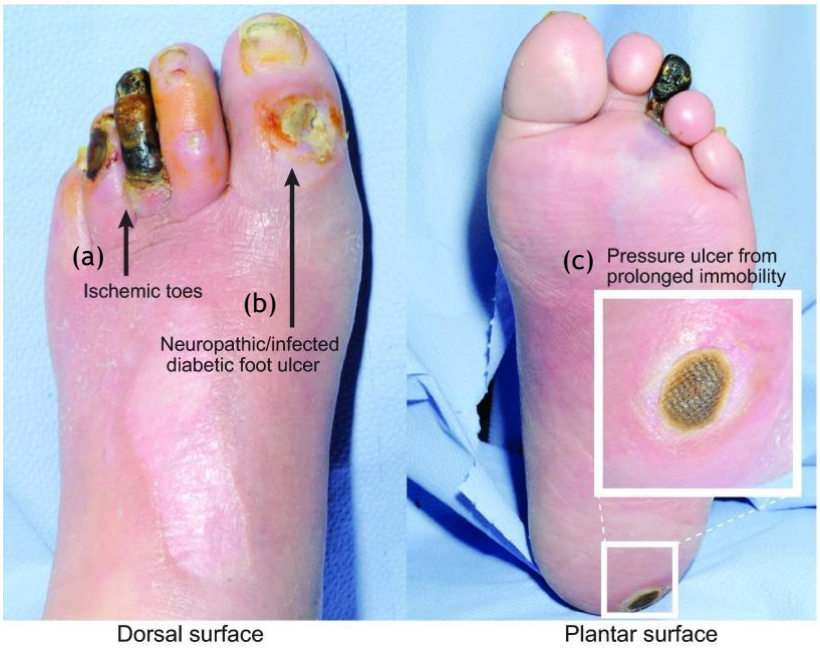

Figure 1 - Three types of chronic wounds can be observed on the same foot of a diabetic patient. On the dorsal surface we can observe, (a) ischemic toes, which are chronic wounds due to either venous or arterial complications and (b) a diabetic foot ulcer is present on the big toe with a bacterial infection. On the plantar surface, (c) a pressure wound can be seen on the heel, due to prolonged forces on the same local. Adapted from (Nunan et al., 2014).

Tissue that are ischemic, necrotic or hypoxic are an ideal environment for the colonization and proliferation of microbial populations. The colonization induces neutrophil and macrophage infiltration in the wound site that will release free oxygen species, cytotoxic enzymes and proteases to the extracellular space to fight the bacterial infection. However, these molecules can destroy cellular and protein components of the extracellular matrix (ECM), which amplifies the inflammatory state of the wound and impairs the normal wound healing pathway (Eming et al., 2007). Microflora associated with chronic wounds is composed of the same resident commensals that exist on the skin of healthy individuals. Some examples are staphylococci, streptococci, enterococci and *Pseudomonas* spp., however, various

organisms coexist in an infected chronic wound and to date there is not a single organism associated to a specific type of chronic wound (Omar et al., 2017). In extreme scenarios, bacterial infections in chronic wounds can lead to a deep tissue infection, where bacteria infect deeper fascia, sometimes reaching bones (Lipsky et al., 2005). To combat infections, health practitioners have numerous topical formulations with antibiotics at their disposal, although there is no clear evidence that with routinely administration of antibiotics the outcome of the wound healing progression is improved. This practice represents a risk as the patient might feel discomfort and the infectious bacteria can develop antibiotic resistance. Products impregnated with silver can also be used to deal with infections, however, silver toxicity is a major side effect of this treatment, as after 1 month of treating patients with dressings that contain silver resulted in raised levels of systemic silver, specifically elderly patients that suffer from malnutrition and anemia (Brouillard et al., 2018).

Diabetes mellitus is a chronic disease that is expected to affect 439 million people in the world by 2030, representing an estimated increase of 98.1% of the worldwide cases from 2010 to 2030 (Whiting et al., 2011). This estimated growth is due to the aging of the population and changes in lifestyle, namely poor diet and healthy choices, such as the lack of sports activities. Diabetes mellitus is a metabolic disorder that is characterized by high levels of glucose in the serum and by problems on the metabolism of carbohydrates, lipids and proteins, due to changes in insulin secretion or action (Intekhab, 2006). It is classified as type 1, type 2 or gestational diabetes, although the latter only affects nearly 14% of pregnant woman, being a risk factor for pregnant woman who have type 2 diabetes (Bertoni et al., 2010; McCance, 2011). Type 1 diabetes, also known as insulin-dependent diabetes mellitus, is responsible for the destruction of pancreatic ß-cells, which makes individuals affected by this disease, completely dependent on insulin exogeneous sources, such as insulin bombs, as they are incapable of producing insulin (Intekhab Ahmed, 2006). Despite being the worst type of diabetes that individuals can suffer, it has a lower incidence as compared to type 2 diabetes incidence of approximately 90% (Wang et al., 2008). Type 2 diabetes, also known as non-insulin-dependent diabetes mellitus, is characterized by insulin resistance, with some individuals also displaying reduced levels of insulin secretion. This type of diabetes is caused by a poor lifestyle, with the risk factors being obesity, sedentary lifestyle and high plasma glucose concentrations during fasting. Contrary to type 1, type 2 diabetes can be prevented or delayed by adopting a healthy nutrition regiment and practicing regular physical activity (Knowler et al., 2002). The high mortality rate observed in diabetic patients is due to their susceptibility to develop coronary heart diseases, strokes, obesity or diabetic nephropathy and neuropathy, being the latter one of the factors associated with diabetic foot ulcers (Fontbonne et al., 1989; Srikanth & Deedwania, 2011). Diabetic foot ulcers are estimated to have a cost of wound treatment of US$8659 for a single patient, with the total medical cost with patients being estimated from US$9 to US$13 billion in the USA (Rice et al., 2014).

While in the European Union (EU) countries costs per patient range from €7539 to US$24,965 (Raghav et al., 2018).

2.2 Wound Healing Pathway and Diabetes disturbances

Skin is the first protection barrier of the body from microorganisms and other external hazards, therefore it is of the upmost importance that skin maintains intact, otherwise homeostasis is compromised. When a superficial wound occurs, various systems are activated on the wound site to clear foreign material and to restore the normal structure and function of the skin, despite never being a total restoration, as for example, the maximum tensile strength of the newly formed skin will be about 70% of unwounded skin (Stadelmann et al., 1998). Nonetheless, most of physiological functions of the skin are returned after the wound healing pathway is completed. The wound healing pathway occurs in five different stages: (i) homeostasis/coagulation, (ii) inflammation, (iii) proliferation, (iv) re-epithelialization and (v) remodeling (Moura et al., 2013), in an orderly and timely manner to complete the skin regeneration and repair (Da Silva et al., 2017). Transition between phases is dependent on maturation and differentiation of keratinocytes, fibroblasts, mast cells and macrophages (Monaco & Lawrence, 2003). The duration of wound healing is dependent on the location, size, depth and type of wound, however, external factors such as temperature, moisture, pressure, and other pathologic factors can influence the progression of wound healing, affecting the treatments applied to the patient and their success in regenerating the wound site (Moura et al., 2013).

After tissue injury, the first step of the wound healing pathway is to form a fibrin plug to re-establish homeostasis, and the secretion of growth factors, such as transforming growth factor beta (TGF-ß), and cytokines, such as the monocyte chemoattractant protein 1 (MCP-1) by the aggregated platelets, recruiting neutrophils and monocytes to the wound site. These growth factors attract keratinocytes and activated fibroblasts from the wound edges to the wound site, which in turn will start to proliferate producing ECM to cover the wound site (Da Silva et al., 2010), and neutrophils and monocytes are able to migrate to the wound site due the increased vasodilatation that creates vascular permeability (Wynn & Barron, 2010). The inflammatory phase has a duration of about three days after the wound appearance and is followed by the proliferative phase (Wilgus, 2008). The newly formed ECM is gradually replaced by a collagenous matrix (mainly composed by collagen type III), due to the enhanced expression of proteases, and angiogenesis. Angiogenic factors, such as fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), promote angiogenesis by stimulating macrophages and endothelial cells to produce fibroblast and vascular endothelial growth factors (Wilgus, 2008). Therefore, granulation tissue has a constant growth until the wound site is completely closed and angiogenesis comes to a halt, where apoptosis of new vessels occurs, denoting the end of the proliferation phase. The granulation tissue is then degraded and dermis regeneration takes place, denoting the re-epithelialization phase (Rodero & Khosrotehrani, 2010), which happens after 2 to 3 weeks from the development of the wound and is characterized by the restoration of collagen type I from the collagen type III, maturation of the wound tissue by cross-linking ECM

components, and a regression of the vascular network (Haukipuro et al., 1990) . The normal wound healing pathway results in a full closure of the wound, and restoration of anatomically and physiological properties of the native tissue.

One major factor in the physiologic conditions that promote wound healing is oxygen supply and oxygen tension in the wound bed, as oxygen will interact with cytokines, be consumed by proliferating cells and will be an effector in the neutrophil respiratory burst (Hunt & Hopf, 1997). Low oxygen supply to the wound bed may result on necrotic tissue, which can facilitate bacterial growth and compromise the efficiency of the primary mechanisms of the immune system. Moreover, low oxygen supply is also accompanied by nutrients deficiency, specially protein intake, but also other important factors, such as vitamins A, C and zinc, which may result in a poor wound repair or regeneration (MacKay & Miller, 2003). In diabetic patients however, the wound healing pathway does not occur in a linear manner and can become stalled in one or more phases, usually resulting in a perpetuated inflammatory phase (Silva et al., 2016). These individuals suffer from peripheral vascular disease and/or diabetic neuropathy (damage to nerve tissue and loss of sensation), a common complication of diabetes that results in the progressive loss of peripheral nerve fibers, due to the underlying peripheral vascular disease that decreases blood flow to the affected local and high glycemic levels (Dyck et al., 1993). Diabetic neuropathy can occur in type 1 and type 2 diabetes, being more frequent in older patients (Casellini & Vinik, 2007). Other conditions such as microvascular disease and increased susceptibility to infection may also be present (Rathur & Boulton, 2005). Consequently, an impaired wound healing in diabetic foot ulcer patients is mostly due to (i) the exacerbated and persistent activity of metallo-proteinases (MMPs) and consequently, low levels of MMP inhibitors, allied to (ii) the low supply of oxygen and nutrients to the wound site due to ischemia and vascular disease (Guo & DiPietro, 2010). Noteworthily, there is also a decrease on the expression of VEGF and PDGF leading to an impairment of macrophages functions and chemotaxis, as well as, a prolonged inflammatory state is developed causing deregulation of the neovascularization phase (Acosta et al., 2008). On the ECM, these patients can suffer from accumulation of components, abnormal migration and proliferation of fibroblasts and keratinocytes and nitric oxide accumulation (Silva et al., 2016).

The wound healing process is a coordinated timely sequence where the major differences between healthy individuals and diabetic individuals are focused on the inflammatory phase, impairing the progression of the process to fully repair or regenerate the wound site (fig. 2). Therefore, there is a need for the development of new wound dressings that can overcome the particular characteristics of chronic wounds in diabetic individuals.

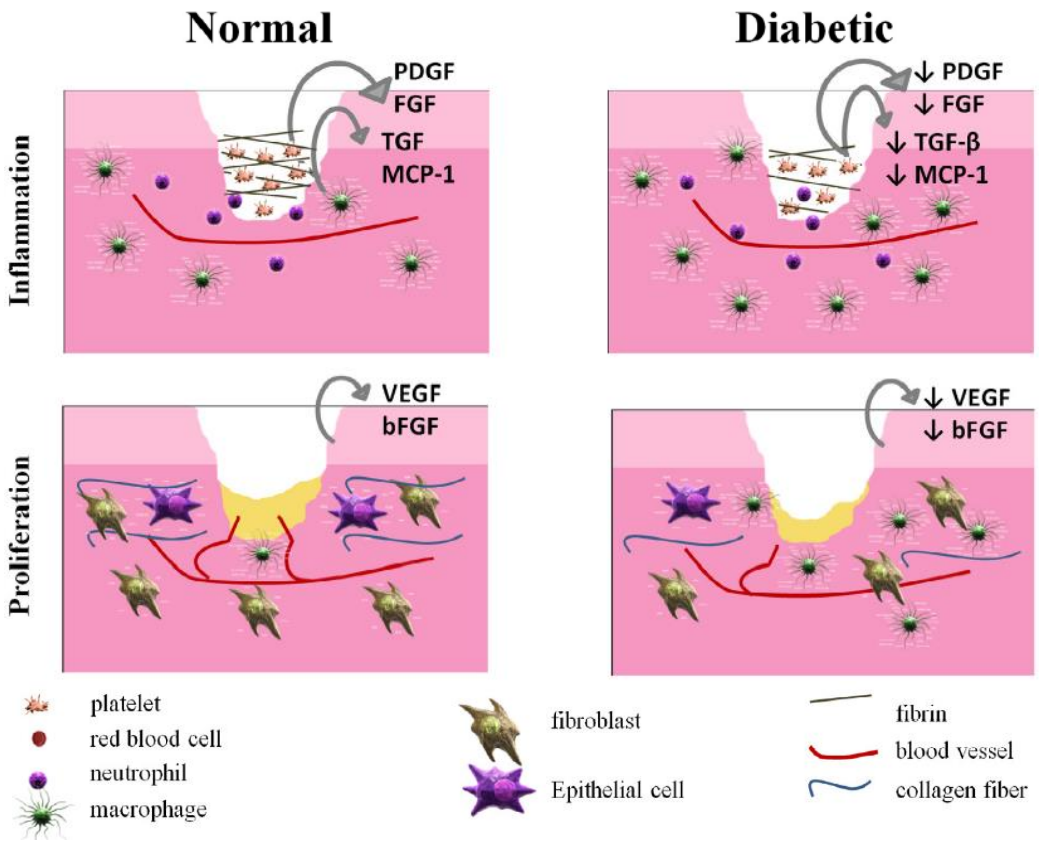

Figure 2 - Comparison between the wound healing pathway of a (left) healthy and a (right) diabetic individual. In the inflammatory phase there is a low expression of cytokines and growth factors due to a poor development of the homeostasis phase, hampering the progression of the wound healing pathway, forming a chronic wound or leading to an incomplete wound closure. Adapted from (Moura et al., 2013).

2.3 Chronic Wounds Treatment and Wound Dressings

Chronic wounds treatment should be a simple process designed to be quick, effective, unpainful to the patient and inexpensive. The procedure starts by cleaning the wound site with a saline solution, since it is cheap and widely available at hospitals and/or health clinics. After cleaning the wound, its debridement is performed, i.e., the removal of all the non-viable tissue, either necrotic or devitalized tissue, by autolytic mechanism or surgical removal. Autolytic removal is when the wound site is treated in a way to promote patient's endogenous enzymes involved in fibrin degradation to remove all the non-viable tissues (Mulder et al., 1993). In spite of the attractiveness of such technique, surgical debridement is proved to be more efficient than autolytic debridement for removing devitalized tissue. Noteworthily, in recent years there is an increasing utilization of larvae/maggot's applications to the wound site. These organisms are capable of performing enzymatic and surgical debridement while being able to eliminate some of the pathogenic microbiota in the wound site and stimulate fibroblasts proliferation, however it is a technique on its early stages and is not a widely applied technique (Cranney & Barton, 1999). Debridement goal in the wound healing treatment is to expose healthy perfused tissue to promote the

proliferation of epithelial cells in the wound bed by removing necrotic debris, which are responsible for creating an environment prone to bacterial infections (Han & Ceilley, 2017). Afterwards, the wound is covered with a dressing that is kept moist. For this purpose, health practitioners can choose between films, gauzes, foams, hydrocolloids, hydrogels, and/or dressings containing silver or alginates. Their application will depend on the type of wound that the patient possesses and in which step of the wound healing pathway the wound is currently in, but their main function is to remove excess fluid from the wound and protect it from bacterial infections (Selvaraj & Santhini, 2015). Wound dressings are usually left for several days at the wound site until they no longer absorb the wound exudates or if they dislodge from the wound site. Some wound dressings might even contain growth factors to promote fibroblasts or epithelial cells proliferation, which in turn improves wound healing, or silver, which is used as a bactericidal agent. If the patient requires additional treatments due to underlying conditions, such as peripheral blood disease, compression stockings or bandages can be used to improve the blood circulation by applying pressure to the veins, helping blood return to the heart (Summary, 2013).

Wound dressings were firstly designed to have a protective function in the wound healing process. However, technology and research in the last decade revealed that a moisture environment in the wound site promotes a faster healing by inducing fibroblasts and keratinocytes proliferation, which in turn promotes collagen synthesis that reduces scar tissue formation due to the low oxygen tension (by activating hypoxic factors) created by occlusive wound dressing (Hali et al., 2000). Likewise, low amounts of exudate in the wound site will allow autolytic debridement, which in turn increases treatment chances of success. Consequently, traditional dry gauzes might not be suitable for an optimal wound healing process, allied with the pain associated in the application and removal of such wound dressings (Han & Ceilley, 2017). An ideal wound dressing must fulfill the following requirements: (i) provide thermal isolation, gaseous exchange and help drainage and debris removal, (ii) be biocompatible and not cause allergic reactions or immune responses, (iii) protect and prevent the wound from bacterial infections, and (iv) easily removed without damaging the wound tissue and peripheral tissue (Halim et al., 2012; Morton & Phillips, 2012). There isn't a wound dressing that can treat all chronic wounds, however, by manipulating the chemical and physical properties of wound dressings it is possible to optimize the same product for more than one type of chronic wound (Boateng et al., 2008; Fonder et al., 2008). Currently, hydrocolloids and hydrogels are the most commonly used wound dressings to treat chronic wounds.

Hydrocolloid dressings are moist wound dressings comprised of a backing material and a layer of hydrophilic or colloidal particles. Backing materials are usually foams, semi-permeable films or non-woven polyester fibers, making these wound dressings capable of being loaded with biocompatible gels made of proteins such as collagen, or

polysaccharides such as cellulose (Moura et al., 2013). Hydrocolloid dressings are suitable for wounds with exudate, since they can absorb any excess liquid and create a moist environment while maintaining water and gaseous exchanges. However, they are not recommended for infected wounds as they can potentially create a hypoxic and moist environment that can lead to autolysis of the necrotic tissue, potentiating the risk of infection (Jeffcoate et al., 2004). Consequently, these wound dressings can be applied on diabetic foot ulcers only if the wound is superficial and no infection is present (McIntosh, 2007).

Hydrogel dressings are comprised of one or more hydrated polymers with a total weight of at least 20% water (Fonder et al., 2008). Their gel-like structure is obtained by the crosslinking of polymers through covalent or non-covalent forces, depending on the desired swelling capacities and conformational structures. The ability of controlling the chemical or physical properties of the gels allows them the property of reversible swelling in aqueous environments at specific pH or ionic strength values (Brandon et al., 2009). Moreover, these wound dressings are more suitable for dried wounds with few exudates, since their application on wounds with excess exudate can cause tissue maceration, which impact wound healing progression (Edwards & Stapley, 2010). In addition, they have minimal interaction with the wound bed, an advantage since the wound healing is not disturbed and the patient pain is minimized.

The wound dressings characteristics mentioned above can be further improved by incorporating bioactive compounds, such as growth factors and other cell growth stimulating drugs or stem cells, by utilizing different synthetic or natural biocompatible polymers in their structure, or a combination of both (Hong et al., 2008; Matsumoto & Kuroyanagi, 2010). Although synthetic polymers are thoroughly described and employed on commercially available wound dressings for the treatment of diabetic foot ulcers, there is an increasing interest on the development of new solutions based on natural polymers that can be used for designing novel wound dressings.

2.4 Natural Polymers for Diabetic Foot Ulcers Dressings

Natural polymers are usually proteins or polysaccharides of microbial, animal or vegetal origin that can mimic the human body ECM components. However, these polymers are susceptible to natural degradation processes by endogenous enzymes (Tabata, 2009). Moreover, there is an associated high cost with these materials as additional steps of purification are needed to avoid the transmission of infection diseases or immunological responses, due to allogenic or xenogeneic characteristics of the source materials. Also, it can exist a batch-to-batch variation from the industrial isolation of these polymers (Malafaya et al., 2007; Sell et al., 2010). Nonetheless, if these polymers are properly used and/or functionalized to overcome the problems mentioned above, new materials can be developed to be suitable for the treatment of diabetic foot ulcers.

One of the first natural polymers to be explored was chitin. Chitin is a cationic linear polysaccharide of N-acetyl-d-glucosamine units linked by ß-(1-4) glycosidic bonds (Kim et al., 2008) that is found in the exoskeleton of arthropods, crustacea, some mollusk and in the cell walls of fungi, being greatly available at a low cost on shrimps and crabs shells (Dai et al., 2011). However, chitin is only soluble at a pH below 6.0 and consequently is insoluble in water. For being solubilized, it needs to be converted in the usable form, chitosan, by a thermochemical deacetylation in an alkaline solution that creates a linear copolymer of d-glucosamine and N-acetyl-D-glucosamine (Dash et al., 2011). The extent of deacetylation and molecular weight is responsible for the chemical, physical and biological properties of chitosan. Chitosan is considered to be a biodegradable (chemical hydrolysis and lysozymes), biocompatible, non-antigenic, non-toxic, bioadhesive, anti-microbial, bioactive and hemostatic compound (Dai et al., 2011; Tessmar & Göpferich, 2007). A noteworthy characteristic of this polymer is the possibility of functionalizing the amino and hydroxyl groups, allowing the development of diverse compounds with different properties. The responsiveness of the polymer to pH changes allows it to be used as a delivery matrix for pharmaceutical bioactive compounds, and the positive net charge at acidic pH values (which are present in the wound environment) enhances the probability of the polymer matrix interacting with proteins, anionic polysaccharides or nucleic acids. Also, chitosan has film-forming properties and demonstrates a strong adhesion to the wound tissue. As such, chitosan is an efficient natural polymer for wound dressings that proved to accelerate wound healing on diabetic foot ulcers (Dash et al., 2011; Madihally, 2011).

Hyaluronic acid (HA) is a linear non-sulfated glycosaminoglycan composed of alternating disaccharide units of α-1,4-D-glucuronic acid and ß-1,3-N-acetyl-D-glucosamine linked by ß(1→3) glycosidic bonds (Tessmar & Göpferich, 2007). It is a major component of ECM from connective tissue, mainly cartilage and synovial fluid. Contrary to chitin it is soluble in water and can produce highly viscous three-dimensional structures through hydrogen bonding. This property can be harnessed to fill the space left in the wound site while absorbing and retaining some exudate. HA is biodegradable, possessing a degradation profile that is directly dependent on the molecular weight of the polymer. Noteworthily, HA degradation products can stimulate endothelial cell proliferation and migration, which in turn modulates the inflammatory processes and angiogenesis (Xu et al., 2007; Vazquez et al., 2003).

Alginate is a polysaccharide composed of ß-D-mannuronate (M-residues) and α-L-guluronate (G-residues) covalently linked in an alternating sequence. Similarly to chitin it has a marine origin, as it is a structural component of marine brown algae, but can be also found in some soil bacteria (Malafaya et al., 2007). The main attraction of alginates is the possibility of forming hydrogels with reversible properties based on the interactions of divalent cations, such as Ca^{2+} or Mg^{2+}, with G-residues of adjacent alginate chains, where these chains are cross-linked by ionic interactions, being an easy

and cheap chemical cross-linking step (D'Ayala et al., 2008). Alginate is biocompatible, has low toxicity, mucoadhesive properties and is sensitive to pH alterations (D'Ayala et al., 2008). However, two problems are associated with these polymers: (i) the uncontrollable degradation profile after the divalent cations being removed from the crosslinking, although this problem can be solved by performing covalent crosslinking with other biopolymers (Han et al., 2010); (ii) the enzymatic degradation of these polymers is slow in mammals and they have an hydrophilic nature, which affects the interactions with ECM proteins (D'Ayala et al., 2008). Nonetheless, alginate-based wound dressings are an attractive treatment for diabetic foot ulcers due to its easy tunable properties, bioactive molecules loading capacity and wound exudate absorption characteristics, allowing the commercialization of various products for wound healing. An example of a commercially available product based on alginate is Algisite M Calcium Alginate Dressing (Smith and Nephew Inc., Australia), which can maintain a good wound healing environment and shows an enhanced wound healing as opposed to other treatments.

Collagen, like HA, exists abundantly in the human body, as a component of the ECM, in twenty-seven different types, with types I to IV the most common. Type I collagen is present in the healthy skin ECM and it is responsible for the strength and integrity of the ECM, supporting cell signaling for cell migration, proliferation or differentiation. Therefore it can be used as a material for wound dressings (Arul et al., 2007; Malafaya et al., 2007). Collagen degradation is mediated by endogenous collagenases, gelatinases and MMPs, but it can be controlled by chemical or physical crosslinking of its polymer chains despite this being a difficult process (Lammers et al., 2009). As it is a major component of the skin ECM, biocompatible, has low toxicity and a low inflammatory response, it is widely used for chronic wound dressings, polymeric solutions or even powders. Nonetheless, gelatin, a product of the denaturation of collagen, is a superior polymer for biomedical applications as the problems with collagen biodegradation and sterilization can be easily resolved. Moreover, the denaturation process of gelatin can create two types of gelatin, alkaline and acidic gelatin, allowing the development of medical devices with different properties based on the surface charge of gelatin, that can be applied as delivery systems for bioactive compounds (Gioffrè et al., 2012). Collagen is the most utilized natural polymer in wound dressings for chronic wounds and there are various commercially available products, such as Unite® Biomatrix (Synovis Orthopedic and WoundCare, Inc.), which is strong and durable, conforms to the wound topology and absorbs exudate, allowing the proliferation of granulation tissue, and Promogran Prisma® Matrix (Systagenix), which protects the wound from bacterial infection, adapts to the wound topology, promotes cell differentiation and is easily applied on the patient, minimizing its pain.

The biopolymers mentioned above are the commonly used materials for developing new solutions for diabetic wounds, but fibrin, silk fibroin and elastin have also been pursued throughout the years as candidates for new wound dressings (Koria et al., 2011; Malafaya et al., 2007; Mandal & Kundu, 2009). Different materials possess different attractive properties, such as a tunable polymer structure that is responsive to the wound physical or chemical conditions, loading capacity, anti-bacterial nature,

enhancement of cell proliferation and reduced cost. However, none of the polymers mentioned above comprise all these characteristics and, consequently, there is a need to search for novel natural polymers to answer to this need.

2.5 Cyanobacteria and their extracellular polymer substances

Cyanobacteria are photoautotrophic prokaryotes and some strains are capable of atmospheric nitrogen fixation. Therefore, it is only necessary minimal culture medium and light to grow these microorganisms, resulting in reduced production costs (Hatzinger & Kelsey, 2005). Another characteristic of these microorganisms is their ability to synthesize extracellular polymer substances (EPS) that can remain associated to the cell surface as capsular polysaccharides (CPS) or be released into the environment as released polysaccharides (RPS). The quantity of EPS produced, as well as its type and chemical composition, are dependent on the strain and the culture conditions (Pereira et al., 2009). These EPS are complex heteropolysaccharides composed of up to 15 different monosaccharides: glucose, galactose, mannose, fructose, ribose, xylose, arabinose, fucose, rhamnose, methyl rhamnose, glucuronic and galacturonic acid, galactosamine, glucosamine and N-acetyl galactosamine (De Philippis & Vincenzini, 1998). The presence of one or two uronic acids or even sulphate groups on EPS composition is responsible for its overall anionic nature (Sutherland, 1994).

RPS can be recovered from the supernatant of cyanobacterial cultures without complex or expensive techniques due to their physicochemical properties, making cyanobacteria an attractive source of inexpensive natural polymers (De Philippis et al.,1998). Within cyanobacterial strains, members of the *Cyanothece* genus, such as *Cyanothece* sp. CCY 0110 (hereafter CCY 0110) and *Cyanothece* sp. VI 22 (hereafter VI 22), are good producers of RPS, since both strains release most of the polymer to the culture medium (Mota et al., 2013). Both strains were isolated from marine environments, which allows the exploitation of an abundant resource, saltwater, compared to the scarcity of freshwater.

CCY 0110 is a unicellular strain isolated from costal waters of Zanzibar, Africa. The RPS can be released to the culture medium in quantities up to 70% of the total EPS produced and are complex macromolecules composed by the following nine different monosaccharides: glucuronic acid, galacturonic acid, arabinose, fucose, rhamnose, xylose, galactose, glucose and mannose. Also, peptides and sulphate groups are present on its constitution (Mota et al., 2013). The uronic acids and sulphate groups present on the RPS contribute to a strong anionic charge, which can electrostatically repulse bacteria, as their membrane surface is predominantly negatively charged (Costa et al., 2019). Other characteristics of this polymer are its amorphous nature and hydrophilic characteristic that also contribute to decreasing bacterial adhesion due to the low surface energy associated with hydrophilic surfaces (Costa et al., 2019). This polymer was previously studied as a biosorbent for heavy metals (Mota et al., 2016) and as a

vehicle for therapeutic proteins and vitamins delivery systems (Leite et al., 2017; Estevinho et al., 2019)

VI 22 is a unicellular strain isolated from tidal pools in Sardinia, Italy and this strain was also described as a strong EPS producer (De Philippis et al., 1998). The RPS are composed by the following seven different monosaccharides: glucuronic acid, galactose, glucose, mannose, xylose, fucose and rhamnose, as well as sulfate groups and some acetate and pyruvate groups (De Philippis et al., 1998). Preliminary results revealed that this polymer could have immunomodulatory activity (data not published), a property that can be harnessed to modulate the inflammatory phase of the wound healing pathway, reducing the required time to the wound site fully repair. However, to date, no research envisioning RPS applications was performed with this *Cyanothece* strain.

Cyanobacterial polymers are attractive for the development of new wound dressings to treat chronic wounds in diabetic patients since they possess all the natural polymer desired properties to fulfill the necessary requisites. However, the use of cyanobacterial polymers in this field is still in its infancy compared to the use of other biopolymers. Therefore, during this work several techniques were performed to evaluate the extent of CCY 0110 and VI 22 RPS solutions in wound healing applications.

Chapter 3

Materials and Methods

3.1 Cyanobacteria Production and RPS Extraction

3.1.1 *Cyanothece* sp. VI 22 Cultivation

Cyanothece sp. VI 22 (available at Centro di Studio dei Microrganismi Autotrofi, CNR, Firenze, Italy) cyanobacterium was grown in 1 L bioreactors (DWK Life Sciences) containing 800 mL of ASN III medium prepared as previously described (Rippka et al., 1979) with magnetic stirring (150 rpm), aeration (1.2 L/min), light intensity of 50 μE $m^{-2}s^{-1}$ in a 16 h light / 8 h dark regiment, at 25 °C. Light intensity was measured with an Electric Quantum Light Meter (Spectrum Technologies, Inc., USA).

3.1.2 Growth Measurements and Carbohydrate Content Determination

Optical density (OD) measurements were performed once a week at 730 nm on a UVmini-1240 UV-vis spectrophotometer (Shimadzu Corp.) as previously described (Anderson & Mcintosh, 1991). One millilitre of culture was collected for OD measurements and when OD > 1, the sample was properly diluted with type II water.

Dry weight (DW) measurements were performed in triplicates using 5 mL of culture. The culture was centrifuged for 10 minutes at room temperature (RT) at 5000 rpm to obtain the cell pellet, which was resuspended in type II water and transferred to previously weighted and dried tubes (overnight at 55 °C). Another centrifugation was performed at maximum speed on a minispin plus microcentrifuge (Eppendorf) and the supernatant was properly discarded. Pellets were dried for 24 h at 55 °C, weighted, and dry weight was calculated – DW: weight (g) tube with pellet at 24 h timepoint - weight (g) tube without pellet at 0 h timepoint.

Carbohydrate content determination was performed in triplicates. For this, 50 mL of culture were dialyzed against a minimum of 10 volumes of type II water for 24 h on continuous stirring in dialysis membranes (12-14 kDa of molecular weight cut-off; Medicell International Ltd). Total carbohydrate content was quantified spectrophotometrically at 488 nm by the phenol-sulphuric acid assay (Dubois et al.,

1956). Carbohydrates released to the culture medium (RPS) were obtained by centrifugation of 5 mL of the dialysed culture for 15 min at RT at 5000 rpm to remove the cells. Then the RPS content present in the supernatant was measured as described above. Capsular polysaccharides (CPS) were obtained by centrifugation of 5 mL of the dialysed culture for 15 min at RT at 5000 rpm, the supernatant was recovered, resuspended on type II water and boiled for 15 minutes at 100 °C, to detach the CPS from the cell surface. Then the CPS content present in the supernatant was measured as described above. Carbohydrate contents are expressed as milligram per litre of culture.

3.1.3 Cyanothece sp. VI 22 RPS Isolation

Cyanothece sp. VI 22 culture was grown until an OD_{730nm} of approximately 2.3. The isolation protocol followed was previously optimized (Mota et al., 2013). Briefly, the culture was dialyzed against a minimum of 10 volumes of type II water for 48 h on continuous stirring in dialysis membranes (12-14 kDa of molecular weight cut-off; Medicell International Ltd). Cells were removed by centrifugation for 15 min at 4 °C at 20 000 x g on a Winti J-26 XPI centrifuge (Beckman Coulter) and the supernatant was precipitated with 2 volumes of absolute ethanol (Aga UN1170) overnight at 4°C. Afterwards, RPS were collected with sterile metal forceps and lyophilized (VirTis BenchTop Pro with Omnitronics™, SP Scientific).

3.2 RPS *in vitro* Experiments

3.2.1 Cyanothece sp. CCY 0110 and *Cyanothece* sp. VI 22 RPS Solution Preparation

Cyanobacteria CCY 0110 and VI 22 RPS solutions were prepared by dissolving the RPS in RPMI 1640 medium or Fibroblast Growth 116-500 Medium, both supplemented with 10% fetal bovine serum (FBS; Invitrogen Life Technologies, UK), for 12 h at RT with agitation. The final concentrations for both RPS solutions were 0.10%, 0.25%, 0.50%, 0.75% and 1.00% (w/v).

3.2.2 In vitro Culture of Human Microvascular Endothelial Cells and Human Dermal Fibroblasts

Human microvascular endothelial cells (HMEC-1; ATCC, UK) were cultured in RPMI 1640 medium, whereas human dermal fibroblasts (HDF; ATCC, UK) were cultured in Fibroblast Growth 116-500 Medium, both supplemented with 10% fetal bovine serum (FBS; Invitrogen Life Technologies, UK). Cells were maintained at 37 ºC in a humidified 5% CO_2 atmosphere. All experiments were performed between passages 4 and 9.

3.2.3 Tetrazolium Reduction Assay

Tetrazolium reduction assay measure the enzymatic activity of viable cells by the incubation with a reagent that is converted to a colored or a fluorescent substrate, producing a signal that can be detected by a spectrophotometer, where the intensity of the signal is directly correlated to the number of viable cells (Sittampalam et al., 2016). Apoptotic or dead cells do not have a stabilized metabolism and, consequently, will produce a lower signal compared to viable cells.

HMEC-1 and HDF cells (2×10^4 cells/ml) were allowed to grow until 70–90% confluence and then incubated with the RPS solutions described in section 3.2.1 CCY 0110 and VI 22 RPS were tested for application in acute conditions. After the 24 h incubation period of cells in contact with RPS, cells were washed twice with PBS X1 and subjected to the MTT assay, as previously described (Guerreiro et al., 2007). Chronic conditions were tested only on the CCY 0110 RPS using the same protocol but after a 72 h incubation period.

3.2.4 Bromodeoxyuridine Assay

The synthetic nucleoside bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU) is a non-radioactive analog of thymidine that can be incorporated into the newly synthetized DNA of replicating cells, instead of the natural occurring thymidine, during S phase of the cell cycle. This synthetic thymidine substitutes a bromine atom for thymidine's CH_3 group, which can also act as an anomalous scatter for X-ray diffractions (Peterson et al., 1996). Consequently, all the cells that undergo cell proliferation while in contact with BrdU will have a synthetic thymidine incorporated in their DNA composition. This synthetic thymidine can be conjugated with specific anti-BrdU antibodies to produce a measurable immunohistochemical signal on a spectrophotometer (Konishi et al., 2011). Moreover, all the new cells can also pass their BrdU incorporated DNA to their daughter cells if the latter undergo replication.

HMEC-1 and HDF cells (1×10^4 cells/mL) were cultured with both RPS by employing the treatment procedures for 24 h described on the MTT assay (Guerreiro et al., 2007). Cells were then washed twice with PBS and subjected to the Cell Proliferation ELISA BrdU kit (Roche, Penzberg, Germany), according to the manufacturer's instructions.

3.2.5 Cell Culture Wound Closure Assay

Cell culture wound closure assay allows the visualization of cell motility at various timepoints and the morphological characteristics, such as lamellipodium formation, tail retraction and directional movement (Zhang et al., 2012).

HMEC-1 and HDF cells (2×10^4 cells/ml) were allowed to grow until 70–90% confluence in RPMI 1640 medium and Fibroblast Growth 116-500 Medium, both supplemented with 10% fetal bovine serum (FBS; Invitrogen Life Technologies, UK). Using a 200 µl

micropipette tip, a longitudinal scratch was performed across the midline of the well. After washing with PBS, CCY 0110 RPS solutions at 0.25% and 0.50% (w/v) concentrations were added to the cells and photomicrographs were taken at 0 h, 16 h and 24 h after the scratch was made, using a phase-contrast inverted microscope (50×magnification) (Zeiss Axiovert S100 - Moticam 2500).

3.3 RPS *in vivo* Experiments

3.3.1 In vivo Biocompatibility Assay

Thirty Wistar rats 8-week old male mice (Charles-River, Spain) were randomly divided into 5 experimental groups (n=6). Animals were maintained under controlled conditions of temperature (23 ± 5 °C), humidity (35 ± 5%), and 12 h light/dark cycles and access to diet and beverages were allowed *ad libitum*. All animal experiments were conducted at the animal house located at the Faculty of Medicine, University of Porto, and were carried out by trained technicians in accordance with the European Community policy for Experimental Animal Studies [European Community law dated from November 24th, 1986 (86/609/CEE) with addendum from June 18th, 2007 (2007/526/CE)]. After isoflurane anesthesia, hair on the dorsal side of the animals was shaved and the skin was cleaned with 70% ethanol. A 5 mm skin biopsy punch was used to create full thickness cutaneous wounds under aseptic conditions. Two wounds were created on the dorsal surface with one on each side of the midline. CCY 0110 RPS solutions at 0.25% and 0.5% (w/v) concentrations, and VI 22 RPS solutions at 0.1% and 0.25% (w/v) concentrations, prepared in deionized water and reticulated with a 1 M $CaCl_2$ solution, were used as treatments as they showed the best cytotoxic and proliferative properties. These treatments and sterile serum were topically applied every second day (50 µl per wound) for one week. Wounds were left open to heal without being covered by any type of wound dressing. After 7 days, the animals were euthanized, and wound tissue was collected for histological and immunohistochemistry studies. Skin wound tissue specimens were fixed in 10% neutral-buffered formalin solution and embedded in paraffin. Histological analyzes were performed in 5 µm tissue sections.

3.4 Statistical Analysis

Data is expressed as mean ± standard deviation (SD). Statistical analysis was performed by Kruskal-Wallis test, using GraphPad Prism 7.0 software. The statistically significant level chosen for all statistical tests was $p<0.05$.

Chapter 4

Results

4.1 *Cyanothece* sp. VI 22 Cultivation and RPS Production

Aiming at developing polymeric solutions based on cyanobacterial released polysaccharides (RPS) for the treatment of chronic wounds, the cyanobacterium *Cyanothece* sp. VI 22 (hereafter VI 22) was grown under previously optimized conditions for a strain of the same genus.(Mota et al., 2013) Optical density (OD), dry weight (DW) and carbohydrate contents were measured to assess cyanobacterial growth and carbohydrate production.

VI 22 growth showed an approximate linear and slow increase overtime and it does not exhibit a typical bacterial growth curve, where an exponential phase is always present (fig. 3).

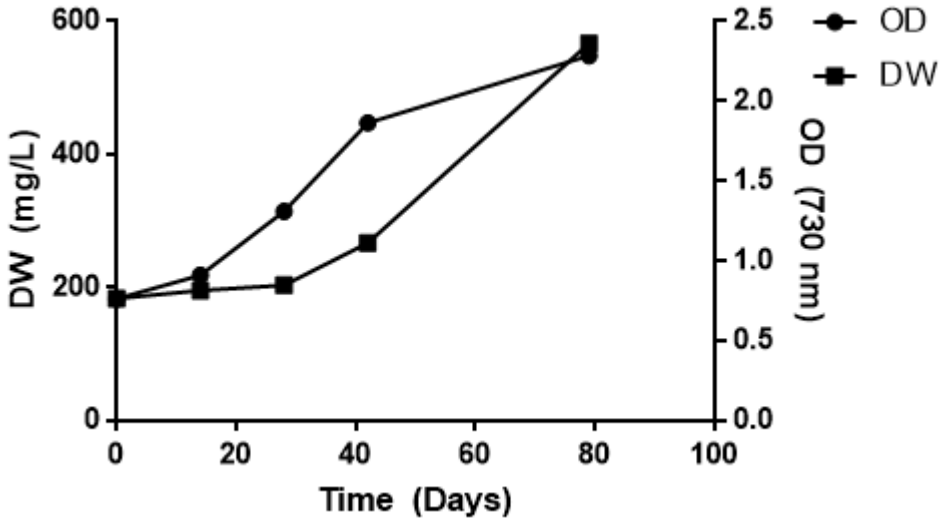

Figure 3 - *Cyanothece* sp. VI 22 (VI 22) growth expressed as OD_{730nm} and DW (mg/L). VI 22 was grown in 1 L bioreactors with the following conditions: 800 ml of ASN III medium, magnetic stirring (150 rpm), aeration (1.2 L/min), light intensity of 50 μE $m^{-2}s^{-1}$ in a 16 h light / 8 h dark regiment, at 25 °C.

Carbohydrates production was evaluated as a quantification of capsular polysaccharides (CPS), released polysaccharides (RPS) and total carbohydrates (CHT). Carbohydrates results were normalized to mg/mg of DW concentration in order to evaluate total carbohydrate production at the cellular level.

Throughout VI 22 culture growth, CPS production showed minimal variations, however in the first 42 days of culture, there was a slight decrease on the RPS and CHT produced, where the initial 0.38 mg/mg DW RPS content dropped to 0.29 mg/mg DW and the 0.45 mg/mg DW CHT content dropped to 0.34 mg/mg DW (fig. 4). Notably, after those 42 days the RPS and CHT production by VI 22 cells had an approximate 3-fold increase on their production where RPS achieved 0.90 mg/mg DW and CHT achieved 1.09 mg/mg DW. Nonetheless, CPS represents about 9% ± 1.8% of CHT and RPS represents about 85% ± 1.4% of CHT throughout the tested time period. Moreover, VI 22 polymer quantity after RPS extraction was 946 mg/L and 2.4 mg/mg DW (data not shown).

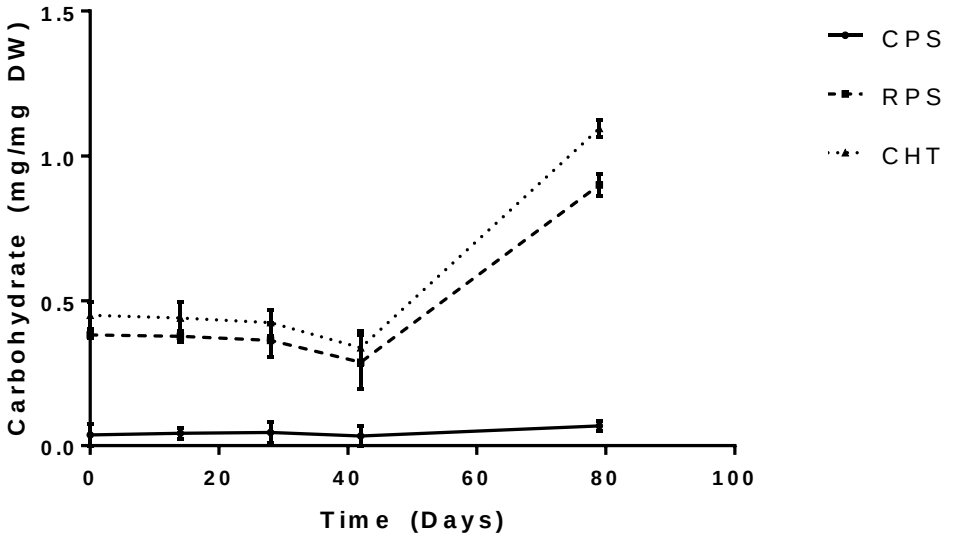

Figure 4 - *Cyanothece* sp. VI 22 (VI 22) capsular polysaccharides (CPS), released polysaccharides (RPS) and total carbohydrates (CHT) production expressed as mg per mg of dry weight (mg/mg DW). VI 22 was grown in 1 L bioreactors with the following conditions: 800 ml of ASN III medium, magnetic stirring (150 rpm), aeration (1.2 L/min), light intensity of 50 μE $m^{-2}s^{-1}$ in a 16 h light / 8 h dark regiment, at 25 °C.

4.2 *Cyanothece* spp. RPS - Cell viability assessment

To evaluate the cytotoxic properties of CCY 0110 and VI 22 RPS solutions on human dermal fibroblasts (HDF) and human microvascular endothelial cells (HMEC-1), the tetrazolium reduction assay (MTT) was employed in acute conditions (24 h) and chronic conditions (72 h).

Regarding the acute effects on the viability of HDF, CCY 0110 RPS solutions have no cytotoxic effects on these cells (fig. 5A). In fact, 0.50% (w/v) RPS solution induce a significant increase on cell viability, suggesting that some interaction between the fibroblasts and the polymer might develop a stimulation of cell metabolism. On the

other hand, VI 22 RPS solutions induce a decrease on cell viability superior to 30%, except with the 0.50% (w/v) RPS solution (fig. 5B).

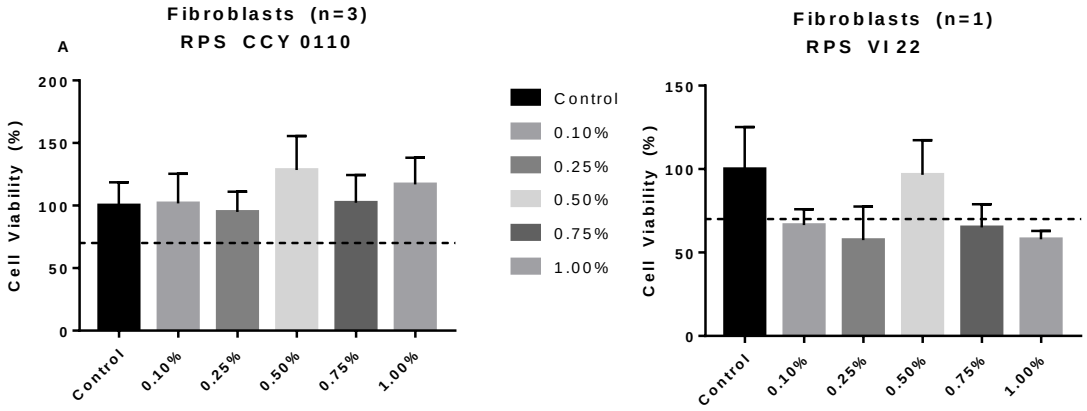

Figure 5 - Acute cytotoxic effects of (A) *Cyanothece* sp. CCY 0110 (CCY 0110) (n=3) and (B) *Cyanothece* sp. VI 22 (VI 22) (n=1) RPS solutions on human dermal fibroblasts (HDF) evaluated by the tetrazolium reduction (MTS) assay. The values are means ± standard deviations.

The results obtained from the acute incubation of RPS solutions with HMEC-1 were in general worse than the results obtained using HDF. Regarding CCY 0110 RPS solutions (fig. 6A), all the tested concentrations induced a decrease of about 30% on cell viability, except the 0.75% and 1.00% (w/v) RPS solutions that presented a decrease less accentuated. Furthermore, VI 22 RPS solutions (fig. 6B) had a very impactful role on HMEC-1 viability, revealing a decrease superior to 50% in all of the tested concentrations, except the 0.25% (w/v) RPS solution which had a decrease of 24%. However, it is noteworthy that this cell type is extremely sensitive to cell culture conditions and small alterations to their environment may greatly impact the cell metabolism.

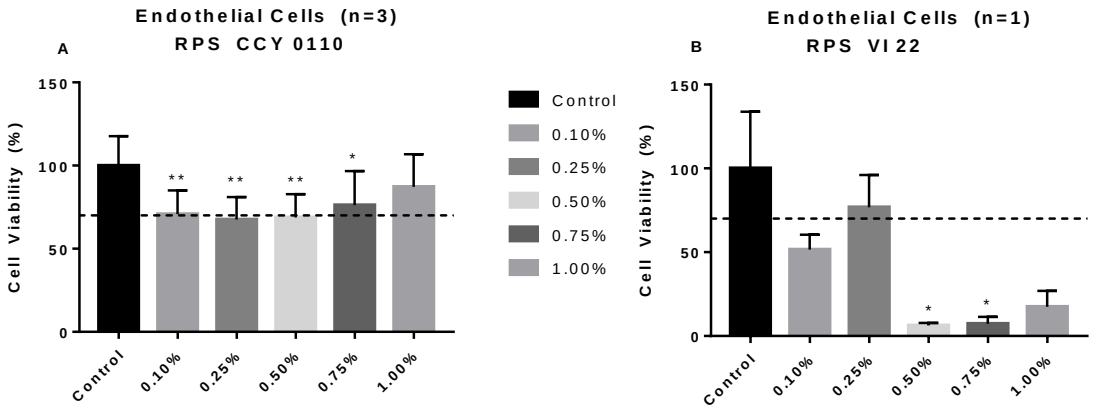

Figure 6 - Acute cytotoxic effects of (A) *Cyanothece* sp. CCY 0110 (CCY 0110) (n=3) and *(B) Cyanothece* sp. VI 22 (VI 22) (n=1) RPS solutions on human microvascular endothelial cells (HMEC-1) evaluated by the tetrazolium reduction (MTS) assay. The values are means ± standard deviations. Statistically significant differences were calculated through Kruskal-Wallis test and are indicated with: * p value ≤ 0.05; ** p value ≤ 0.01.

The results concerning the chronic effects of CCY 0110 RPS solutions when incubated with HDF suggest that a prolonged contact has a slight negative impact on cell viability regarding 0.25% and 0.75% (w/v) RPS solutions (fig. 7A). Noteworthy, regarding the other RPS solutions tested, there was an increase in cell viability, as observed in the acute effects. On the other hand, HMEC-1 cells when in contact with CCY 0110 RPS solutions for 72 h denote a significant reduction of cell viability superior to 50% (fig. 7B).

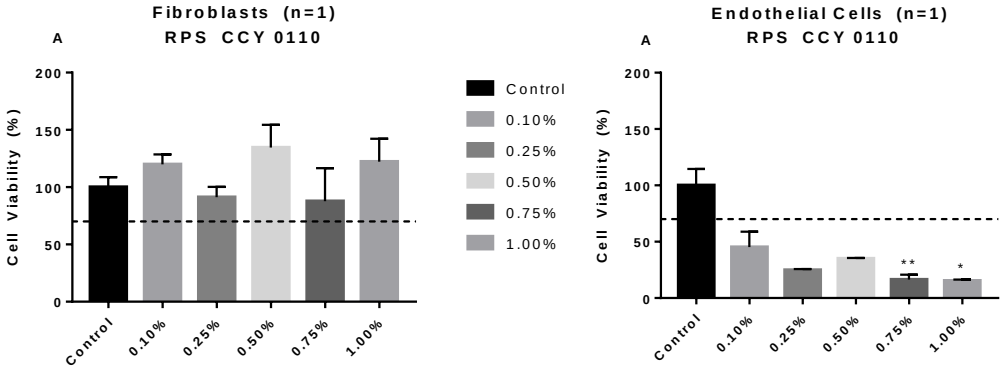

Figure 7 - Chronic cytotoxic effects of (A) *Cyanothece* sp. CCY 0110 (CCY 0110) (n=1) RPS solutions on human dermal fibroblasts (HDF) and human microvascular endothelial cells (HMEC-1) evaluated by the tetrazolium reduction (MTS) assay. The values are means ± standard deviations. Statistically significant differences were calculated through Kruskal-Wallis test and are indicated with: * p value ≤ 0.05; ** p value ≤ 0.01.

4.3 *Cyanothece* spp. RPS - Cell Proliferation Assessment

The bromodeoxyuridine assay (BrdU) was performed in order to investigate the anti-proliferative or pro-proliferative effects of CCY 0110 and VI 22 RPS solutions on HDF and HMEC-1 cells. Regarding CCY 0110 RPS solutions proliferative properties on HDF, there is an increase in all RPS concentrations, apart from the 0.75% (w/v) concentration (fig. 8A). Notably, HDF had a progressive increase on cell proliferation as the RPS solution reached 0.50% (w/v) concentration and a following drop on cell proliferation. However, VI 22 RPS solutions in contact with HDF cells provokes a progressive decrease on cell proliferation as the RPS concentration increases, therefore the only VI 22 RPS concentrations that produce a lower anti-proliferative effect are the 0.10% and 0.25% (w/v) (fig. 8B).

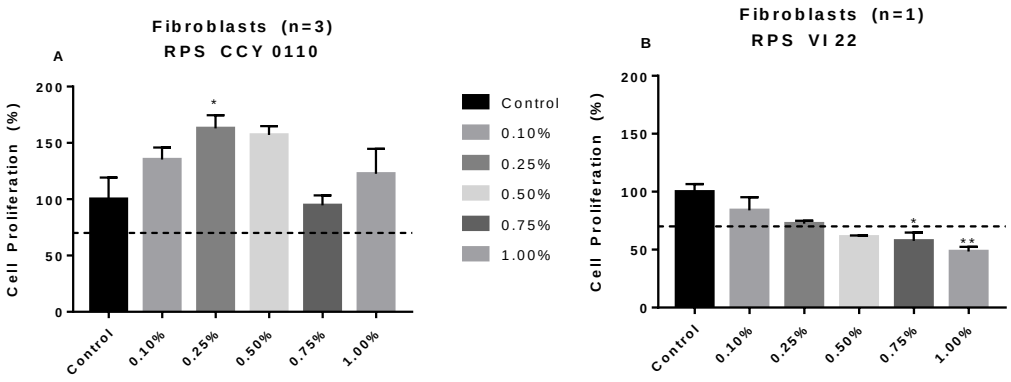

Figure 8 - Proliferative effects of (A) *Cyanothece* sp. CCY 0110 (CCY 0110) (n=3) and *Cyanothece* sp. VI 22 (VI 22) (n=1) RPS solutions on human dermal fibroblasts (HDF) evaluated by the bromodeoxyuridine (BrdU) assay. The values are means ± standard deviations. Statistically significant differences were calculated through Kruskal-Wallis test and are indicated with: * p value ≤ 0.05; ** p value ≤ 0.01.

The BrdU results of CCY 0110 RPS solutions in contact with HMEC-1 cells (fig. 9A) show that all CCY 0110 RPS solutions impact endothelial cell proliferation, except the 0.10% and 0.50% (w/v) concentrations, suggesting that the CCY 0110 RPS has anti-proliferative properties towards endothelial cells. However, CCY 0110 RPS at a concentration of 0.10% (w/v) shows pro-proliferative properties, whereas the 0.50% (w/v) has a decrease of 26% in cell proliferation. Notably, VI 22 RPS solution (fig. 9B) had a similar decrease on cell proliferation as observed in the test with fibroblast cells, in particular for the 0.75% (w/v) and 1.00% (w/v) RPS solution. Nonetheless, at an RPS concentration of 0.10% (w/v), this polymer promotes endothelial cell proliferation and the decrease that is observed in the 0.25% (w/v) concentration is not significant, suggesting that lower VI 22 RPS concentrations do not have anti-proliferative effects on endothelial cells.

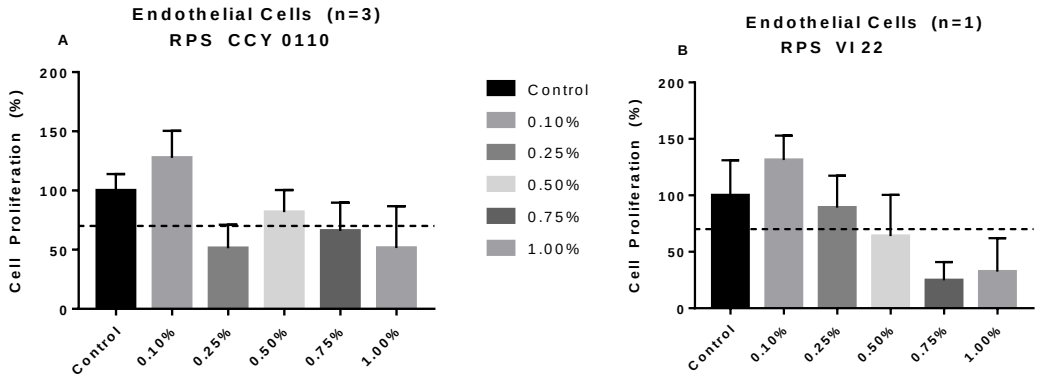

Figure 9 - Proliferative effects of (A) *Cyanothece* sp. CCY 0110 (CCY 0110) (n=3) and *Cyanothece* sp. VI 22 (VI 22) (n=1) RPS solutions on human microvascular endothelial cells (HMEC-1) evaluated by the bromodeoxyuridine (BrdU) assay. The values are means ± standard deviations.

4.4 *Cyanothece* sp. CCY 0110 RPS - Wound Closure Assessment

Cell migration when in contact with CCY 0110 RPS solutions was assed by performing the cell wound closure assay in HDF and HMEC-1 cells. At the time that these tests were conducted, not enough VI 22 RPS was available, consequently, only the CCY 0110 RPS was tested.

An injury was formed (t=0 h) in the group without RPS treatment (control) and the groups treated with 0.25% and 0.5% (w/v) CCY 0110 RPS solutions, creating a hiatus (fig. 10). After 16 h of performing the injury, fibroblasts were able to migrate towards the injury site and occupy the hiatus, although RPS treated groups showed a slightly lower unoccupied area as compared to the control group. Nonetheless, after 24 h from the injury being performed, fibroblasts were able to almost completely occupy the hiatus and move freely within the RPS solution. Moreover, their stretched phenotype was unaltered throughout their migration, which is indicative of healthy viable cells. The closure of the injured site was quantified throughout the experiment (fig. 11). Results indicate that indeed there is a slight decrease on fibroblasts migration in the groups treated with RPS solutions after 16 h, however, after 24 h no significant differences are observable between the groups.

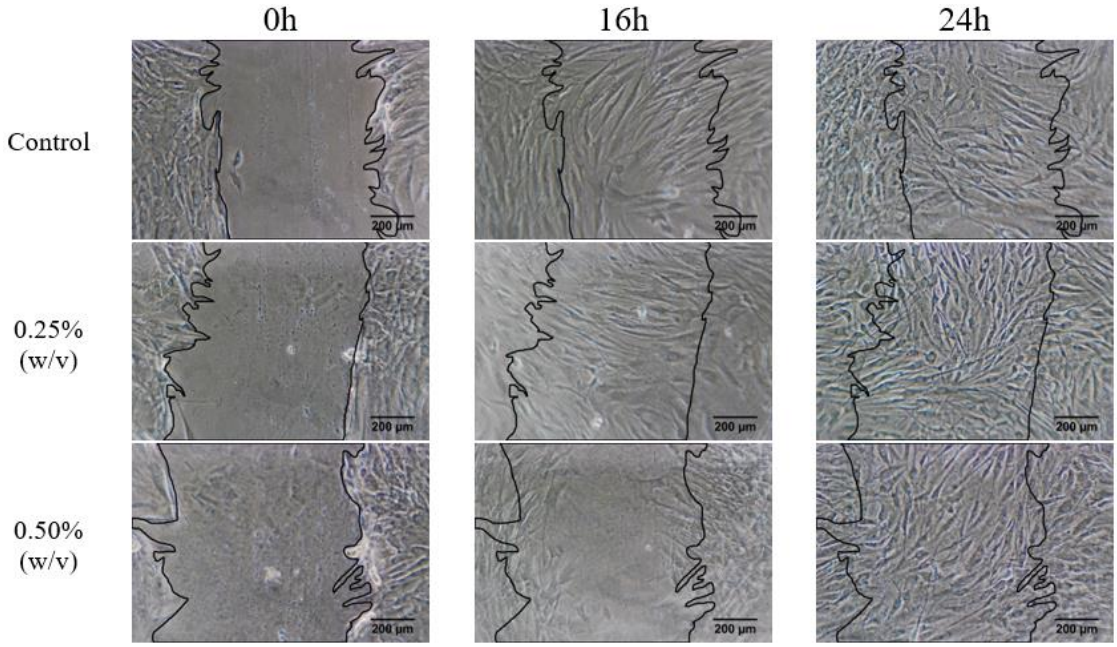

Figure 10 - Phase-contrast inverted microscope micrographs (50x magnification) of human dermal fibroblasts (HDF) migration in contact with *Cyanothece* sp. CCY 0110 (CCY 0110) RPS solutions at 0.25% and 0.5% (w/v) concentrations, and without CCY 0110 RPS solutions (control), towards an injured site.

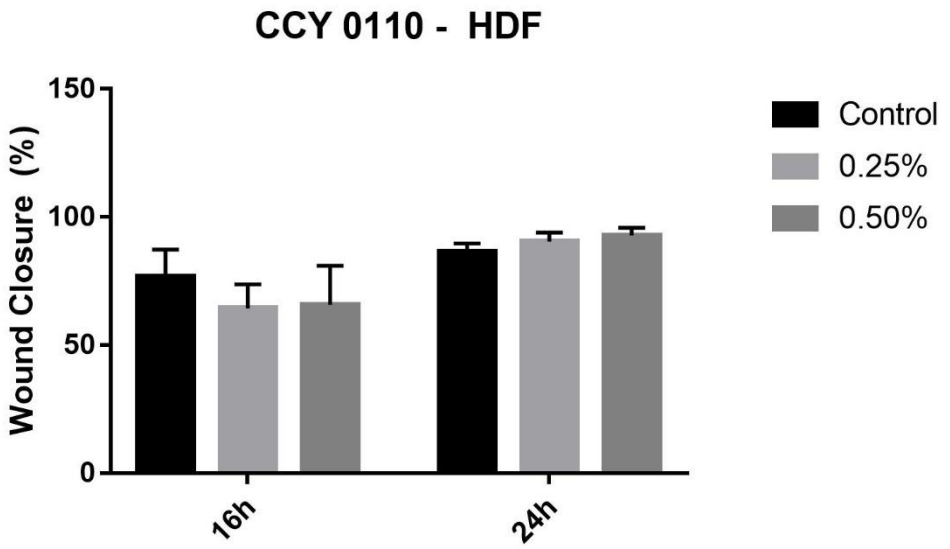

Figure 11 - Wound closure of human dermal fibroblasts (HDF) in contact with *Cyanothece* sp. CCY 0110 (CCY 0110) RPS solutions at 0.25% and 0.50% (w/v) concentrations, and without CCY 0110 RPS solutions (control), after 16 h and 24 h (n=3). The values are means ± standard deviations.

Regarding results of CCY 0110 RPS solutions in contact with HMEC-1, similar results were observed relatively to the cell culture wound closure assay of fibroblasts (fig. 12). Endothelial cell migration towards the hiatus seems to be hindered by the 0.50% (w/v) CCY 0110 RPS solution after 16 h of the injury being performed. However, after 24 h the hiatus has a low unoccupied area, suggesting that endothelial cells migration is not affected by the presence of CCY 0110 RPS solutions. Moreover, endothelial cells characteristic phenotype is maintained throughout the migration process with their stretched appearance while experiencing motility, and rounded shape when stationary. The closure of the injured site was quantified throughout the experiment (fig. 13). Results show that no clear difference is observable in the migration endothelial cells when incubated with CCY 0110 RPS solutions or without RPS treatments.

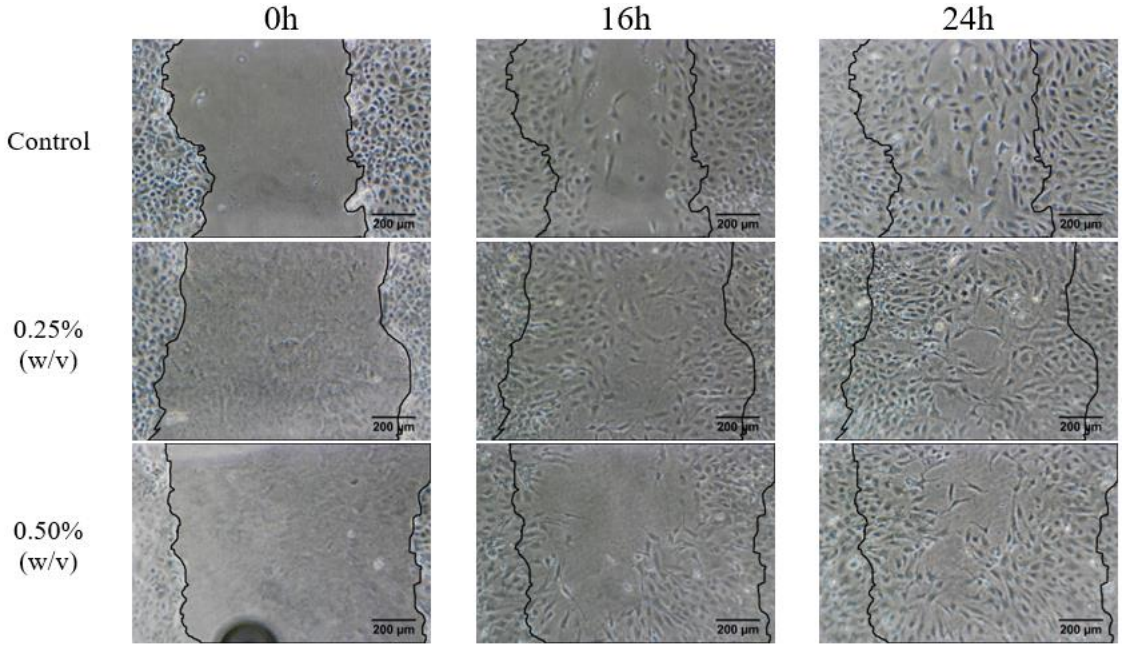

Figure 12 - Phase-contrast inverted microscope micrographs (50x magnification) of human microvascular endothelial cells (HMEC-1) migration in contact with *Cyanothece* sp. CCY 0110 (CCY 0110) RPS solutions at 0.25% and 0.5% (w/v) concentrations, and without CCY 0110 RPS solutions (control), towards an injured site.

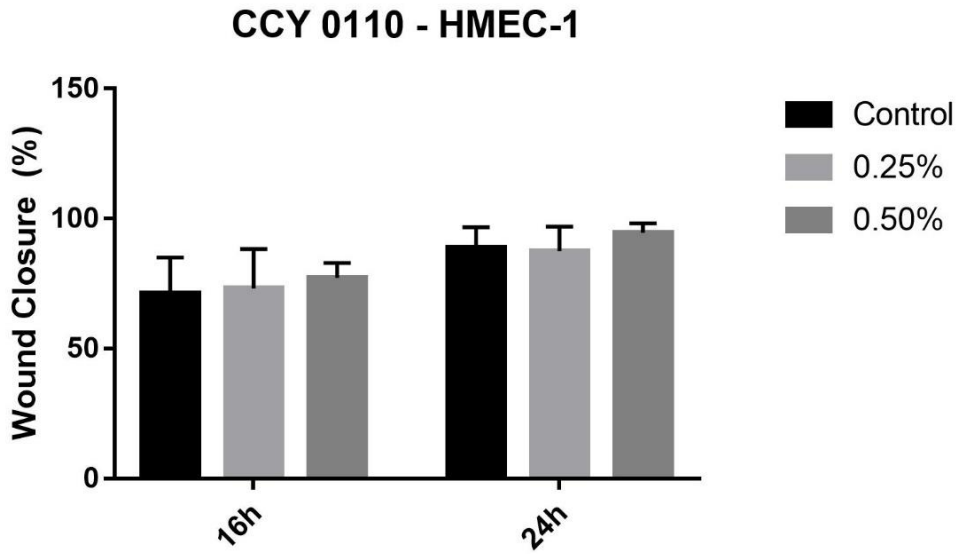

Figure 13 - Wound closure of human microvascular endothelial cells (HMEC-1) in contact with *Cyanothece* sp. CCY 0110 (CCY 0110) RPS solutions at 0.25% and 0.50% (w/v) concentrations, and without CCY 0110 RPS solutions (control), after 16 h and 24 h (n=3). The values are means ± standard deviations.

4.5 *Cyanothece* spp. RPS - *in vivo* Experiments

Cell cytotoxicity and proliferative results from fibroblasts and endothelial cells in contact with CCY 0110 and VI 22 RPS solutions indicated two optimal concentrations for both RPS, 0.25% (w/v) and 0.50% (w/v) for CCY 0110 and 0.10% (w/v) and 0.25% (w/v) for VI 22, where their cytotoxic and proliferative effects were least impactful. These RPS were used for *in vivo* viability tests in healthy Wistar rats.

Wounds with 6 mm size in the dorsal region of the rat were created to mimic an injury (data not shown). While RPS solutions were applied to the wound site, rats did not demonstrate distress reactions such as scratching and licking of the wound site or surrounding tissues. The reticulation with $CaCl_2$ allowed the RPS solutions to be applied without overflowing to surround tissues or dislodged due to rat natural movement, in fact, when rats made movements that hinged the skin, the RPS solutions remained within wound limits. Approximately after 3 minutes from treatment application, the RPS solution was absorbed by the wound bed tissue and a semi-translucid film was observable at the wound bed (data not shown). On the second day of treatment applications, wound sizes were reduced by 1 mm in all experimental groups (fig. 14). No differences were observable on the wound size, edges or color except scab formation, which was more preeminent on both RPS solutions comparatively to the control group. After four days of the wounds being made, wound size reduced to 4 mm in all tested groups and no further differences were observed. On the last day before being euthanized, the wound size decreased to a diameter of 3 mm in all experimental

groups, all wounds showed granulocyte tissue in the wound edges and the scab size was reduced.

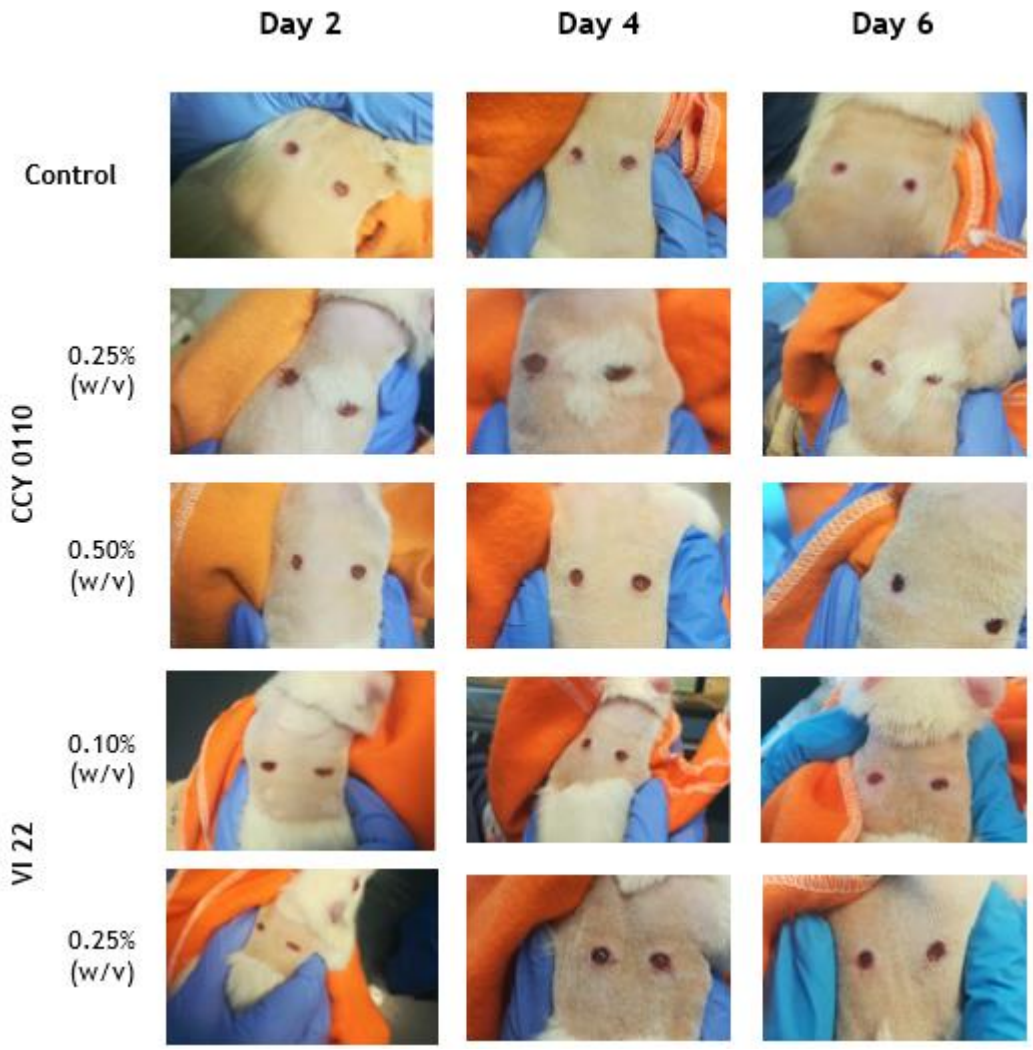

Figure 14 - Wound healing progression on healthy Wistar rats treated with serum (control) and with *Cyanothece* sp. CCY 0110 (CCY 0110) RPS solutions at 0.25% and 0.50% (w/v), and *Cyanothece* sp. VI 22 (VI 22) RPS solutions at 0.10% and 0.25% (w/v) prepared in deionized water and reticulated with 1 M $CaCl_2$ solution. Representative images (not to scale).

Chapter 5

Discussion

5.1 *Cyanothece* sp. VI 22 Cultivation and RPS Production

In this work, 1 L cultures of *Cyanothece* sp. VI 22 (VI 22) were grown in bioreactors following previously optimized conditions. To evaluate culture growth, optical density (OD) and dry weight (DW) measurements were performed as they are expressed to be good indicators of growth performance (Mota et al., 2013). $OD_{730\ nm}$ is a parameter routinely used to monitoring growth of unicellular cyanobacterial cultures (Anderson & Mcintosh, 1991), while DW is important to correlate the growth of different cyanobacterial strains and to allow data normalization.

VI 22 culture showed a slow linear growth with no exponential phase, which is a common occurrence in cyanobacterial strains that produce large amounts of extracellular polymeric substances (EPS) (Pereira et al., 2011). Compared to the *Cyanothece* sp. CCY 0110 (CCY 0110) grown in bioreactors, the VI 22 culture growth was approximately half as fast (Mota et al., 2013). Another culture medium is described to be ideal to grow VI 22 (Colica, 2009), however our preliminary results showed an even slower growth as compared to the ASN III medium used in this work. Therefore, other culture conditions, such as light intensity and temperature, need to be tested to determine the optimal growth conditions for VI 22 growth.

Carbohydrate content was assessed by the phenol-sulphuric acid method commonly used (Dubois et al., 1956). The CHT and RPS content had an approximate 2-fold increase after the VI 22 culture was grown for more than 2 months, consequence of the growth increase and the RPS accumulation in the culture medium over time. Moreover, the majority of carbohydrates are being produced as RPS, which positively impacts the polymer extraction as RPS are the easiest polysaccharidic fraction to recover from the culture medium.

During the polymer extraction protocol, the culture medium is dialysed to remove the salts and the RPS are precipitated with ethanol, as its polarity and temperature helps the recovery of the polymer (Patel et al., 2013). The obtained quantity of VI 22 polymer was 0.6-fold lower than the amount that is usually obtained of RPS produced by CCY 0110 using the same culture conditions and polymer extraction methods, so the strategy for its recovering from the culture medium should be optimized (e.g. test other alcohols). Therefore, with the current results, CCY 0110 is a more suitable candidate to be used as a material for wound dressings since its growth and polymer production is already optimized.

5.2 *Cyanothece* spp. *in vitro* Results

When wound dressings enter in contact with cells in a chronic wound injury, interactions between them are established and will dictate the fate of the cell by possible modifications of the cell metabolism, proliferation capacity or motility function. To avoid complications of the applied treatment to chronic wound patients, a thorough analysis of cytotoxic and proliferative effects of the wound dressing material needs to be conducted with *in vitro* tests, since they are highly specific, efficient, relatively fast and affordable. One of the most commonly employed *in vitro* test in research for analysis of cytotoxic effects is the tetrazolium reduction assay (MTS). CCY 0110 and VI 22 RPS solutions were studied by n MTS assay using human dermal fibroblasts (HDF) and human microvascular endothelial cells (HMEC-1). Results here obtained suggest that CCY 0110 RPS can promote cell viability without functionalization, while VI 22 RPS presents a tenue balance between cytotoxic and non-cytotoxic effects at different concentrations. Regarding the results using endothelial cells, the CCY 0110 polymer lead to a significant decrease on cell viability at the concentrations that showed better results using fibroblasts (0.5%). Despite this, the decrease is still within the 30% margin that is stipulated by the ISO 10993-5 (Biological evaluation of medical devices — Part 5: Tests for in vitro cytotoxicity), and as such, the CCY 0110 RPS solutions can still be used at concentrations up to 0.5% (w/v), although an impact on the endothelial cell's metabolism might occur, which can have implications on the angiogenesis during the proliferative phase in wound healing. Contrary to the results obtained using fibroblasts, VI 22 RPS has significant cytotoxic effects in concentrations superior to 0.5% (w/v) suggesting that VI 22 RPS are not an ideal choice to be used as a possible wound dressing since angiogenesis is destabilized. However, strategies to improve endothelial cell survival when in contact with cyanobacterial RPS can be pursued to ensure a healthy wound healing. Moreover, endothelial cells are reported to be very susceptive to changes in their environment (Costa et al., 2017), therefore a viability decrease regarding these cells in comparison with fibroblasts was expected.

The chronic effects on cell viability by CCY 0110 RPS were also studied. In this test, fibroblasts and endothelial cells were left in contact with CCY 0110 RPS solutions for 72 h, simulating the application of a wound dressing based on this material in a real treatment context. In agreement with the acute results, RPS solutions enhance cell viability, however, unexpectedly endothelial cells did not react properly with CCY 0110 RPS in chronic conditions, showing up to 50% decrease in all the tested RPS concentrations. Proliferative phase only starts after three days from the onset of the chronic wound and although angiogenesis is present throughout the wound healing process, it has more relevance in maintaining the perfusion of granulation tissue to deliver nutrients and oxygen that are essential for the highly metabolic tissue since new extracellular matrix (ECM) is being produced. Consequently, if new blood vessels

are not formed in the granulation tissue it will become devitalized, developing into necrotic tissue in extreme cases and bringing the wound healing process to a halt. These results further support the functionalization of cyanobacterial RPS to become less hostile to endothelial cells.

One of the most commonly employed *in vitro* tests in research for cell proliferation is the bromodeoxyuridine assay (BrdU). An enhanced fibroblast proliferation was observed in all CCY 0110 RPS solutions, except using 0.75% (w/v) that had a slight decrease. Regarding VI 22 RPS solutions, an inversely relation between RPS concentration and cell proliferation was observed, with an increase in RPS concentration leading to a decrease in fibroblast viability. Low VI 22 RPS concentrations are the only solutions that have a decrease in cell proliferation lower than 30%.

In agreement with the available bibliography, the results here obtained corroborate that CCY 0110 RPS can stimulate fibroblast proliferation without the need of growth factors or other biomolecules, however, functionalization strategies for VI 22 RPS need to be pursued for being capable of sustaining cell proliferation. The significant decrease on fibroblast chronic proliferation using VI 22 RPS can be a problem that cannot be overlooked as fibroblasts are one of the two major cell types responsible for the wound closure. Fibroblasts produce new ECM and have a major role on the development of granulation tissue. Therefore, if fibroblasts are not able to replicate, they cannot produce ECM on sufficient quantities to follow the progression of newly formed tissue, making it impossible for new cells to progress towards the injured site to completely cover the wound as there is no mechanical support. Regarding endothelial cells, the CCY 0110 RPS shows anti-proliferative properties in almost all tested concentrations with the 0.5% (w/v) RPS solution having the lowest impact on endothelial cell replication. All the VI 22 RPS concentrations except the 0.1% (w/v) showed anti-proliferative properties when in contact with endothelial cells. The viability results of endothelial cells treated with 0.1% (w/v) VI 22 RPS showed a decrease of nearly 30%, however, the same cells are stimulated to replicate. Although this result seems contradictory, other work focusing on angiogenesis modulation with natural molecules (xanthohumol and 8-prenylnaringenin) denoted an inverse correlation between cell viability and cell proliferation (Costa et al., 2017). These results suggest that at lower concentrations, VI 22 RPS has a low impact on endothelial cell replication and therefore can sustain angiogenesis during wound healing without requiring some sort of functionalization as a wound dressing material. Several authors already described some natural polymers, such as chitosan (Dash et al., 2011), alginate (D'Ayala et al., 2008), hyaluronic acid (Vazquez et al., 2003) and collagen (Gioffrè et al., 2012), as neither cytotoxic or anti-proliferative towards fibroblasts or endothelial cells. Taken together, these works suggest that naturally occurring polymers are good candidates for wound dressing materials due to their intrinsic biocompatibility, biodegradability and environmentally friendly extraction methods. However, the concentrations that can be used in contact with each cell have always to be studied since can vary with the characteristics of the polymer.

Taken together, cell viability and proliferation results from CCY 0110 and VI 22 RPS indicate the two optimal concentrations for the utilization of cyanobacterial RPS in human cells: 0.25% and 0.5% (w/v) for CCY 0110; 0.1% and 0.25% (w/v) for VI 22. One of the most commonly employed *in vitro* tests in research to evaluate cell migration is the cell culture wound closure assay. By applying the CCY 0110 RPS 0.25% and 0.5% (w/v) solutions, the fibroblasts showed a slight increase in the total area covered of the injury compared to the control group, where no treatment was performed. Therefore, what can be clearly stated is that the CCY 0110 RPS solutions do not block the motility of fibroblasts in all directions, since fibroblasts have their lamellipodia subducted and expanded within the RPS solutions. Moreover, fibroblast typical stretched phenotype is maintained throughout the 24 h period, suggesting that cell viability is not affected and that cells are able to interact with their environment to move and proliferate without interferences from the CCY 0110 RPS solutions. Similar results were obtained with endothelial cells, since they showed a typical rounded shape in a stationary state and a stretched shape while in motility across the injured site. These results suggest that in accordance to cytotoxic and proliferative results obtained through tests using fibroblasts, 0.25% and 0.5% (w/v) CCY 0110 RPS solutions do not alter cell morphology of cell basic function, as expected based on results published using other natural polymers (Mayol et al., 2014). Interestingly, no increased closure of the injured site was observable, therefore the enhanced viability and proliferation of these cells in contact with 0.5% (w/v) RPS solution was not translated to the cell culture wound closure assay. Regarding endothelial cells, the slight decrease on cell viability and proliferation that was observed in CCY 0110 RPS solutions appears to not be reproduced in this assay.

5.3 *Cyanothece* spp. *in vivo* Results

Considering the *in vitro* results, 0.25% and 0.5% (w/v) CCY 0110 RPS solutions and 0.1% and 0.25% (w/v) VI 22 RPS solutions were selected for the *in vivo* tests. For this, healthy Wistar rats were used as a wound model where injuries were performed on dorsal back and RPS solutions were applied ever other day during a full week. During the treatment application, rats did not show distress signals or any attempt to lick or scratch the wound site, which is an expected reaction as the RPS solution is delivered in a liquid state and adapts to the topology of the wound site, without overflowing to the surrounding tissue, and CCY 0110 or VI 22 RPS are not expected to have irritating properties. Interestingly, after 3 min of administrating the RPS solutions they seemed to be absorbed by the wound bed tissue, however a histologic analysis should be performed to confirm. Other authors performed experiments using animals that were treated with wound dressings based on natural polymers, wounds needed to be covered with an additional gauze to prevent the wound dressing from being removed from the wound site (Mayol et al., 2014; Park et al., 2009). However, in this work, the utilization of other gauzes or wound dressings was not necessary as an adequate consistency was obtained through the reticulation of the RPS solution with calcium chloride, which was previously described as a reticulation mechanism to tune the CCY 0110 RPS mesh size and to control the delivery of proteins (Leite et al., 2017). Wound scabs of rats that were treated with both RPS solutions formed more rapidly and had an increased size

as compared to rats without RPS solution treatments, indicating that the solvent of the RPS solution is absorbed by the wound tissue and the RPS possibly stays in the wound bed, covering the wound site and helping the formation of the scab.

Wounds were performed with a surgical punch and their initial diameter was 6 mm. During the 7 days of treatment, wounds had a diameter decrease of 1 mm every two days in all wounds treated with RPS solutions or saline solution (control) reaching 3 mm. Consequently, the wound healing process in rats treated or not with RPS has no differences regarding the time to heal. This observation was unexpected for the CCY 0110 RPS, since *in vitro* tests indicated this polymer was able to promote the fibroblast activity and proliferation, and therefore an increased ECM deposition and fibroblast migration towards the wound site, accelerating the wound healing process and closing the wounds. Unexpectedly, wounds treated with VI 22 RPS had similar results to wounds treated with CCY 0110 RPS despite the *in vitro* cytotoxicity and proliferation tests. The natural polymers chitosan and alginate have been already used to develop wound dressings (Lee et al., 2012). Chitosan hydrogels prepared by a heat treatment were applied in mice during ten days and were able to accelerate the wound healing process in about 40% in comparison to a commercial dressing (Mayol et al., 2014). Interestingly, Park et al. also showed that chitosan scaffolds loaded or not with basic fibroblast growth factor (bFGF) could accelerate wound healing in the first seven days after injury as compared to gauze control (Park et al., 2009). Lee et al. showed that full excision wounds in diabetic mice treated with a hydrogel film of alginate, chitosan and poly-γ-glutamic acid had a higher wound closure rate in the first two weeks as compared to wounds treated with a commercially available product or alginate hydrogel films only. This result is thought to be attributed to the chitosan capability of attracting neutrophils to the wound site without inducing excessive inflammation (Lee et al., 2012). Therefore, considering the bibliography, it is possible to conclude that natural polymers modified by simple treatments/mixtures with other polymers are able to enhance the wound healing process. Therefore, as in this work no modifications were employed in the RPS solutions, it is expected that the results obtained can be significantly and easily improved, in order to successfully enhance the wound healing rate while maintaining the attractive natural characteristics of the RPS.

Chapter 6

Conclusion and Future Perspectives

This work aimed to developed wound dressings based on released polysaccharides (RPS) from cyanobacteria, *Cyanothece* sp. CCY 0110 (CCY 0110) and *Cyanothece* sp. VI 22 (VI 22), in order to promote a more rapidly wound healing process in chronic wounds of patients suffering from diabetic foot ulcers.

The first part of this work was to establish a culture of VI 22 in 1 L bioreactors and assess its growth and carbohydrate production. In general, VI 22 culture growth was slow and, consequently the contents of capsular polysaccharides (CPS), released polysaccharides (RPS) and total carbohydrates (CHT) were also low. Only over 2 months, it was possible to observe an increase in growth and carbohydrate production. These results suggest that the growth conditions need to be optimized to enhance VI 22 growth and RPS production, in particular, to maximize the amount of polymer obtained and available for study its applications.

In the second part of this work, different type of cells cytotoxic, proliferative and cell migration effects were studied in the presence of CCY 0110 and VI 22 RPS solutions. The CCY 0110 RPS solution is not cytotoxic for fibroblasts and promotes cell viability and proliferation at a concentration of 0.50% (w/v), however endothelial cells seem to have a poorly reaction towards the RPS. Accordingly, applying CCY 0110 RPS for chronic periods of time has a significant cytotoxic effect for endothelial cells, suggesting that longer periods of exposition may impair angiogenesis (more replicates should be performed to confirm these results).

On the other hand, 0.50% (w/v) VI 22 RPS solution has the least acute cytotoxic effect towards fibroblasts, but regarding endothelial cells this property is observed at a concentration of 0.25% (w/v). In general, the VI 22 RPS hampered the proliferation of fibroblast cells and endothelial cells, being this last at concentrations higher than 0.25% (w/v). Moreover, fibroblasts and endothelial cells are able to move freely within 0.25% and 0.50% (w/v) CCY 0110 RPS solutions without their phenotype being modified. Taken together these results suggest that 0.25% and 0.50% (w/v) CCY 0110 RPS solutions, and 0.10% and 0.25% (w/v) VI 22 RPS solutions are the optimal concentrations since are the least cytotoxic and anti-proliferative when in contact with fibroblasts or endothelial cells.

These properties were assessed in an *in vivo* biocompatibility assay, which indicate that CCY 0110 and VI 22 RPS are not able to enhance wound healing and promote a faster wound closure in comparison to wounds treated with saline solution. These polymers were only able to promote a faster and accentuated scab formation during

the first 4 days of treatment, which was possibly due to the deposition of RPS on the wound bed. Despite this observation, CCY 0110 RPS can be further enhanced by functionalization strategies to promote a heightened fibroblast and endothelial cell metabolism and growth, in order to improve the wound healing process and provide a good option for patients with diabetic foot ulcers: a treatment that is painless, affordable, easy to apply and capable of preventing wound infection.

Taken all results in consideration, complementary tests should be performed, such as the immunohistochemistry of rat tissues (e.g. skin and kidney) to evaluate the immunity systemic response and the wound site histopathology, in order to further understand the interactions of the RPS with the organism. In addition, VI 22 RPS cytotoxic, proliferative and migration results need to be confirmed with more replicates. Importantly, optimal VI 22 culture conditions should be studied in order to maximize its growth and consequently the carbohydrate production, as well as the polymer extraction method should be optimized, in order to increase the amount of RPS extracted. Functionalization strategies, such as growth factor incorporation or physical/chemical reticulation, of the cyanobacterial RPS should be pursued to enhance the cytotoxic and proliferation properties of these polymers when in contact with fibroblasts and endothelial cells, in order to promote a heightened response from the wound site and a faster wound closure.

Bibliography

Acosta, J. B., Garcia Del Barco, D., Cibrian Vera, D., Savigne, W., Lopez-Saura, P., Guillen Nieto, G., & Schultz, G. S. (2008). The pro-inflammatory environment in recalcitrant diabetic foot wounds. *International Wound Journal*, *5*(4), 530–539.

Anderson, S. L., & Mcintosh, L. E. E. (1991). Light-Activated Heterotrophic Growth of the Cyanobacterium Synechocystis sp . Strain PCC 6803 : a Blue-Light-Requiring Process. *Journal of Bacteriology*, *173*(9), 2761–2767.

Arul, V., Kartha, R., & Jayakumar, R. (2007). A therapeutic approach for diabetic wound healing using biotinylated GHK incorporated collagen matrices. *Life Sciences*, *80*(4), 275–284.

Bertoni, A. G., Burke, G. L., Owusu, J. a, Carnethon, M. R., Vaidya, D., Barr, R. G., Jenny, N. S., Ouyang, P., Rotter, J. I. (2010). Inflammation and the Incidence of Type 2 Diabetes. *Diabetes Care*, *33*(4), 804–810.

Boateng, J. S., Matthews, K. H., Stevens, H. N. E., & Eccleston, G. M. (2008). Wound Healing Dressings and Drug Delivery Systems: A Review. *Journal of Pharmaceutical Sciences*, *97*(8), 2892–2923.

Brandon V. Slaughter, Shahana S. Khurshid, Omar Z. Fisher, Ali Khademhosseini, N. A. P. (2009). Hydrogels in Regenerative Medicine. *Advanced Materials*, *21(1)*, 3307–3329.

Brouillard, C., Bursztejn, A.-C., Latarche, C., Cuny, J.-F., Truchetet, F., Goulle, J.-P., & Schmutz, J.-L. (2018). Silver absorption and toxicity evaluation of silver wound dressings in 40 patients with chronic wounds. *Journal of the European Academy of Dermatology and Venereology : JEADV*, *32*(12), 2295–2299.

Casellini, C. M., & Vinik, A. I. (2007). Clinical manifestations and current treatment options for diabetic neuropathies. *Endocrine Practice*, *13*(5), 550–566.

Colica, G. (2009). *Use of Microorganisms in the Removal of Pollutants from the Wastewater* (10th ed.). Firenze: Firenze University Press.

Costa, B., Mota, R., Parreira, P., Tamagnini, P., L. Martins, M. C., & Costa, F. (2019). Broad-Spectrum Anti-Adhesive Coating Based on an Extracellular Polymer from a Marine Cyanobacterium. *Marine Drugs*, *17*(4), 243.

Costa, R., Rodrigues, I., Guardão, L., Lima, J. Q., Sousa, E., Soares, R., & Negrão, R. (2017). Modulation of VEGF signaling in a mouse model of diabetes by xanthohumol and 8-prenylnaringenin: Unveiling the angiogenic paradox and metabolism interplay. *Molecular Nutrition and Food Research*, *61*(4), 1–12.

Cranney, M., & Barton, S. (1999). Performance indicators for primary care groups. Performance of these indicators is critical. *BMJ (Clinical Research Ed.)*, *318*(7186), 804–805.

D'Ayala, G. G., Malinconico, M., & Laurienzo, P. (2008). Marine derived polysaccharides for biomedical applications: Chemical modification approaches. *Molecules*, *13*(9), 2069–2106.

Da Silva, L., Carvalho, E., & Cruz, M. T. (2010). Role of neuropeptides in skin inflammation and its involvement in diabetic wound healing. *Expert Opinion on Biological Therapy*, *10*(10), 1427–1439.

Dai, T., Tanaka, M., & Huang, Y. (2011). Chitosan preparations for wounds and burns: antimicrobial and wound-healing effects. *Expert Reviews*, *9*(7), 857–880.

Dash, M., Chiellini, F., Ottenbrite, R. M., & Chiellini, E. (2011). Chitosan - A versatile semi-synthetic polymer in biomedical applications. *Progress in Polymer Science (Oxford)*, *36*(8), 981–1014.

De Philippis, R., Margheri, M. C., Materassi, R., & Vincenzini, M. (1998). Potential of unicellular cyanobacteria from saline environments as exopolysaccharide producers. *Applied and Environmental Microbiology*, *64*(3), 1130–1132.

De Philippis, R., & Vincenzini, M. (1998). Exocellular polysaccharides from cyanobacteria and their possible applications. *FEMS Microbiology Reviews*, *22*(3), 151–175.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric Method for Determination of Sugars and Related Substances. *Analytical Chemistry*, *228*(3), 350–356.

Dyck, P. J., Kratz, K. M., Karnes, J. L., Litchy, W. J., Klein, R., Pach, J. M., Wilson, D. M., O'Brien, P. C., Melton, L. J. (1993). The prevalence by staged severity of various types of diabetic neuropathy, retinopathy, and nephropathy in a population-based cohort: The rochester diabetic neuropathy study. *Neurology*, *43*(4), 817–824.

Edwards, J., & Stapley, S. (2010). Debridement of diabetic foot ulcers. *Cochrane Database of Systematic Reviews*, (1).

Eming, S. A., Krieg, T., & Davidson, J. M. (2007). Inflammation in wound repair: Molecular and cellular mechanisms. *Journal of Investigative Dermatology*, *127*(3), 514–525.

Estevinho, B. N., Mota, R., Leite, J. P., Tamagnini, P., Gales, L., & Rocha, F. (2019). Application of a cyanobacterial extracellular polymeric substance in the microencapsulation of vitamin B12. *Powder Technology*, *343(1)*, 644–651.

Fonder, M. A., Lazarus, G. S., Cowan, D. A., Aronson-Cook, B., Kohli, A. R., & Mamelak, A. J. (2008). Treating the chronic wound: A practical approach to the care of nonhealing wounds and wound care dressings. *Journal of the American Academy of Dermatology*, *58*(2), 185–206.

Fontbonne, A., Eschwège, E., Cambien, F., Richard, J.-L., Ducimetière, P., Thibult, N., Warnet, J. -M., Claude, J. -R., Rosselin, G.-E. (1989). Hypertriglyceridaemia as a risk factor of coronary heart disease mortality in subjects with impaired glucose tolerance or diabetes. *Diabetologia*, *32*(5), 300–304.

Frykberg, R. G., & Banks, J. (2015). Challenges in the Treatment of Chronic Wounds. *Advances in Wound Care*, *4*(9), 560–582.

Gioffrè, M., Torricelli, P., Panzavolta, S., Rubini, K., & Bigi, A. (2012). Role of pH on stability and mechanical properties of gelatin films. *Journal of Bioactive and Compatible Polymers*, *27*(1), 67–77.

Grey, J. E., & Enoch, S. (2006). ABC of wound healing: Pressure ulcers. *The BMJ*, *332*(6), 472–475.

Grey, J., Harding, K., & Enoch, S. (2006). ABC of healing: Venous and arterial leg ulcers. *BMJ (Clinical Research Ed.)*, *332*(7537), 347–350.

Guerreiro, S., Monteiro, R., Martins, M. J., Calhau, C., Azevedo, I., & Soares, R. (2007). Distinct modulation of alkaline phosphatase isoenzymes by 17 ß -estradiol and xanthohumol in breast cancer MCF-7 cells. *Clinical Biochemistry*, *40*, 268–273.

Guo, S., & DiPietro, L. A. (2010). Critical review in oral biology & medicine: Factors affecting wound healing. *Journal of Dental Research*, *89*(3), 219–229.

Halim, A. S., Khoo, T. L., & Saad, A. Z. M. (2012). Wound bed preparation from a clinical perspective. *Indian Journal of Plastic Surgery : Official Publication of the Association of Plastic Surgeons of India*, *45*(2), 193–202.

Han, G., & Ceilley, R. (2017). Chronic Wound Healing: A Review of Current Management and Treatments. *Advances in Therapy*, *34*(3), 599–610.

Han, J., Zhou, Z., Yin, R., Yang, D., & Nie, J. (2010). Alginate-chitosan/hydroxyapatite polyelectrolyte complex porous scaffolds: Preparation and characterization. *International Journal of Biological Macromolecules*, *46*(2), 199–205.

Harding, K. G., Jones, V., & Price, P. (2000). Topical treatment: which dressing to choose. *Diabetes/Metabolism Research and Reviews*, *16*(1), 47–50.

Hatzinger, P., & Kelsey, J. (2005). Biodegradation. In *Encyclopedia of Soils in the Environment, 1(3), 250-271.*

Haukipuro, K., Melkko, J., Med, C., Risteli, L., & Ph, D. (1990). Synthesis of Type I Collagen in Healing Wounds in Humans. *Annals of Surgery*, *213*(1), 75–80.

Hong, H. J., Jin, S. E., Park, J. S., Ahn, W. S., & Kim, C. K. (2008). Accelerated wound healing by smad3 antisense oligonucleotides-impregnated chitosan/alginate polyelectrolyte complex. *Biomaterials*, *29*(36), 4831–4837.

Hunt, T. K., & Hopf, H. W. (1997). Wound healing and wound infection. What surgeons and anesthesiologists can do. *The Surgical Clinics of North America*, *77*(3), 587–606.

Intekhab Ahmed, B. G. (2006). Diabetes mellitus. *Clinics in Dermatology*, *24(1)*, 237–246.

Järbrink, K., Ni, G., Sönnergren, H., Schmidtchen, A., Pang, C., Bajpai, R., & Car, J. (2017). The humanistic and economic burden of chronic wounds: A protocol for a systematic review. *Systematic Reviews*, *6*(1), 1–7.

Jeffcoate, W. J., & Harding, K. G. (2003). Diabetic foot ulcers. *The Lancet*, *361(1)*, 1545–1551.

Jeffcoate, W. J., Price, P., & Harding, K. G. (2004). Wound healing and treatments for people with diabetic foot ulcer. *Diabetes/Metabolism Research and Reviews*, *20*(1), 78–89.

Kim, I. Y., Seo, S. J., Moon, H. S., Yoo, M. K., Park, I. Y., Kim, B. C., & Cho, C. S. (2008). Chitosan and its derivatives for tissue engineering applications. *Biotechnology Advances*, *26*(1), 1–21.

Knowler, W. C., Barrett-Connor, E., Fowler, S. E., Hamman, R. F., Lachin, J. M., Walker, E. A., & Nathan, D. M. (2002). Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. *New England Journal of Medicine*, *346*(6), 393–403.

Konishi, T., Takeyasu, A., Natsume, T., Furusawa, Y., Hieda, K., & Tunel, D. (2011). Visualization of Heavy Ion Tracks by Labeling 3 ' -OH Termini of Induced DNA Strand Breaks. *Journal of Radiation Research*, *440*, 433–440.

Koria, P., Yagi, H., Kitagawa, Y., Megeed, Z., Nahmias, Y., Sheridan, R., & Yarmush, M. L. (2011). Self-assembling elastin-like peptides growth factor chimeric nanoparticles for the treatment of chronic wounds. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(3), 1034–1039.

Lammers, G., Tjabringa, G. S., Schalkwijk, J., Daamen, W. F., & van Kuppevelt, T. H. (2009). A molecularly defined array based on native fibrillar collagen for the assessment of skin tissue engineering biomaterials. *Biomaterials*, *30*(31), 6213–6220.

Lazarus, G. S., Cooper, D. M., Knighton, D. R., Margolis, D. J., Percoraro, R. E., Rodeheaver, G., & Robson, M. C. (1994). Definitions and guidelines for assessment of wounds and evaluation of healing. *Wound Repair and Regeneration*, *2*(3), 165–170.

Lee, Y. H., Chang, J. J., Yang, M. C., Chien, C. T., & Lai, W. F. (2012). Acceleration of wound healing in diabetic rats by layered hydrogel dressing. *Carbohydrate Polymers*, *88*(3), 809–819.

Leite, J. P., Mota, R., Durão, J., Neves, S. C., Barrias, C. C., Tamagnini, P., & Gales, L. (2017). Cyanobacterium-Derived Extracellular Carbohydrate Polymer for the Controlled Delivery of Functional Proteins. *Macromolecular Bioscience*, *17*(2), 1–8.

Lipsky, B. A., Berendt, A. R., Deery, H. G., Embil, J. M., Joseph, W. S., Karchmer, A. W., LeFrock, J. L., Lew, D. P., Mader, J. T., Norden, C., Tan, J. S. (2005). Diagnosis and Treatment of Diabetic Foot Infections. *Journal of the American Podiatric Medical Association*, *95*(2), 183–210.

MacKay, D., & Miller, A. L. (2003). Nutritional Support for Wound Healing. *Alternative Medicine Review*, *8*(4), 359–377.

Madihally, S. (2011). Processing chitosan for tissue regeneration. *Current Trends Polymer Science*, *15(1)*, 83–88.

Malafaya, P. B., Silva, G. A., & Reis, R. L. (2007). Natural-origin polymers as carriers and scaffolds for biomolecules and cell delivery in tissue engineering applications. *Advanced Drug Delivery Reviews*, *59*(4–5), 207–233.

Mandal, B. B., & Kundu, S. C. (2009). Cell proliferation and migration in silk fibroin 3D scaffolds. *Biomaterials*, *30*(15), 2956–2965.

Matsumoto, Y., & Kuroyanagi, Y. (2010). Development of a wound dressing composed of hyaluronic acid sponge containing arginine and epidermal growth factor. *Journal of Biomaterials Science, Polymer Edition*, *21*(6), 715–726.

Mayol, L., De Stefano, D., Campani, V., De Falco, F., Ferrari, E., Cencetti, C., Matricardi, P., Maiuri, L., Carnuccio, R., Gallo, A., Maiuri, M. C., De Rosa, G. (2014). Design and characterization of a chitosan physical gel promoting wound healing in mice. *Journal of Materials Science: Materials in Medicine*, *25*(6), 1483–1493.

McCance, D. R. (2011). Pregnancy and diabetes. *Best Practice and Research: Clinical Endocrinology and Metabolism*, *25*(6), 945–958.

McIntosh, C. (2007). Are hydrocolloid dressings suitable for diabetic foot ulcers? *Wound Essentials*, 2(1), 170–172.

Monaco, J. A. L., & Lawrence, W. T. (2003). Acute wound healing: An overview. *Clinics in Plastic Surgery*, *30*(1), 1–12.

Morton, L. M., & Phillips, T. J. (2012). Wound healing update. *Seminars in Cutaneous Medicine and Surgery*, *31*(1), 33–37.

Mota, R., Guimarães, R., Büttel, Z., Rossi, F., Colica, G., Silva, C. J., Santos, C., Gales, L., Zille, A., De Philippis, R., Pereira, S. B., Tamagnini, P. (2013). Production and characterization of extracellular carbohydrate polymer from *Cyanothece* sp. CCY 0110. *Carbohydrate Polymers*, *92*(2), 1408–1415.

Mota, R., Rossi, F., Andrenelli, L., Pereira, S. B., De Philippis, R., & Tamagnini, P. (2016). Released polysaccharides (RPS) from *Cyanothece* sp. CCY 0110 as biosorbent for heavy metals bioremediation: interactions between metals and RPS binding sites. *Applied Microbiology and Biotechnology*, *100*(17), 7765–7775.

Moura, L. I. F., Dias, A. M. A., Carvalho, E., & de Sousa, H. C. (2013). Recent advances on the development of wound dressings for diabetic foot ulcer treatment—A review. *Acta Biomaterialia*, *9*(7), 7093–7114.

Mulder, G., Jones, R., Cederholm-Williams, S., Cherry, G., & Ryan, T. (1993). Fibrin Cuff Lysis in Chronic Venous Ulcers Treated With a Hydrocolloid Dressing. *International Journal of Dermatology*, *32*(4), 304–306.

Mustoe, T. A., O'Shaughnessy, K., & Kloeters, O. (2006). Chronic wound pathogenesis and current treatment strategies: a unifying hypothesis. *Plastic and Reconstructive Surgery*, *117*(7), 35-41.

Nunan, R., Harding, K. G., & Martin, P. (2014). Clinical challenges of chronic wounds: searching for an optimal animal model to recapitulate their complexity. *Disease Models & Mechanisms*, *7*(11), 1205–1213.

Omar, A., Wright, J., Schultz, G., Burrell, R., & Nadworny, P. (2017). Microbial Biofilms and Chronic Wounds. *Microorganisms*, *5*(1), 9.

Park, C. J., Clark, S. G., Lichtensteiger, C. A., Jamison, R. D., & Johnson, A. J. W. (2009). Accelerated wound closure of pressure ulcers in aged mice by chitosan scaffolds with and without bFGF. *Acta Biomaterialia*, *5*(6), 1926–1936.

Patel, A. K., Laroche, C., Marcati, A., Ursu, A. V., Jubeau, S., Marchal, L., Petit, E., Djelveh, G., Michaud, P. (2013). Separation and fractionation of exopolysaccharides from *Porphyridium cruentum*. *Bioresource Technology*, 145(1), 345–350.

Pereira, S., Micheletti, E., Zille, A., Santos, A., Moradas-Ferreira, P., Tamagnini, P., & De Philippis, R. (2011). Using extracellular polymeric substances (EPS)-producing cyanobacteria for the bioremediation of heavy metals: do cations compete for the EPS functional groups and also accumulate inside the cell? *Microbiology (Reading, England)*, *157*(2), 451–458.

Pereira, S., Zille, A., Micheletti, E., Moradas-Ferreira, P., De Philippis, R., & Tamagnini, P. (2009). Complexity of cyanobacterial exopolysaccharides: Composition, structures, inducing factors and putative genes involved in their biosynthesis and assembly. *FEMS Microbiology Reviews*, *33*(5), 917–941.

Peterson, M. R., Harrop, S. J., McSweeney, S. M., Leonard, G. A., Thompson, A. W., Hunter, W. N., & Helliwell, J. R. (1996). MAD Phasing Strategies Explored with a Brominated Oligonucleotide Crystal at 1.65A Resolution. *Journal of Synchrotron Radiation*, *3*(1), 24–34.

Posnett, J., & Franks, P. (2008). The burden of chronic wounds in the UK. *Nursing Times*, 104(1), 44–45.

Raghav, A., Khan, Z. A., Labala, R. K., Ahmad, J., Noor, S., & Mishra, B. K. (2018). Financial burden of diabetic foot ulcers to world: a progressive topic to discuss always. *Therapeutic Advances in Endocrinology and Metabolism*, *9*(1), 29–31.

Rathur, H. M., & Boulton, A. J. M. (2005). Recent advances in the diagnosis and management of diabetic neuropathy. *Journal of Bone and Joint Surgery - Series B*, *87*(12), 1605–1610.
Reiber, G. E., Lipsky, B. A., & Gibbons, G. W. (1998). The burden of diabetic foot ulcers. *American Journal of Surgery*, *176*(2), 5-10.
Rice, J. B., Desai, U., Cummings, A. K. G., Birnbaum, H. G., Skornicki, M., & Parsons, N. B. (2014). Burden of diabetic foot ulcers for medicare and private insurers. *Diabetes Care*, *37*(3), 651–658.
Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M., & Stanier, R. Y. (1979). Generic Assignments, Strain Histories and Properties of Pure Cultures of Cyanobacteria. *Microbiology*, *111*(1), 1–61.
Rodero, M. P., & Khosrotehrani, K. (2010). Skin wound healing modulation by macrophages. *International Journal of Clinical and Experimental Pathology*, *3*(7), 643–653.
Sell, S. A., Wolfe, P. S., Garg, K., McCool, J. M., Rodriguez, I. A., & Bowlin, G. L. (2010). The use of natural polymers in tissue engineering: A focus on electrospun extracellular matrix analogues. *Polymers*, *2*(4), 522–553.
Selvaraj Dhivya, V. V. P., & Santhini, E. (2015). Wound dressins - a review. *BioMedicine*, *5*(4), 24–28.
Silva, L., Cerqueira, M., Correlo, V., Reis, R. L., & Marques, A. (2016). Engineered hydrogel-based matrices for skin wound healing. In *Wound Healing Biomaterials*, 2(1), 227–250.
Srikanth, S., & Deedwania, P. (2011). Primary and Secondary Prevention Strategy for Cardiovascular Disease in Diabetes Mellitus. *Cardiology Clinics*, *29*(1), 47–70.
Stadelmann, W. K., Digenis, A. G., & Tobin, G. R. (1998). Physiology and healing dynamics of chronic cutaneous wounds. *American Journal of Surgery*, *176*(2), 26-38.
Summary, E. (2013). Chronic Venous Ulcers: A Comparative Effectiveness Review of Treatment Modalities. *Agency for Healthcare Research and Quality*, *127*(127), 1–23.
Sutherland, I. W. (1994). Structure-function relationships in microbial exopolysaccharides. *Biotechnology Advances*, *12*(2), 393–448.
Tabata, Y. (2009). Biomaterial technology for tissue engineering applications. *Journal of the Royal Society Interface*, *6*(3), 311–324.
Tessmar, J. K., & Göpferich, A. M. (2007). Matrices and scaffolds for protein delivery in tissue engineering. *Advanced Drug Delivery Reviews*, *59*(5), 274–291.
Thomas A. Wynn, L. B. (2010). Macrophages: Master Regulators of Inflammation and Fibrosis. *Bone*, *30*(3), 245–257.
Vazquez, J. R., Short, B., Findlow, A. H., Nixon, B. P., Boulton, A. J. M., & Armstrong, D. G. (2003). Outcomes of hyaluronan therapy in diabetic foot wounds. *Diabetes Research and Clinical Practice*, *59*(2), 123–127.
Walshe, C. (1995). Living with a venous leg ulcer: a descriptive study of patients'experiences. *Journal of Advanced Nursing*, 22(1), 1092–1100.
Wang, J., Luben, R., Khaw, K. T., Bingham, S., Wareham, N. J., & Forouhi, N. G. (2008). Dietary energy density predicts the risk of incident type 2 diabetes the european prospective investigation of cancer (EPIC)-norfolk study. *Diabetes Care*, *31*(11), 2120–2125.
Whiting, D. R., Guariguata, L., Weil, C., & Shaw, J. (2011). IDF Diabetes Atlas: Global estimates of the prevalence of diabetes for 2011 and 2030. *Diabetes Research and Clinical Practice*, *94*(3), 311–321.
Wilgus, T. A. (2008). Immune cells in the healing skin wound: Influential players at each stage of repair. *Pharmacological Research*, *58*(2), 112–116.
Xu, H., Ma, L., Shi, H., Gao, C., & Han, C. (2007). Chitosan–hyaluronic acid hybrid film as a novel wound dressing:in vitro andin vivo studies. *Polymers for Advanced Technologies*, *18*(11), 869–875.