UNIVERSIDADE DA BEIRA INTERIOR
Ciências

Development of a new bioactive membrane to be used for the treatment of skin injuries

Déborah Simões

Dissertação para obtenção do Grau de Mestre em
Química Medicinal
(2º ciclo de estudos)

Orientador: Prof. Doutor Ilídio Joaquim Sobreira Correia
Co-orientador: Mestre Sónia Alexandra Pereira Miguel

Covilhã, junho de 2018

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“Mes chers parents, je pars
Je vous aime mais je pars.
Vous n’aurez plus d’enfant.
Ce soir.

Je ne m’enfuis pas, je vole,
Comprenez bien, je vole,
Sans fumée, sans alcool
Je vole, je vole.”

Sardou, Je vole

À mes chers parents qui ont toujours cru en moi et à mon frère qui me fait toujours rire

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Resumo

As infeções da pele e dos tecidos moles (IPTMs) têm sido associadas a altas taxas de morbidade e mortalidade. Apesar do sucesso alcançado no tratamento de algumas IPTMs, aquelas que afetam o tecido subcutâneo, a fáscia ou o músculo retardam o processo de cicatrização e podem conduzir a situações que comprometem a vida do paciente. No sentido de contornar/evitar estas condições patológicas, torna-se fundamental desenvolver revestimentos com propriedades antimicrobianas. Recentemente, o desenvolvimento de pensos que incorporam agentes antimicrobianos surgiu como uma alternativa inovadora na redução da colonização bacteriana e infeção da ferida, com o intuito de melhorar o processo de cicatrização. Nesta tese, foi produzido e caracterizado um novo revestimento de pele com atividade bactericida. Neste contexto, foram produzidas membranas de policaprolactona (PCL) funcionalizadas com produtos resultantes da reação de maillard (PRM), recorrendo à técnica de eletrofiação. A funcionalização de nanofibras de PCL com PRM permitiu a produção de membranas com propriedades mecânicas, caráter hidrofílico e porosidade adequadas que permitem uma boa absorção do exsudado da ferida, assim como a troca de nutrientes e gases. Além disso, as membranas de PCL modificadas por PRM foram capazes de inibir o crescimento de *Staphylococcus aureus* e *Pseudomonas aeruginosa*, sem induzir qualquer efeito citotóxico em células de fibroblastos humanos. Estes resultados sugerem que as membranas em estudo têm um elevado potencial para serem aplicadas no processo de cicatrização.

Palavras-chave

Cicatrização de feridas; eletrofiação; infeções da pele e tecidos moles; penso antimicrobiano; produtos de reação de maillard.

Resumo alargado

A pele é o maior órgão do corpo humano e reveste toda a superfície do nosso organismo. A pele atua como uma barreira protetora contra agentes potencialmente agressivos provenientes do meio externo. Além disso, a pele participa ativamente no controlo da temperatura corporal, na homeostase de fluídos, na sensação e na vigilância imunológica. Contudo, lesões na pele podem ocorrer como consequência de cortes superficiais (induzidos por incisões e abrasões), podem ser causadas por queimaduras, perfurações e doenças (por exemplo, diabetes, úlceras venosas, etc), ou podem surgir de procedimentos cirúrgicos.

Atualmente, as lesões cutâneas representam uma das maiores preocupações de saúde a nível mundial. Quando ocorre uma lesão na pele, a sua função protetora fica comprometida, pelo que o processo de cicatrização é imediatamente ativado de modo a impedir a entrada de material estranho, nomeadamente bactérias, e restaurar a estrutura da pele. O processo de cicatrização requer a ação coordenada de várias células inflamatórias, quimiocinas, citocinas, moléculas da matriz extracelular (MEC) e o transporte de nutrientes para o local da ferida. Geralmente, lesões superficiais com pequena área de tecido afetado, requerem pouca ou nenhuma intervenção no seu tratamento. No entanto, em algumas circunstâncias, como, por exemplo, feridas crónicas, queimaduras e feridas diabéticas, os profissionais enfrentam muitos desafios, sendo o desenvolvimento de infeções o mais comum.

As infeções de pele e dos tecidos moles (IPTMs) estão entre as infeções bacterianas mais comuns, sendo responsáveis por 10% dos internamentos hospitalares por infeções nos Estados Unidos da América. Na primeira fase do processo infecioso, as bactérias gram-positivas, tais como a *Staphylococcus aureus*, são os principais organismos envolvidos. Quando o sistema imunitário é incapaz de remover estes organismos, a infeção procede para a fase final, onde é verificada a presença de bactérias gram-negativas como, por exemplo, *Pseudomonas aeruginosa*. Estas bactérias causam danos tecidulares, deterioração de fatores de crescimento e componentes da MEC, que são essenciais para o processo de cicatrização. Deste modo, têm vindo a ser desenvolvidos revestimentos com atividade antimicrobiana com o objetivo de prevenir a colonização microbiana das lesões, ao mesmo tempo que conferem suporte à migração e proliferação de fibroblastos.

Entre os diferentes tipos de revestimentos antimicrobianos que existem, as membranas produzidas pelo processo de eletrofiação revelaram-se uma abordagem terapêutica interessante, uma vez que são formadas por nanofibras capazes de imitar a estrutura da MEC da pele. Estas fibras exibem uma elevada relação superfície-volume e porosidade, características essenciais para a adesão e proliferação celular.

As propriedades exibidas pelas membranas são dependentes dos materiais usados na sua produção. A policaprolactona (PCL) tem sido amplamente utilizada, uma vez que apresenta excelentes propriedades mecânicas, estabilidade térmica e um perfil de degradação ajustável. Porém, este polímero apresenta algumas limitações (ex. caráter hidrofóbico) que prejudicam as interações células-biomaterial, e consequentemente o processo de cicatrização. De modo a melhorar as suas propriedades biológicas, a PCL tem sido modificada ou funcionalizada com agentes bioativos, nomeadamente fatores de crescimento, vitaminas e produtos naturais. Para além disso, a PCL tem sido conjugada com diversos agentes antimicrobianos, tais como antibióticos, prata e produtos naturais, com o intuito de produzir membranas com atividade bactericida.

Presentemente, estão a ser abordadas novas estratégias para desenvolver potenciais agentes antimicrobianos. Os produtos da reação de maillard (PRM) possuem atividade antioxidante, anti-inflamatória e antimicrobiana. Assim sendo, no presente trabalho as membranas de PCL foram funcionalizadas com PRM, glucose-arginina (GA) e frutose-arginina (FA), para melhorar as suas propriedades biológicas e dotar as membranas de PCL de atividade antimicrobiana. Após otimização da reação e do processo de produção das membranas, procedeu-se à caracterização físico-química das mesmas. Os resultados obtidos revelaram que as membranas foram capazes de promover a adesão e proliferação celular, assim como inibir o crescimento bacteriano. Estas propriedades são fundamentais para uma futura utilização destas membranas no revestimento de feridas e para acelerar o processo de cicatrização.

Abstract

Skin and soft tissue infections (SSTIs) have high rates of morbidity and mortality associated. Despite the successful treatment of some SSTIs, those affecting the subcutaneous tissue, fascia, or muscle delay the healing process and can lead to life-threatening conditions. Therefore, it is fundamental to develop antimicrobial wound dressings to deal with such pathological situations. Recently, wound dressings loaded with antimicrobial agents emerged as viable options to reduce wound bacterial colonization and infection, in order to improve the healing process. In this thesis, a new antimicrobial wound dressing was produced and characterized. In this context, polycaprolactone (PCL) nanofibrous membranes functionalized with biosynthesized maillard reaction products (MRPs) were produced using an electrospinning apparatus. The functionalization of PCL nanofibers with MRPs allowed the production of membranes with the mechanical, wettability and porosity features required for wound exudate absorption as well as nutrients and gas exchange. Furthermore, MRPs-modified PCL membranes were also able to inhibit *Staphylococcus aureus* and *Pseudomonas aeruginosa* growth, without inducing any cytotoxic effect to human fibroblast cells. These findings support the potential use of the produced membranes in the healing process.

Keywords

Antimicrobial wound dressing; electrospinning; maillard reaction products; skin and soft tissue infections; wound healing.

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Acronyms

A.iwoffi	Acinetobacter iwoffii
AMPS-NA^+	2-acrylamido-2-methylpropane sulfonic acid sodium salt
ATR-FTIR	Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy
B.cereus	*Bacillus cereus*
B.subtilis	*Bacillus subtilis*
BC	Bacterial cellulose
C.freundii	Citrobacter freundii
CA	Cellulose Acetate
CLSM	Confocal Laser Scanning Microscopy
CMCS	Carboxymethyl Chitosan
CMGG	Carboxymethyl Guar Gum
CS	Chitosan
DHBA	2,3-dihydroxybenzoic acid
DMEM-F12	Dulbecco's Modified Eagle's Medium
DPPH	2,2-Diphenly-1-Picrylhydrazyl
E.aerogenes	*Enterobacter aerogenes*
E.coli	*Escherichia coli*
E.faecalis	Enterococcusi faecalis
ECM	Extracellular Matrix
EDA	Ethylenediamine
ETDA	Ethylenediaminetetraacetic Acid
FA	Fructose-Arginine
FBS	Fetal Bovine Serum
GA	Glucose-Arginine
GMs	Gelatin Microspheres
HNTs	Halloysite Nanotubes
K.pneumoniae	*Klebsiella pneumoniae*
MMSA	Methicillin susceptible *Staphylococcus aureus*

MR	Maillard Reaction
MRPs	Maillard Reaction Products
MRSA	Methicillin resistant *Staphylococcus aureus*
MTS	3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4 sulfophenyl)-2H-tetrazolium
NHDF	Normal Human Dermal Fibroblasts
NIPAAm	N-isopropyl acrylamide
OAlg	Oxidized Alginate
OD	Optical Density
P.aeruginosa	*Pseudomonas aeruginosa*
P.mendocina	*Pseudomonas mendocina*
P.vulgaris	*Proteus vulgaris*
PBS	Phosphate-buffered Saline solution
PCD	ß-cyclodextrin polymer
PCL	Polycaprolactone
PEI	Polyethyleneimine
PEO	Polyethylene oxide
PHEA	Poly(2-hydroxyethylacrylate)
PI	Propidium iodine
PLA	Poly(lactic acid)
PLGA	Polylactic-co-glycolic acid
Plur	Pluronic F127
PP	Polypropylene
PSSA-MA	Poly(styrene sulfonic acid-co-maleic acid)
PU	Polyurethane
PVA	Polyvinyl alcohol
PVP	Polyvinylpyrrolidone
ROS	Reactive Oxygen Species
RT	Room Temperature
S.haemolyticus	*Staphylococcus haemolyticus*
S.aureus	*Staphylococcus aureus*

S.epidermidis	*Staphylococcus epidermidis*
S.pyogenes	*Streptococcus pyogenes*
S.typhi	*Salmonella typhi*
S.typhimurium	*Salmonella typhimurium*
SA	Sodium Alginate
SEM	Scanning Electron Microscopy
SF	Silk Fibroin
SSTIs	Skin and Soft Tissue Infections
TFE	Trifluoroethanol
V.vulnificus	*Vibrio vulnificus*
WVTR	Water Vapor Transmission Rate
ZN	Zein

Chapter I - Introduction

1. Introduction

Skin is the largest and outermost organ that covers the entire body. Therefore, above all, skin´s primary function is to protect underlying muscles, bones, ligaments and internal organs from external biological, chemical, mechanical and physical agents [1, 2]. Furthermore, skin is also involved in sensation, temperature regulation, immunological surveillance, prevention of water loss (dehydration) and synthesis of vitamin D3 [3]. However, the structure and functions performed by this organ can be affected by cuts, burns, surgical incisions or illnesses, such as diabetes [4]. After skin structure be compromised, its structure and functions must be re-established, as soon as possible to ensure the body homeostasis. To accomplish that, the wound healing process begins almost immediately after a skin injury occurs, in order to avoid the risk of bacterial contamination [5]. Non-healing wounds usually appear after this type of contamination occur [4].

Skin and soft tissue infections (SSTIs) are the most common type of infections and they affect approximately 14 million people every year in the United States [6, 7]. Depending on the etiology and severity of the microbial invasion, SSTIs can range from minor superficial to life-threatening infections [8]. In the initial stage of the infectious process, gram-positive organisms such as *Staphylococcus aureus* (*S.aureus*) and *Streptococcus pyogenes* (*S.pyogenes*) are the dominant organisms involved, while gram-negative organisms like *Escherichia coli (E.coli)* and *Pseudomonas aeruginosa (P.aeruginosa)* are only found in later stages of the process, i.e. when a chronic wound is developed [7].

In a healthy human being, infection is avoided, by activating the immune system for abolishing the invading pathogens. In this process, macrophages initiate the migration to the wound site and subsequently perform phagocytosis of the pathogens (which are destroyed in a phagolysosome or by nitric oxide production). In a later stage of infection, the immune response is performed by the activation of lymphocytes T helper which secrete interferon-γ and CD40 ligand to coordinate the immune adaptive and humoral response to kill and remove the invading bacteria [9]. However, if the immune system is not able to remove the pathogen, infection occurs and causes the deterioration of granulation tissue, growth factors and extracellular matrix components (collagen, elastin and fibrin), thus compromising the normal wound healing process [10, 11]. Therefore, it is fundamental to develop wound dressings that are capable of preventing bacteria penetration into the wound or avoid microorganisms' growth. In order to accomplish a fully-functional antimicrobial dressing, three key points must be considered: (1) production method; (2) the materials used to produce the dressing (that may have intrinsic antibacterial activity) and/or (3) selection of an appropriate antimicrobial agent to be incorporated within the dressing [12, 13].

1.1. Skin structure

Skin has an average surface area of 1.6 - 2 m^2 and accounts for approximately 16% of total body weight [14]. Anatomically, skin is highly complex and is composed by three connected layers, epidermis, dermis and hypodermis [15]. All skin's layers are responsible to provide strength and flexibility and perform the multiple functions of the skin [14]. As shown in Figure 1, the outer-most layer, the epidermis is responsible for providing a barrier to protect the body from any infection [16]. Dermis, skin's middle layer, consists of connective tissues which cushion the body from stress and strain. Epidermis and dermis are separated by a basement membrane, also known as the extracellular matrix (ECM), acting as a consistent and dynamic interface [17, 18]. The inner-most layer is the hypodermis, that contains larger blood vessels and nerves; it insulates the body and provides mechanical protection against physical shock [19]. Moreover, various appendages are associated with the skin layers such as hair follicles, nails, sweat and sebaceous glands, sensory receptors, lymphatic and blood vessels, all of which play essential functions [20, 21]. A detailed representation of the human skin structure is provided in Figure 1.

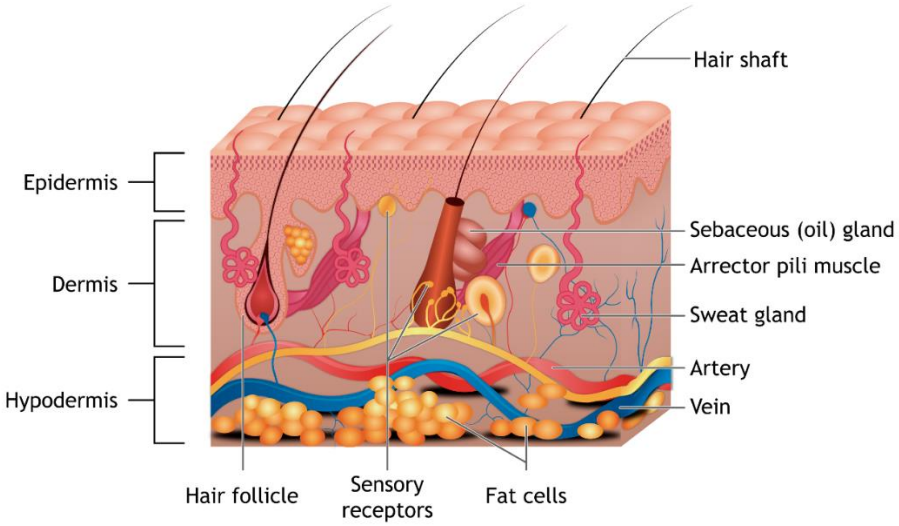

Figure 1. Schematic representation of the skin's structure.

In order to ensure skins' functions, it is important to emphasize cells communication and role across the layers of skin. The predominant cell type of epidermis is the keratinocyte (90-95%), which is involved in the production of keratin, a major structural protein [22]. Additional epidermal cells include the melanocytes (pigment-producing cells), the Langerhans cells (mobile, dendritic antigen-presenting cells, important to the immune barrier), and the Merkel cells (cells with both neuroendocrine and epithelial features that play a sensory role) [20, 23]. The dermis' cellular components include fibroblast, adipocytes, macrophages and mast cells. Fibroblasts are the main cell type present in the thick layers of the skin and they are responsible for the production and deposition of ECM components, e.g. collagen, elastic fibers, glycosaminoglycan and glycoproteins [24, 25].

1.2. Wound pathophysiology and the wound healing process

Wounds occur when a tissue is disrupted or the cellular integrity is compromised due to mechanical, physical or metabolism-related issues [12]. According to the duration and nature of the healing process, skin wounds can be classified as acute or chronic. An acute wound occurs suddenly, as a consequence of abrasions, avulsions, burns, incisions, lacerations and punctures (Figure 2A), and have associated an healing time that is dependent on the size and number of layers of skin that have been affected [26, 27]. Under normal physiological conditions, the restoration of the epidermal structure is highly efficient, however when a chronic wound occur, it is characterized by displaying a defective healing process, that do not allow skin to be repaired in an orderly and timely manner [28]. Based on etiology, the Wound Healing Society classifies chronic wounds into four categories: pressure, diabetic, venous and arterial insufficiency ulcers (Figure 2B) [29]. Bacterial colonization usually occurs in chronic wounds and it is considered as a primary cause of chronic inflammation [30].

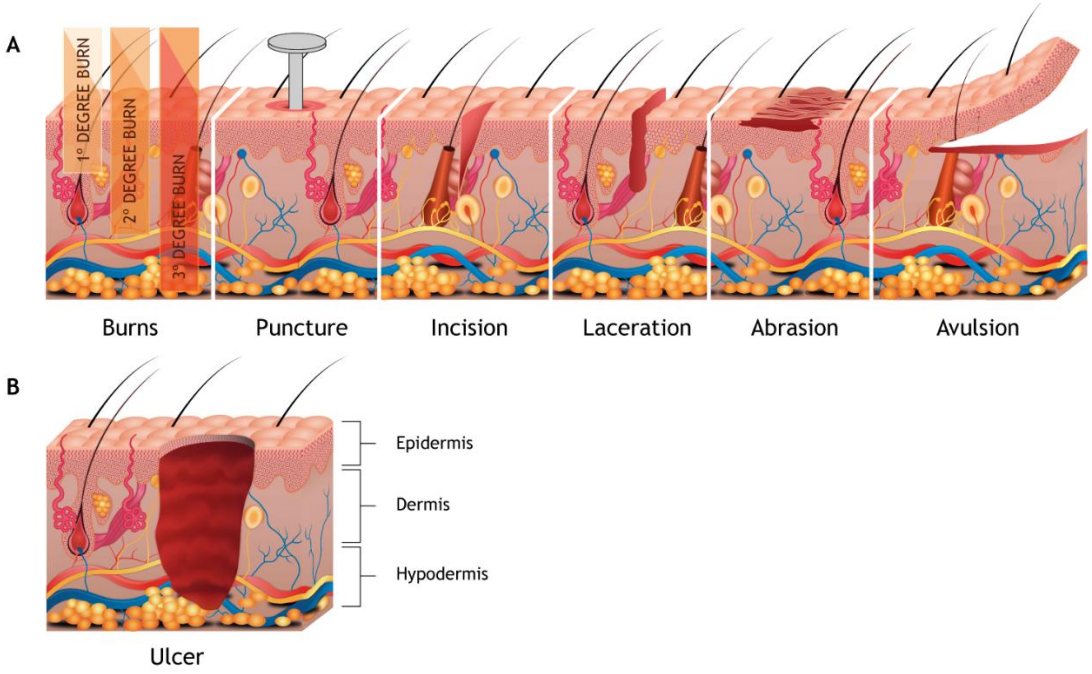

Figure 2. Representation of acute (A) and chronic (B) skin wounds.

The healing process comprises a cascade of precisely synchronized events in which are involved both resident and migratory cell populations, extracellular matrix components and soluble mediators [31]. This process includes five distinct phases: hemostasis, inflammation, migration, proliferation and remodeling [32]. In the first phase, hemostasis, a fibrin clot is formed to prevent blood loss through vasoconstriction as well as to avoid microbial contamination [33]. The inflammatory phase begins almost simultaneously with hemostasis and it involves the recruitment of neutrophils (that engulf bacteria and decontaminate the wound through proteases and antimicrobial peptides secretion and by producing reactive oxygen intermediates), monocytes/macrophages (monocytes differentiate into macrophages to remove apoptotic neutrophils and other cells and secrete cytokines and multiple growth

factors), and lymphocytes that exert a specific response against microbes (B-lymphocytes produce antibodies, while T-lymphocytes secrete cytokines involved in cytolytic activity) [33-35]. The migration and proliferative phases begin with fibroblast migration to the wound site and differentiation into myofibroblasts to produce extracellular matrix components like fibronectin, hyaluronic acid, collagen and proteoglycan, that are involved in the production of extracellular matrix (ECM), new blood vessels and re-epithelization [33]. Maturation, or remodeling, is the last stage of the wound healing process and in this phase all processes that were activated after injury are ceased [32].

In order to ensure an effective wound healing process, it is fundamental to maintain a controlled set conditions at the wound site (i.e. oxygenation, temperature and high availability of vitamins, minerals, and trace elements) that sustain the complex cellular activity during this process [36]. However, chronic wounds, burns, diabetic ulcers and post-surgical wounds have extended healing times and, in some cases, even fail. For example, burn wounds usually display high levels of exudate, which provides a moist and nutrient-rich environment that promotes bacterial growth, namely *Pseudomonas* species [37]. These bacteria produce virulence factors that mediate a number of processes like adhesion, nutrient acquisition, leucocyte killing and bloodstream invasion. Furthermore, these microorganisms are also able to produce endotoxins that promote pro-inflammatory cytokines expression, such as interleukin-1 and tumor necrosis factor-α, that ultimately lead to wound inflammation [38-40]. Wounds exhibiting an extended inflammation, show a high content of metalloproteinases (MMPs) that are involved in the degradation of ECM components, thus avoiding the formation of the granulation tissue and consequently delaying the healing [11, 41]. On the other hand, patients suffering from Diabetes mellitus (DM) have an impaired protective sensation and altered pain response, which makes them vulnerable to trauma and extrinsic forces. Diabetic wounds are characterized by their dry and keratinized aspect, that usually crack or suffer fissures more easily, leading to an extended healing time [42]. Therefore, patients with DM are predisposed to cutaneous infections occur, namely those caused by *S.pyogenes* and *S.aureus* [43].

1.3. Wound dressings displaying antimicrobial activity

In 1987, Gristina came up with the expression “race for the surface” to describe the competition that occurs between cells and bacteria for colonize a surface. Bacteria are inherently favored in this event, due to is natural ability to colonize both biological and non-biological surfaces [44]. An open wound is a favorable niche for microbial colonization [45]. Generally, the majority of infected wounds present polymicrobial and are usually contaminated by pathogens found in the surrounding environment, i.e. endogenous microbes living in the mucous membranes, and by the microflora available on the adjacent skin [46]. In the initial stages of chronic wound formation, gram-positive organisms, specifically *S.aureus*, are predominant. In the later stages, gram-negative *E.coli* and *Pseudomonas* species are observed

and tend to invade deeper layers of skin causing significant tissue damage. Furthermore, *Staphylococci* and *Streptococci* species are also found in 50% of chronic wounds [7].

Nowadays, bacterial contamination of skin wounds are responsible for the high rates of morbidity and mortality [47]. To address this health issue, different labs around the world started to develop antimicrobial wound dressings to prevent wound contamination [48]. A schematic representation of a polymeric antimicrobial dressing, designed to act as a physical barrier that protects the wound from microbial invasion and supports fibroblasts migration and differentiation, is presented in Figure 3.

The wound dressings develop up to now have been produced with different materials (synthetic or natural) and with various physical forms (sponges, hydrogels, hydrocolloids, films). These different formulations have distinct properties that make them suitable for the treatment of a particular type of wound. For example, sponges exhibit a huge porosity, provide thermal insulation and sustain a moist environment at the wound site. Nonetheless, the sponges are mechanically weak, may provoke skin maceration and are unsuitable for the treatment of third-degree burns or wounds with dry eschar [49, 50]. On the other hand, hydrogels are characterized by their capacity to store high amounts of water within their 3D polymeric network, which allow them to provide a moist environment to the wound. However, hydrogels display weak mechanical properties, thus demanding a secondary dressing [51, 52]. Furthermore, hydrocolloids are easily removed by saline or sterilized water, non-adherent, present high density and are painless dressings. Nevertheless, hydrocolloids display some disadvantages that may limit their use, i.e. they may be cytotoxic, display an unpleasant odor, present a low mechanically stability and maintain an acid pH at the wound site [53, 54]. Films used as wound dressings are impermeable to bacteria, allow healing monitorization and are painless. However, this type of dressing are hard to handle, adhere to the wound bed and cause exudate accumulation [26, 53].

The unsuitability of these dressings to fully restore skin structure and functions, made scientists be focused on the development of new dressings. Among them, nanofiber-based dressings displaying similar features to the native skin emerged as an interesting approach.

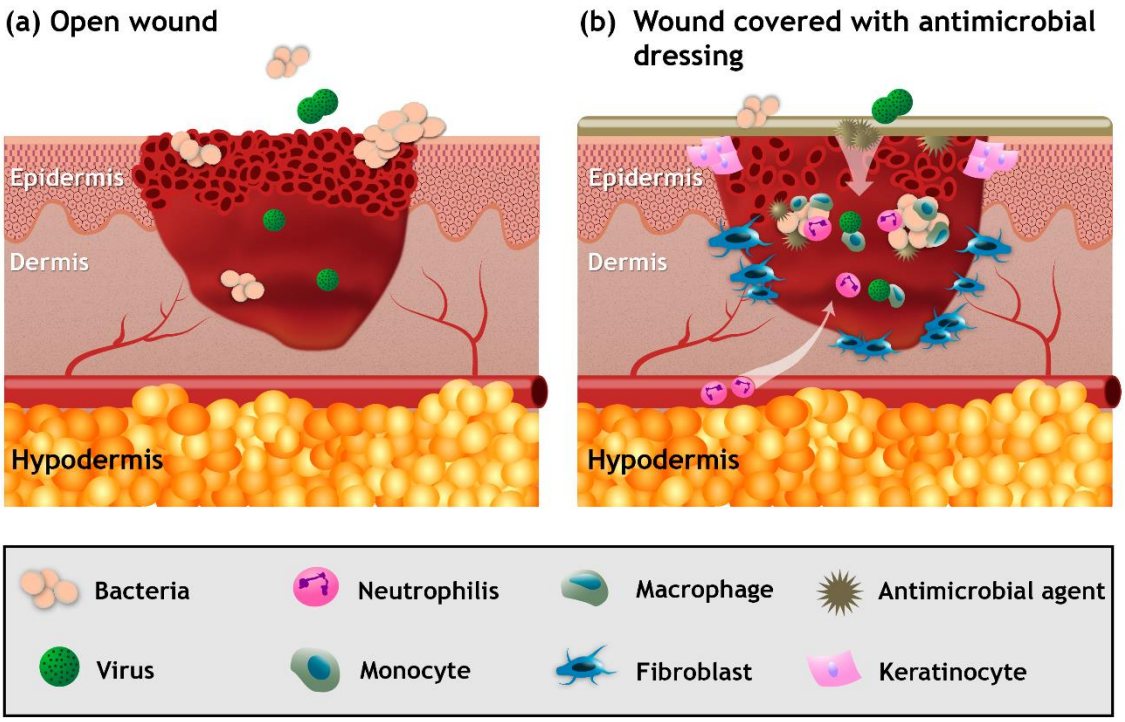

Figure 3. Representation of a contaminated open wound (a) and the healing process in a wound covered with antimicrobial wound dressing (b): The open wound is vulnerable to bacterial contamination, leading to an extended inflammatory phase and an increased expression of metalloproteinases that are involved in the degradation of ECM components and also inhibit the formation of new granulation tissue. When the antimicrobial dressing was used to cover the wound bed, it acts as a physical barrier to prevent pathogens entrance into the wound or to kill the invading microorganisms. In addition, the antimicrobial dressing supports the healing process by stimulating the immune system and fibroblast/keratinocyte migration.

1.4. Nanofibrous membranes produced through electrospinning

Nanofibers display a high surface-to-volume ratio, flexibility in surface functionalities, superior mechanical properties and high porosity. These features are essential for cell adhesion and proliferation [2, 55, 56]. So far, different techniques have been used to produce nanofibers, such as electrospinning, phase separation and self-assembly [57]. Among them, electrospinning due to its simplicity, versatility, and low cost, provides a straightforward electrohydrodynamic mechanism to produce nanofibers with diameters from 2 nm to several micrometers [58, 59].

A conventional electrospinning setup is simple and easily accessible (as represented in Figure 4). It is typically composed of four major components: a high-voltage power supply, a syringe pump, a capillary needle (the spinneret), and a collector [60]. Under the influence of an electric field, a pendent droplet of the polymer solution at the tip of the needle is deformed into a conical shape, known as Taylor Cone [61, 62]. An increased electric field generates a critical value where the repulsive electrostatic forces overcome the surface tension and a polymer jet is created from the needle tip in the form of nanofibers which are then deposited on the collector [63, 64]. If the jet is collected in a stationary collector, non-woven meshes composed of randomly oriented nanofibers are obtained [65]. The production of electrospun

nanofibrous membranes is dependent on the properties of the precursor solution (e.g. surface tension, conductivity, viscosity and solvent selection), processing variables (e.g. voltage, flow rate and the distance between the capillary and the collector) and environmental conditions (e.g. temperature and humidity) [66].

Up to now, different studies report the use of electrospun membranes as wound dressings. To confer to these membranes bactericidal activity, various antimicrobial agents such as antibiotics, silver nanoparticles and natural products have been incorporated in their structure [67-69].

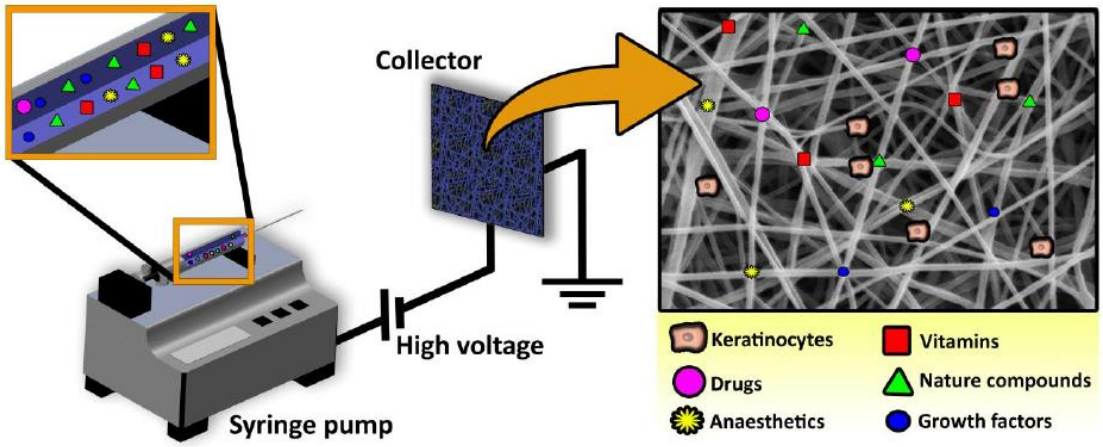

Figure 4. Representation of a conventional electrospinning apparatus that is usually used in the production of nanofibrous meshes. These membranes can be incorporated with bioactive molecules for enhanced healing.

1.5. Antibacterial agents used to functionalize wound dressings

1.5.1. Antibiotics

The discovery of natural compounds that exhibit antimicrobial activity was a major breakthrough in the treatment of infectious diseases, like SSTIs [70]. Although thousands of antibiotics are known, less than 1% are currently in use in the clinic, due to toxicity related issues or lack of uptake by the host cells [71]. Up to now, only aminoglycosides [72], beta-lactams [73], glycopeptides [74-76], quinolones [77], sulphonamides [78, 79] and tetracyclines [80, 81] have been used to produce wound dressings displaying antimicrobial activity. The incorporated antibiotics can interfere with a function/feature of the bacteria structure or on their metabolic pathways through one of the following four mechanisms (see Figure 5 further details):

1. **Inhibition of bacterial cell wall synthesis:** ß-lactams and glycopeptides are among the classes of antibiotics that interfere specifically with the cell wall biosynthesis [82]. For example, ß-lactams (including penicillins, carbapenems and cephalosporins) block the crosslinking of peptidoglycan units, by inhibiting the peptide bond formation reaction catalyzed

by Penicillin Binding Proteins (PBPs). Contrariwise, glycopeptides antibiotics (i.e. vancomycin) inhibit peptidoglycan synthesis, by binding peptidoglycan units and by blocking transglycosylase and PBPs activity [83]. Such events will impact on the shape of the bacteria and eventually lead to their lysis due to the high internal osmotic pressure [84].

2. Blockage of key metabolic pathways: The presence of folate pathway in many pathogenic microorganisms and its absence in mammals, has made this pathway an attractive target for antimicrobial drugs [85]. Some antibiotics mimic the folic acid structure (e.g. sulphonamides) and allow their competitive binding to bacterial enzymes. Such interferes with the production of DNA, RNA and proteins, leading to the disruption of bacteria proliferation [86].

3. Interference on protein synthesis: Antibiotics that interfere with protein synthesis can be divided into two subclasses: the 50S and 30S inhibitors. According to the data available in literature, only the 30S inhibitors have been used to treat skin infections. Aminoglycosides (i.e. streptomycin) and tetracyclines are antibiotics that act as 30S ribosome-inhibitors, by obstructing the access of aminoacyl-tRNAs to the ribosome [86, 87].

4. Inhibition of nucleic acids synthesis: Some antibiotics have the capacity to interfere with nucleic acid synthesis, by inhibiting the replication or transcription processes. Antibiotics that inhibit nucleic acid synthesis usually target topoisomerase II and topoisomerase IV of bacteria and RNA polymerase activity, thereby preventing the production of mRNA [82, 86]. The quinolone group of synthetic antimicrobial drugs acts by converting their targets (DNA gyrase and topoisomerase IV), into enzymes that fragment the bacterial chromosome [88].

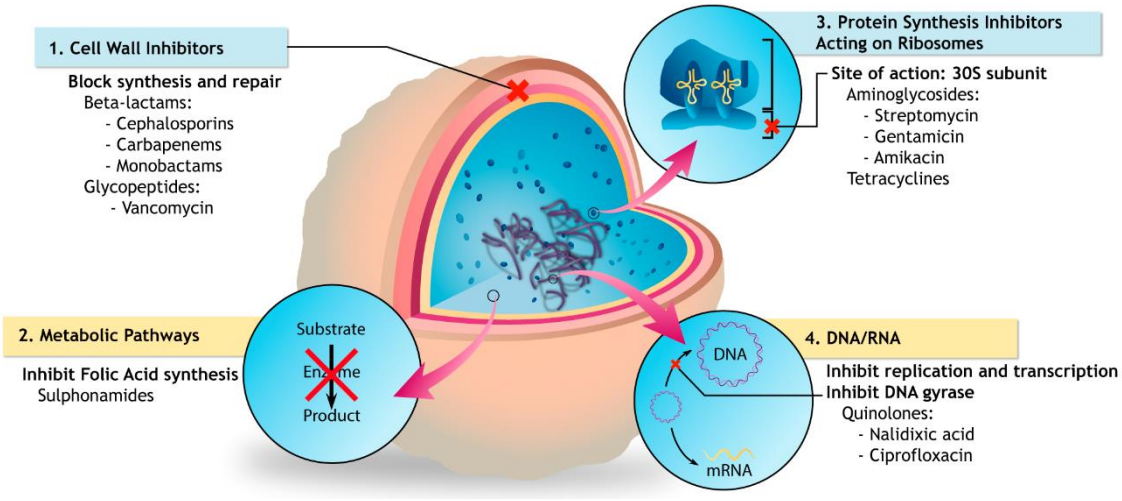

Figure 5. Representation of the different targets of antibiotic agents within bacteria.

Among the different antibiotics incorporated so far in wound dressings, tetracycline [89], ciprofloxacin (CIP) [90, 91], gentamicin [92] and sulfadiazine [78, 93] have been the most used. García *et al*. developed films based on chitosan (CS), at concentration of 1% (w/v), and modified-hybrids CS-weisocyanate as functional carriers of CIP. The obtained results revealed that these films loaded with antibiotic were able to inhibit *S.aureus* and *P. aeruginosa* growth, and the films' inhibitory activity was proportional to the antibiotic content loaded within their

structure [94]. Moreover, Heyu Li and co-workers produced electrospun fibers with thermoresponsive polymers, (poly(N-isopropylacrylamide) (PNIPAAm) and poly (l-lactic acid-co-ε-caprolactone) (PLCL)) at different ratios with a total concentration of polymer of 10% (w/v) and loaded them with CIP to confer antibacterial activity to the fibers. Their results showed that CIP-loaded fibers displayed similar inhibitory growth effect against *E.coli* and *S.aureus* [95].

On the other hand, Wei Shao and colleagues developed a tetracycline hydrochloride-loaded bacterial cellulose (BC) composite membrane. The antibacterial activity of the produced composite was investigated by both disc diffusion method and plate count method against *E.coli*, *S.aureus* and *Bacillus subtilis* (*B.subtilis*) [81]. The composite membrane presented a higher inhibitory effect against *E.coli* (45.7 mm) than for *S.aureus* (38.5 mm) and *B.subtilis* (34 mm). Regarding the plate counting method, the wound dressing reduce *E.coli* growth by 99.98%, while for *S.*aureus and *B.subtilis* the membrane was able to completely inhibit their growth [81]. Furthermore, Chen *et al*. fabricated an alginate-CS hydrogel dressing loaded with gelatin microspheres containing tetracycline hydrochloride to be used as a skin substitute. The bactericidal activity assays showed that the composite gel dressing was able to inhibit *E.coli* and *S.aureus* growth [89]. Recently, a semisynthetic derivative of tetracycline (glycylcyclines) was approved for the treatment of SSTIs. Tigecycline was the first member of this new class of antibiotics and presents a similar mechanism of action to that exhibited by tetracycline (30S ribosome-inhibitors), however it was designed to avoid the bacteria efflux-mediated resistance mechanisms [96, 97]. Dhanalakshmi and colleagues encapsulated tigecycline within chitosan nanoparticles (CNPs), and used them for treating infected chronic wounds [98]. Subsequently, Nimal *et al*. incorporated tigecycline loaded chitosan nanoparticles into a chitosan-platelet-rich plasma (PRP) hydrogel. The produced system showed an improved antibacterial activity against *S.aureus* [99].

Monteiro *et al*. produced a gentamicin-loaded liposome immobilized at the surface of CS nanofibers mesh (NFM), to confer this electrospun membrane antibacterial activity. The obtained results showed that the produced mesh was able to inhibit *E.coli, P.aeruginosa* and *S.aureus* growth [92]. Fajardo *et al*. incorporated silver sulfadiazine (AgSD) into CS/chondroitin sulfate (CHI) film to improve their applicability as a wound dressing. The antibacterial activity of the CHI/CS/AgSD was evaluated through the determination of their capacity to inhibit *P.aeruginosa* and *S.aureus* growth. The produced film presented an inhibitory growth effect against both bacteria, especially against *P.aeruginosa* [100].

Table 1 summarizes several studies where different antibiotics have been incorporated in wound dressings to improve their bactericidal activity.

Table 1. Wound dressings functionalized with antibiotics.

	Antibiotic	Wound dressing	Materials	Tested bacteria	Ref.
Beta-lactams	Ceftadizime	Electrospun membrane	SF/Gelatin	*P.aeruginosa*	[101]
		Film	Collagen/CMGG /EDA	*S.aureus* *P.aeruginosa*	[102]
	Ampicillin	Electrospun membrane	PCL	*S.aureus* *K.pneumoniae*	[69]
		Hydrogel	PVA/SA	*E.coli*	[103]
	Cefazolin	Electrospun membrane	Gelatin	*S.aureus*	[104]
Aminoglycosides	Streptomycin	Electrospun membrane	PU/CA/ZN	*E.coli* *S.aureus* *S.typhimurium* *V.vulnificus* *B.subtilis*	[105]
		Hydrogel	PVA/Cellulose	*S.aureus* *E.coli*	[106]
	Gentamicin	Electrospun membrane	CS	*P.aeruginosa* *S.aureus* *E.coli*	[92]
	Neomycin	Electrospun membrane	PSSA-MA/PVA	*S.aureus* *E.coli*	[107]
Quinolones	Ciprofloxacin	Electrospun membrane	PVP; PU/Dextran	*E.coli* *B.subtilis* *S.aureus* *S.typhimurium* *V.vulnificus*	[90, 91]
	Levofloxacin	Sponge	CS/PHEA	MSSA MRSA *P.aeruginosa*	[108]
	Norfloxacin	Film	CS	*S.aureus* *B.cereus* *E.coli* *K.pneumoniae*	[109]
	Moxifloxacin	Electrospun membrane	PVA/SA	*P.aeruginosa* *S.aureus*	[110]
Sulphonamides	Sulfadiazine	Film	BC/SA; CS/CHI	*E.coli* *C.albicans* *S.aureus*	[79, 100]
		Sponge	CS	*E.coli* *C.albicans* *S.aureus* *B.subtilis*	[93]
		Electrospun membrane	PCL/PVA	*S.aureus*	[78]
	Sulfanilamide	Fiber	Alginate	*E.coli* *S.aureus*	[111]
Tetracyclines	Doxycycline	Film	Collagen/DHBA/ GMs	*P.aeruginosa*	[80]
	Tetracycline hydrochloride	Membrane	BC	*E.coli* *S.aureus* *B.subtilis*	[81]
		Hydrogel	OAlg/CMCS/GMs	*E.coli* *S.aureus*	[89]

Glycopeptides	Vancomycin	Hydrogel	SF/GMs	*E.coli* *S.aureus*	[76]
		Film	Alginate/HNTs/ Gelatin	*S.epidermidis* *S.aureus* *S. haemolyticus* *S.pneumoniae* *S.pyogenes* *E.faecalis*	[75]

Despite of several antibiotics being available to treat skin infections, their recurrent use can trigger bacterial resistance [112]. In literature, various studies report that an improper use of antibiotics leads to the development of new resistance mechanisms by bacteria, thus causing their global dissemination [113]. More than 70% of the bacteria that are responsible for wound infections display resistance to at least one of the antibiotics used in the clinic [114]. Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci are two multi-resistant bacteria that are involved in skin infections [113]. Such type of infections are a major health issue, since vancomycin belongs to the latest generation of antibiotics and it is assumed to be the most effective agent against *S.aureus* [114].

The number of multidrug resistant bacteria is increasing at an alarming rate, i.e. bacteria are gaining resistance to all known classes of natural and synthetic antibiotics leading to an urgent need for new therapeutic alternatives [115]. Nanomedicine tools, particularly nanoparticles, constitute a different approach for the development of new antimicrobial agents [116].

1.5.2. Nanoparticles as potential antimicrobial agents

Based on the data available in literature, nanoparticles (NPs) are regarded as promising alternatives to conventional antibiotics, since they display bactericidal activity against a large number of strains and are able to minimize the undesirable side effects of drugs and do not trigger microbial resistance [117, 118]. Due to the intrinsic properties displayed by NPs, they have been used in different therapeutic approaches that aim to circumvent the problems associated with the acquisition of resistance to antibiotics by bacteria.

NPs alone can perform their bactericidal effect by direct contact with the bacteria cell wall, through the release of toxic metal ions or by the generation of Reactive Oxygen Species (ROS) [119]. When NPs are in contact with bacterial cells walls, the positively-charged NPs are attracted by the negatively-charged groups found in bacteria surfaces (lipopolysaccharides in gram-negative and teichoic acid/peptidoglycan in gram-positive). Then, van der Waals forces, receptor-ligand, and hydrophobic interactions are established and the cell wall permeability is altered through the formation of “pores” at bacteria surface, leading to its disruption and consequent loss of intracellular components [120]. At the same time, NPs can also cross the cell wall and affect metabolic pathways, or even target mitochondria and cause its disruption

and, consequently, induce ROS production [121]. In addition, NPs can also affect proton efflux pumps resulting in a serious pH deregulation and on the variation of membrane's surface charge [119]. Furthermore, NPs can likewise interact with DNA, lysosomes, ribosomes, and enzymes, leading to oxidative stress, electrolyte imbalance, enzyme inhibition, protein deactivation and variations in the gene expression profile [122]. Figure 6 presents the main bacterial targets of NPs and also some examples of NPs that have been used in the treatment of skin infections.

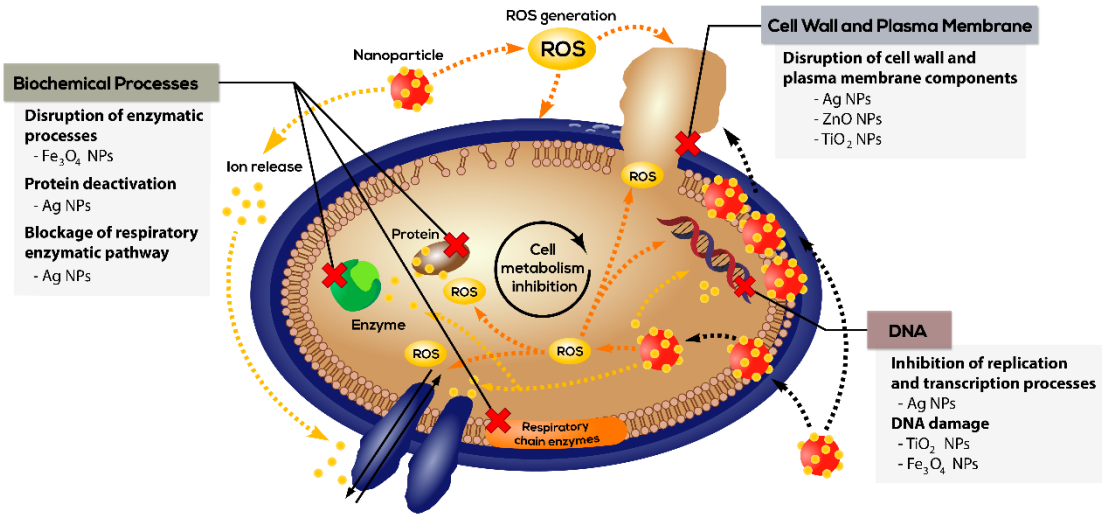

Figure 6. Representation of NPs targets in the bacteria and some examples of NPs that have been used as antimicrobial agents.

Among the available NPs, silver nanoparticles (AgNPs) have gained considerable attention owing to their broad inhibitory activity towards nearly 650 species of microbes, and more importantly, against antibiotic resistant bacteria [123]. With the advancement of nanotechnology, the scientific community was able to enhance the antimicrobial properties of silver, and consequently decrease silver NPs minimum inhibitory concentration (MIC), as well as reduce the possible interference of AgNPs in the wound healing process [124]. Such developments trigger the use of AgNPs in the production of wound dressings and their subsequent introduction in the market [125]. Acticoat®, Aquacel Ag® and Silvasorb® are examples of AgNPs containing dressings [126].

In 2013, Jian Wu and collaborators developed a new method to produce a BC hybrid gel-membranes containing AgNPs. The antibacterial activity of AgNPs-BC membranes was investigated against gram-negative (*E.coli and P.aeruginosa)* and gram-positive (*S.aureus)* bacteria. For comparative purposes, they also used a commercial silver-containing dressing (Coloplast®Ag non-adhesive foam dressing). The obtained results revealed that the composite membranes loaded with AgNPs inhibited the growth of all tested bacteria. No inhibitory effect was observed for the pure BC membrane (control), thus demonstrating the crucial role of AgNPs in conferring antimicrobial properties to the produced dressings. In comparison to the results obtained for the commercial dressing, no significant difference was observed, meaning that the antimicrobial dressing produced in this study has potential to be used as an effective wound

dressing [127]. In another study, Augustine *et al*. produced a polycaprolactone (PCL) electrospun membranes loaded with AgNPs to be used as wound dressing. The fabricated membranes showed excellent antibacterial activity against both *S.aureus* and *E.coli* [67].

In recent studies, the antimicrobial properties of metallic nanocomposites were combined with natural products, in order to increased their antibacterial effect and biocompatibility [128]. Anisha and their co-workers developed an antimicrobial sponge composed by CS, Hyaluronic acid (HA) and nano silver (nAg) to treat diabetic foot ulcers. The produced sponges showed antimicrobial effect against *E.coli*, *S.aureus*, *P.aeruginosa* and *K.pneumonia*. Furthermore, those sponges loaded with higher nAg concentrations (0.005%, 0.01% and 0.02%) were the most effective in reducing the *in vitro* growth of MRSA [129].

Further studies where AgNPs and other metal nanoparticles have been used to confer antimicrobial properties to the wound dressings are summarized in Table 2.

Table 2. Examples of nanoparticles with bactericidal activity that have been incorporated in wound dressings.

Type of Nanoparticle	Wound dressing	Materials	Tested bacteria	Ref.
Iron Oxide (Fe_3O_4) nanoparticles	Electrospun Membrane	CS/Gelatin	*E.coli* *S.aureus*	[130]
Titanium Dioxide ($Ti0_2$) nanoparticles	Composite	CS/human ECM sheet; CS/PVP	*E.coli* *S.aureus* *B.subtilis* *P.aeruginosa*	[131, 132]
	Electrospun Membrane	PVA/Plur/PEI	*E.coli* *S.aureus* *S.typhi*	[133]
Zinc Oxide (ZnO) nanoparticles	Hydrogel	CS; SA/gum acacia	*E.coli* *S.aureus* *B.cereus*	[134, 135]
	Composite	BC	*E.coli* *S.aureus* *P.aeruginosa* *C.freundii*	[136]
Silver (Ag) nanoparticles	Sponge	SF/CMCS	*S.aureus* *P.aeruginosa*	[137]
	Hydrogel	AMPS; PVP	*S.aureus* *P.aeruginosa* *S.epidermidis* *MRSA* *E.coli* *A.iwoffii* *B.cereus* *S.pyogenes*	[138, 139]

Despite of the bactericidal activity presented by NPs, some studies report that the same properties that make NPs so unique (small size, large surface area, chemical composition, solubility and geometry) could also be hazard to the human health, i.e. due to their size, NPs can easily enter the human body and cross various biological barriers, reaching the most

sensitive organs and disrupt the cell normal biochemical pathways [121, 140]. Costa *et al.*, demonstrated that AgNPs decrease Wistar rats tissues (brain, skeletal muscle, heart and liver) mitochondrial respiratory chain complexes I, II, III, and IV activity [141]. Furthermore, Botelho *et al.* demonstrated that TiO_2 NPs induce tumor-like phenotypes in human gastric epithelial cells [142].

Regardless of the several studies available in literature, the cytotoxic profile of a particular NP must be characterized in deeper detail for a particular therapeutic purpose [143]. One strategy that is currently being followed to reduce the possible toxicity associated with NPs use, is based on the obtainment of these carriers from natural sources, namely plant extracts, to be incorporated in wound dressings.

1.5.3. Natural products

Nowadays, an increasing number of wound dressings have been functionalized with compounds obtained from natural sources, to increase their antimicrobial activity [144, 145]. In the past years, several polymer/essential oils including cinnamaldehyde [146], *Thymus vulgaris* [147], chamomilla [148], *Mentha piperita* [149], and *Eremanthus erythropappus* [150] have been incorporated in wound dressings. For example, Zhang *et al.*, studied the effect of tea tree and manuka essential oils on the mechanical properties and antibacterial activity of electrospun polylactic acid (PLA) fibers. The results obtained demonstrated that natural extracts can be used to confer wound dressings antibacterial activity [151]. Table 3 summarizes some studies where natural products displaying bactericidal activity were incorporated into wound dressings.

Table 3. Wound dressings containing natural antibacterial agents isolated from plants.

Natural products	Wound dressing	Materials	Tested bacteria	Ref.
Henna (*Lawsonia inermis*)	Electrospun Membrane	CS/PEO; Gelatin/ Oxidized Starch	*E.coli* *S.aureus*	[152, 153]
St John's-wort EO (*Hypericum perforatum)*	Film	CS	*E.coli* *S.aureus*	[154]
	Electrospun Membrane	PCL	*E.coli* *S.aureus*	[155]
Curcumin	Composite	PVA	*E.coli* *S.aureus* *B.subtilis* *P.vulgaris* *E.faecalis* *S.epidermidis* *K.pneumoniae* *E.aerogenes* *P.mendocina*	[156]
	Electrospun Membrane	CA/PVP	*S.aureus*	[157]

Aloe vera	Electrospun Membrane	PLGA	*S.epidermidis* *S.aureus*	[158]
Thymol	Electrospun Membrane	PCL/PLA	*E.coli* *S.aureus*	[147]

1.5.3.1. Honey

Honey has been regarded since the ancient times as a natural healing agent. Due to its antimicrobial activity and capacity to perform the topical nutrition to the wound, debriding activity, minimize inflammation and stimulate angiogenesis, granulation, wound contraction and epithelialization, honey has been incorporated into wound dressings [159, 160]. Different honey-impregnated dressings are already available in the market, like MediHoney®, Activon Tulle®, Algivon® and Actilite® [160].

Honey's antimicrobial activity has been attributed to its acidity, low water content, and presence of antimicrobial substances such as hydrogen peroxide, antimicrobial peptide bee defensin-1, flavonoids, and phenolic acids [161-164]. The acidic character exhibited by honey results from the presence of gluconic acid and some authors believe that the acidic pH of honey may aid macrophages to kill bacteria and prevent microbial biofilm formation [165, 166]. In turn, the low water content (<20%) provides an unfavorable environment for microorganism survival and growth. High osmolarity inhibits microbial growth, since water molecules are chemically tied to the sugar molecules, leading to an inappropriate environment for organisms survival [167, 168]. Lastly, the production of hydrogen peroxide by honey is responsible for bacterial growth inhibition, i.e. hydrogen peroxide is able to react with the cell wall, lipids, proteins and nucleic acids available in bacteria [169, 170]. In Figure 7 are illustrated the bacterial activities exhibited by honey.

However, honey in the presence of catalase (an enzyme that degrades hydrogen peroxide) displays a decreased antimicrobial activity [159]. To surpass this drawback, Manuka Honey (MH), which is obtained from the manuka tree (Leptospermum scoparium), unlike other honeys, contains a non-peroxide component, that is not degraded by catalase, and is able to sustain its antibacterial activity in biologic fluids [159, 160]. MH inhibits the growth of a broad range of microorganisms (including gram-positive strains such as MRSA and *S.pyogenes*, as well as gram-negative strains like *E.coli*, *Proteus mirabilis (P.mirabilis)*, *Enterobacter cloacae*, and *P.aeruginosa*) and avoids biofilm formation at the wound site [171]. Furthermore, Packer *et al*. have described that methylglyoxal (MGO), a component of MH, interferes with ribosome and its translational capacity (see further details in Figure 7) [159].

Bulman *et al*. incorporated MGO as a functional antibacterial agent into polyvinyl alcohol fibers. The obtained results confirmed that the fibers containing MGO exhibited bactericidal activity against *E.coli* and *S.aureus* [172]. Moreover, Yang *et al.*, incorporated MH in an electrospun

membrane produced with silk fibroin (SF), for being used as an antimicrobial wound dressing. The SF/MH fibrous matrices presented antibacterial activity against both gram-positive (MRSA and *S.aureus*) and gram-negative (*E.coli* and *P.aeruginosa*) bacteria [173].

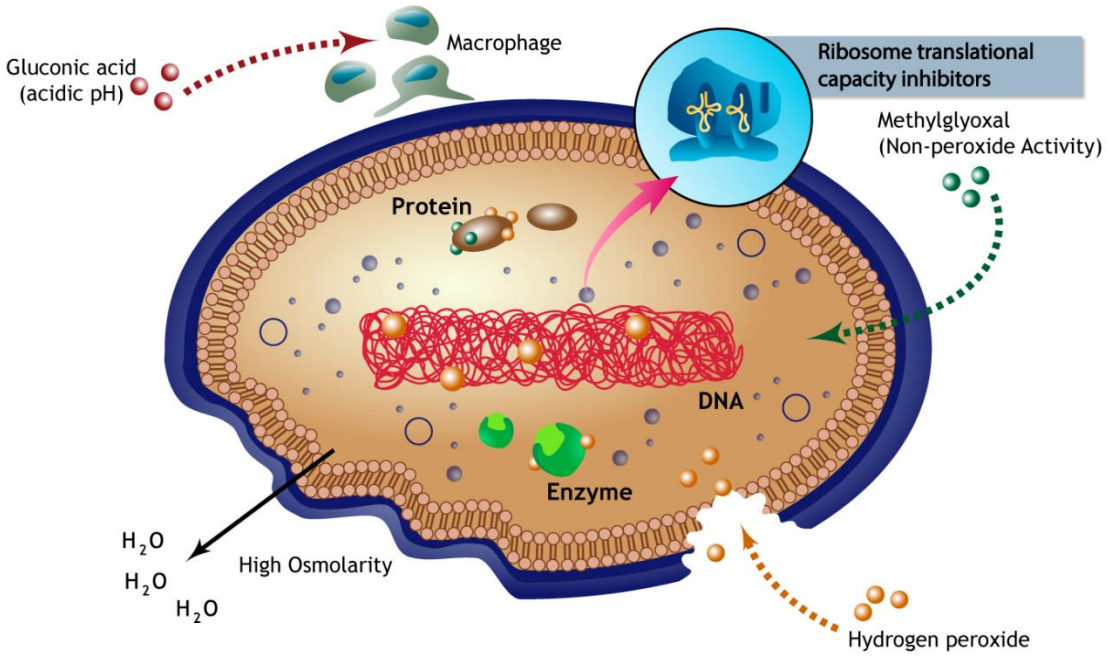

Figure 7. Representation of the mechanisms proposed to explain the bactericidal activity exhibited by Honey and MH (non-peroxide activity).

1.5.3.2. Essential oils

In addition to honey, essential oils (EOs) have been incorporated as antibacterial agents in bioactive wound dressings. Essential oils, also called volatile natural mixtures, are plant secondary metabolites that exhibit antioxidant, antiviral, anticancer, insecticidal, anti-inflammatory, anti-allergic and antimicrobial properties [174].

Different authors stated that the antimicrobial activity of EOs that are usually incorporated in wound dressings is attributed to phenolic compounds, specifically to thymol and carvacrol [159, 160, 171]. Kavoosi *et al.*, reported that EOs attack the phospholipids present in the cell membranes and the lipids available on the cell wall of the bacteria, leading to an increased permeability and ultimately to cell lysis. Such, results in the cytoplasm leakage, pH decrease, and loss of cellular processes, like ATP biosynthesis, DNA transcription and protein synthesis. Moreover, Altiok *et al*. described that EOs disturbs the function of the cytoplasmic membrane, by disrupting the active transport of nutrients through the cell membrane, and coagulation of bacteria cell contents [175]. In Figure 8 are illustrated the mechanisms through which EOs exert their antimicrobial activity.

Amongst the different EOs components, cinnamaldehyde, geraniol, thymol analogues, menthol and carvacrol (a major ingredient of Zataria multiflora EO) are the most used for antibacterial

purposes. Liakos *et al.*, incorporated 1% and 5% of EOs (cinnamon, lemongrass and peppermint) in cellulose-based fibrous dressings and they noticed that the fibrous dressings were able to inhibit the growth of *E.coli*, even when small amounts of EOs were used [149]. In another study, Liakos *et al*. prepared polymeric composite films with sodium alginate (NaAlg) incorporating different EOs (chamomile blue, cinnamon, lavender, tea tree, peppermint, eucalyptus, lemongrass and lemon oils) at different concentrations (16%, 50% and 66%). The produced dressings were able to inhibit *E.coli* growth [176].

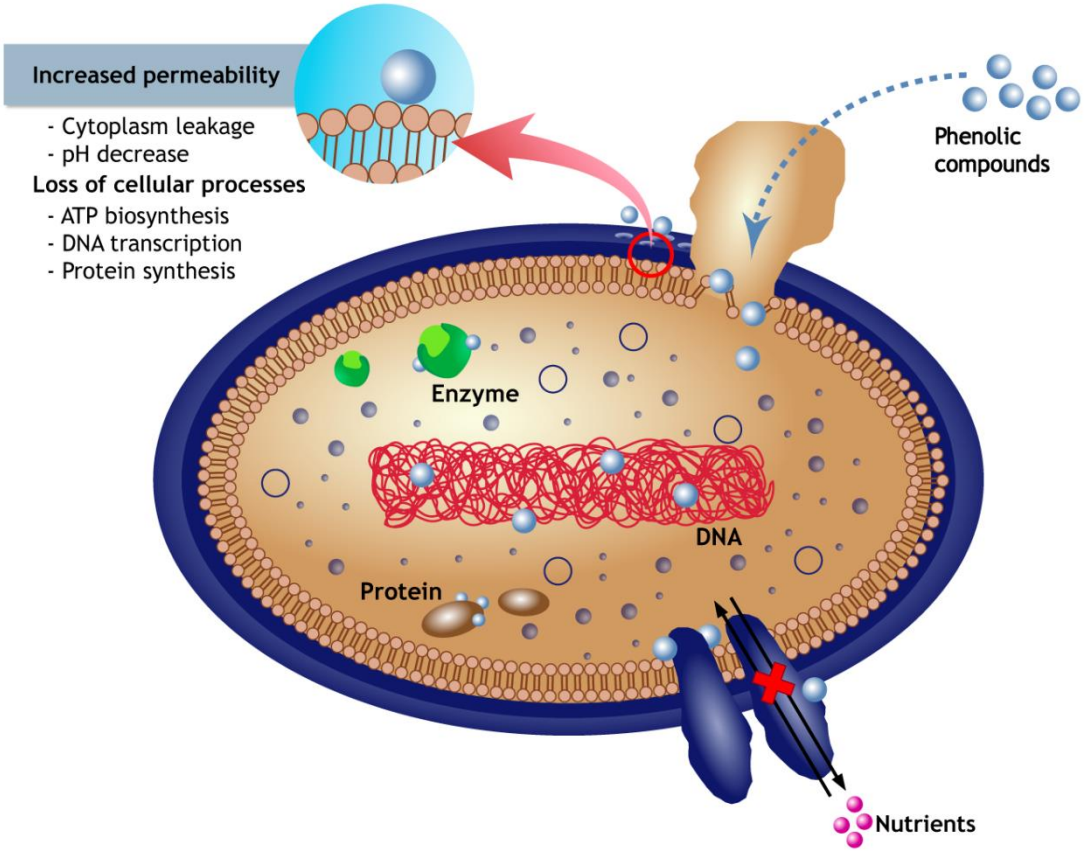

Figure 8. Representation of phenol (active component of essential oils) targets in bacteria.

1.5.3.3. Chitosan

Chitosan (CS) and its derivatives display a high antimicrobial activity against fungi, bacteria, algae and viruses [177]. In literature there are at least three mechanisms proposed for explaining the antibacterial activity of CS (depicted in Figure 9) [178]. The most accepted mechanism proposes that CS antimicrobial activity results from the electrostatic interactions occurring between the positively-charged groups of chitosan (amine groups of glucosamines) and the negatively-charged groups available on the bacterial cell wall (surface components like peptidoglycans) [179]. This electrostatic interaction can affect the permeability of the cell wall, thus causing internal osmotic imbalances and consequently inhibit the growth of microorganisms. On the other hand, the electrostatic interactions can induce hydrolysis of the microorganisms' cell wall, prompting the leakage of intracellular electrolytes [178]. The second

proposed mechanism involves the formation of a polymeric envelope around bacteria, leading to the inhibition of cell exchanges and nutrients absorption [179]. The last mechanism includes the chelation of trace metals and oligo-elements that are essential for bacterial growth, i.e. the amino groups of CS might interact with essential trace metals and thereby inhibit the production of toxins and microbial growth [178, 179]. These specific properties triggered its use in the production of wound dressings available in the market, such as HidroKi®, Patch®, Chitopack®, Tegasorb® and KytoCel® [180].

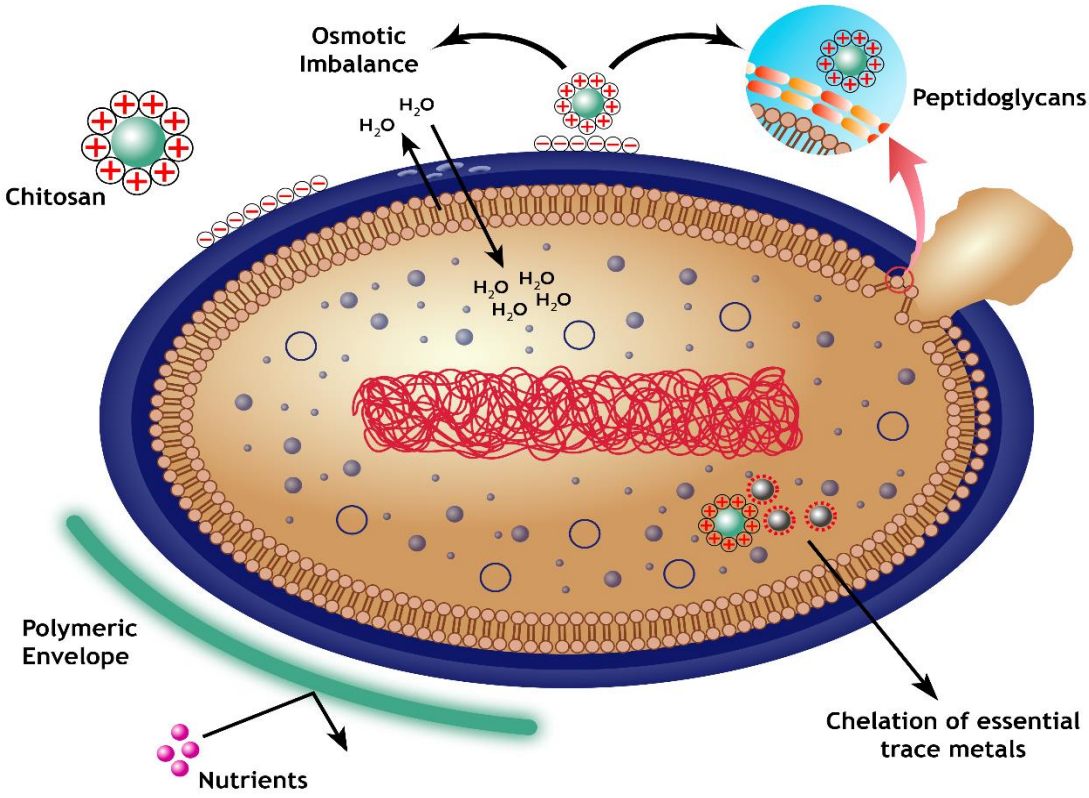

Figure 9. Representation of the mechanisms proposed to explain the antibacterial activity of Chitosan.

Antunes *et al*. produced an electrospun membrane comprised by deacetylated/arginine modified chitosan (CS-A) to be used as a wound dressing. In this study, CS was modified with arginine to increase the number of positively-charged groups available on material's surface, to enhance its electrostatic interaction with bacteria cell wall. The obtained results highlight the importance of coupling arginine residues to CS, since the modified CS exhibit a much higher antimicrobial activity [181]. Furthermore, Yuan *et al*. prepared a CS and polyethylene oxide (PEO) nanofibrous meshes for wound healing application. The electrospun mesh was able to significantly reduce the bacterial colonies attached to the membrane surface. In addition, those materials with a higher concentration of chitosan present a greater bactericidal effect [182]. Other studies where CS has also been used for the production of antimicrobial wound dressings are summarized in Table 4.

Table 4. Chitosan-based wound dressings for skin regeneration applications.

Chitosan-based wound dressing	Materials	Tested bacteria	Ref.
Membrane	CS	*P.aeruginosa* *S.aureus*	[183]
	CS/BC	*E.coli* *S.aureus*	[184]
	CS/PP/NIPAAm/CG	*S.aureus*	[185]
	CS/SF	*E.coli* *S.aureus*	[186]
	CS/sericin	*E.coli* *B.subtilis*	[187]
	CS/CS-glucan	*E.coli* *K.pneumoniae* *B.subtilis* *S.aureus*	[188]
	CS/Aloe vera	*E.coli* *S.aureus*	[180]
Hydrogel	CS/Agarose	*E.coli* *S.aureus*	[189]
Sponge	CS/PCD	*S.aureus*	[190]
	CS/PVA	*E.coli*	[191]
Film	CS/PVP/nanocellulose	*S.aureus* *P.aeruginosa*	[192]
	CS/Hyaluronan	*E.coli* *S.aureus*	[193]

1.6. Maillard reaction products: a new approach to functionalize electrospun membranes for an improved healing process

The maillard reaction (MR) is a non-enzymatic browning reaction involving the condensation between a carbonyl group of reducing sugars, aldehydes or ketones (e.g. glucose and fructose), and an amino group of amino acids, proteins or peptides (e.g. arginine), resulting in the production of maillard reaction products (MRPs), that generate aroma, flavor and color in food [194, 195]. Several authors proved that MRPs possess antioxidant [196, 197], anti-inflammatory [198, 199] and antimicrobial activity [200-202]. As a result of their interesting properties, MRPs are considered a promising approach for wound dressing functionalization, and consequently for an improved healing process [203, 204].

Several mechanisms have been proposed to explain the antibacterial activity of the MRPs [200]. The most accepted mechanism proposes that MRPs act as anionic hydrophilic compounds that bind to iron and form stable complexes [205]. As consequence, the bioavailability of the extracellular iron is disturb causing a negative impact on different cellular processes, such as DNA replication, cell growth, proliferation, ATP generation and respiration of bacteria [206]. The remaining proposed mechanisms include cell integrity disruption and hydrogen peroxide activity. Hydrogen peroxide is produced during the MR and it may remain in the final solution in residual concentrations, acting as an important contributor to the antimicrobial activity of MRPs, i.e. it reacts and disrupts the cell wall, lipids, proteins and nucleic acids in bacteria [169, 170, 200, 207].

Herein, biosynthesized MRPs, glucose-arginine and fructose arginine, were incorporated within PCL electrospun nanofibers structure, to enhance their biological properties and endows them with antimicrobial activity.

1.7. Aims

In the present thesis, biosynthesized MRPs were incorporated within electrospun membranes for improving the wound healing performance (Figure 10). The specific goals of this study were:

- Produce MRPs derived from glucose-arginine (GA) and fructose-arginine (FA) model systems;
- Functionalize PCL polymer with the produced MRPs;
- Produce MRPs-modified PCL membranes;
- Evaluate and characterize the physicochemical properties of the produced membranes;
- Evaluate and characterize the biological properties of the produced membranes;
- Evaluate and characterize the antimicrobial and antioxidant properties of the produced membranes.

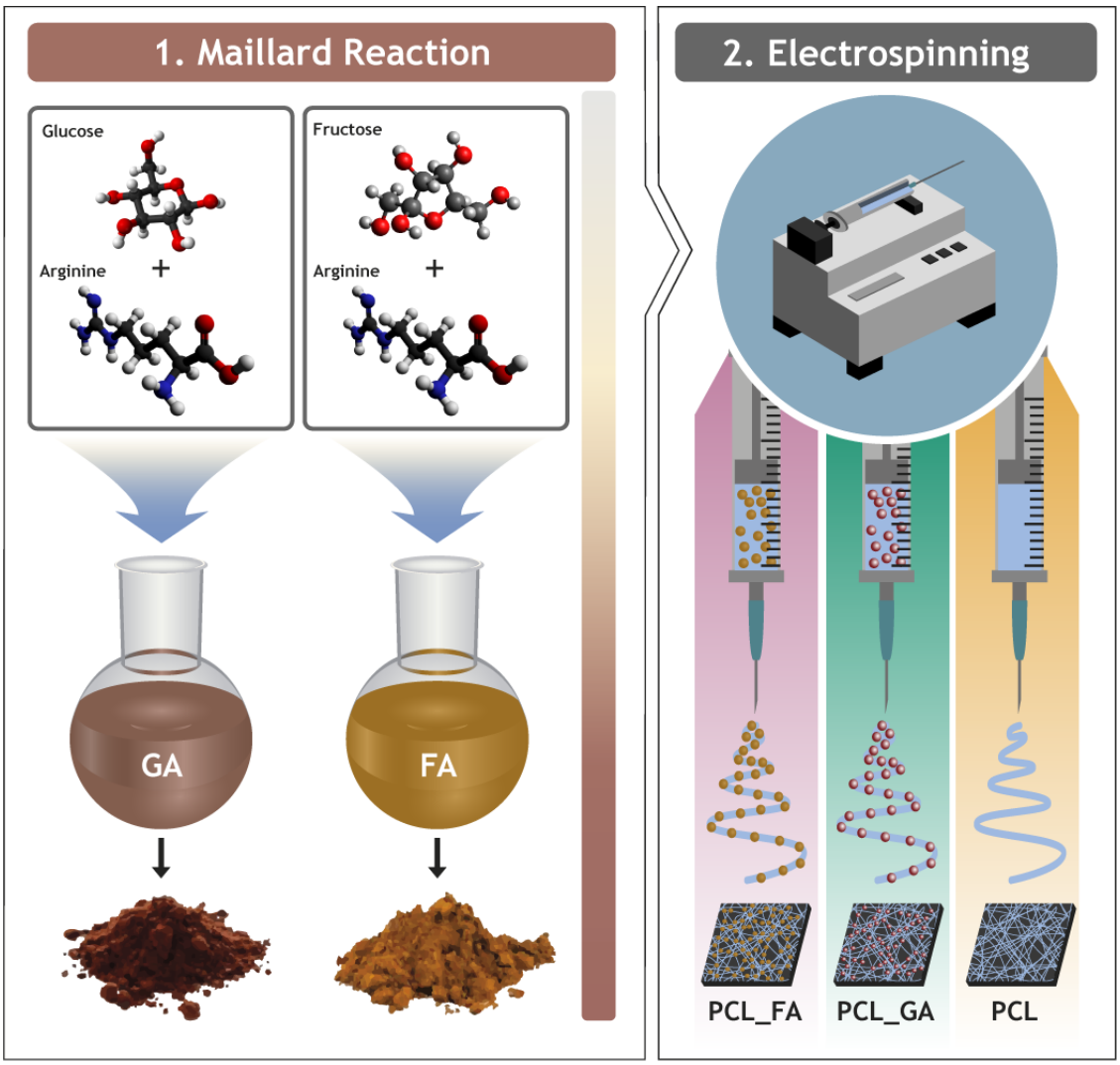

Figure 10. Schematic representation of the experimental setup used to produce the MRPs-modified PCL membranes. The MRPs were prepared from aqueous glucose-arginine (GA) and fructose-arginine (FA) model systems at pH 10.64, heated at 100ºC for 60 min, and then freeze-dried (A). The MRPs were blend with PCL polymer and electrospun (B).

Chapter II – Materials and Methods

2. Materials and Methods

2.1. Materials

D-Glucose anhydrous, D-Fructose and 3,3,3 Trifluoroethanol (TFE) was supplied by Acros Organics (Jersey City, NJ, USA). Fetal bovine serum (FBS) free from any antibiotic was purchased from Biochrom AG (Berlin, Germany). Normal Human Dermal Fibroblasts (NHDF) cells were acquired from PromoCell (Labclinics, S.A., Barcelona, Spain). 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2 (4-sulfophenyl)-2H-tetrazolium (MTS) was bought from Promega (Madison, WI, USA). L-arginine, Dulbecco's modified Eagle's medium (DMEM-F12), Ethylenediaminetetraacetic acid (EDTA), Gentamicin, Glutaraldehyde, LB Broth, Phosphate-buffered saline solution (PBS), Sodium hydroxide (NaOH), PCL (80,000 Da) and Trypsin were purchased from Sigma-Aldrich (Sintra, Portugal). Quant-iT Pico Green dsDNA assay kit was obtained from ThermoFisher Scientific (Waltham, MA, USA). *Staphylococcus aureus* clinical isolate (*S. aureus*) ATCC 25923 and *Pseudomonas aeruginosa (P.aeruginosa)* obtained from a human sample were used as models of prokaryotic organisms to evaluate the bactericidal activity exhibited by the produced membranes. Propidium iodine buffer was gotten from Invitrogen (Carlsbad, Califórnia, EUA) and Calcein AM was supplied by Calbiochem (Merck Millipore, Oeiras, Portugal).

2.2. Methods

2.2.1. Preparation of the sugar-amino acid model Maillard reaction products (MRPs)

MRPs were synthesized following a slightly modified version of the protocol previously described by Wu *et al*. [202]. Equimolar (0,01 mol) amounts of glucose-arginine and fructose-arginine were separately dissolved in Milli-Q water. After adjusting the pH of both solutions to 10.7, the solutions were transferred to a flask and refluxed in an oil bath at 100ºC for 1 h. The final solutions were freeze-dried for further use.

2.2.2. Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy analysis of the MRPs

Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR) was used to study the final composition of the MRPs. The samples' spectra were obtained with an average of 128 scans, a spectral width ranging from 400 and 4000 cm^{-1}, and a spectral resolution of 4 cm^{-1}, using a Nicolet iS10 FTIR spectrophotometer (Thermo Scientific, Waltham, MA, USA).

All the components used for the maillard reaction (glucose, fructose and arginine) were also analyzed in pure state for further comparison purposes.

2.2.3. Production of electrospun MRPs-modified PCL nanofibrous membranes

Different nanofibrous membranes composed of PCL, PCL_GA and PCL_FA polymeric blends were produced using a conventional electrospinning apparatus. The system setup was comprised of a high voltage source (Spellman CZE1000R, 0-30 kV), an automatic precision syringe pump (KDS-100), a plastic syringe with a stainless-steel needle (21 Gauge) and an aluminum foil connected to a copper collector, at a working distance of 14 cm. To accomplish the production of the PCL_GA and PCL_FA membranes, a PCL solution (9% w/v) was initially prepared by dissolving PCL in TFE 80% (v/v). Then 25 mg/mL of GA or FA MRPs were added to PCL solution and maintained under stirring for 15 to 20 min. After homogeneous solutions be obtained, they were electrospun at a constant flow rate of 2.5 mL/h and an applied voltage of 25 kV. Additionally, PCL membranes were also produced as described above, for comparison purposes.

2.2.4. Evaluation of the morphological, physical and mechanical properties of the produced electrospun membranes

2.2.4.1. Characterization of the surface morphology and composition of the produced membranes

Scanning electron microscopy (SEM) analysis of the produced membranes was used to characterize nanofibers' surface morphology and fibers' diameters distribution. Samples were initially mounted onto aluminum stubs using Araldite glue, and sputter-coated with gold using a Quorum Q150R ES sputter coater (Quorum Technologies Ltd, Laughton, East Sussex, UK). SEM images were acquired using a Hitachi S-3400N Scanning Electron Microscope (Hitachi, Tokyo, Japan) at an accelerating voltage of 20 kV. The average diameter of electrospun nanofibers was measured using ImageJ (Scion Corp., Frederick, MD).

Furthermore, ATR-FTIR spectra were acquired to characterize the chemical composition of the produced membranes, following the protocol described above (section 2.2.2).

2.2.4.2. Assessment of the mechanical properties of the membranes

The mechanical properties of PCL, PCL_GA and PCL_FA membranes were evaluated using a Shimadzu AG-X Tensile Testing Machine (Tokyo, Japan), at room temperature (RT), under wet and dry conditions, following the guidelines established by Standard Test Method for Tensile

Properties of Polymer Matrix Composite Materials (ASTM standard D3039/D3039M) [208]. To perform this assay, samples (n=5) with a width of 2 cm, gauge length of 6 cm and thickness ranging from 0.15 to 0.3 mm were used. The length between the clamps was set to 2 cm and the speed of testing was set to 15 mm/min. For the wet conditions, membranes were immersed in a PBS solution (pH = 5.5), for 24 h at 37ºC. Load-extension data was recorded and the stress-strain curve of the membranes was assessed by applying Equations (1) and (2), respectively:

$$Stress = \sigma = \frac{F}{A} \quad (1)$$

$$Strain = \varepsilon = \frac{\Delta l}{L} \quad (2)$$

where F is the applied force; A is the cross-sectional area; Δl is the change in length; and L is the length between the clamps.

2.2.4.3. Evaluation of the membranes' porosity

The total porosity of the membranes was determined using a fluid displacement method adapted from Miguel *et al*. [209]. In this assay, five specimens were weighed and then immersed in absolute EtOH for 1 h. Subsequently, the samples were reweighed and the porosity of the membranes was assessed by determining the amount of ethanol absorbed by the membranes, through Equation (3):

$$Porosity\ (\%) = \frac{W_s - W_d}{D_{ethanol} \times V_{membrane}} \times 100 \quad (3)$$

where W_d is the initial weight of dry membrane, W_s is the weight of the swollen membrane, $D_{ethanol}$ is the density of the ethanol at RT and $V_{membrane}$ is the volume of the swollen membrane.

2.2.4.4. Characterization of the swelling profile of the produced membranes

The swelling behavior of the produced membranes was evaluated by immersing them into PBS (pH = 5.5), at 37ºC, under stirring (40 rpm) [209]. At predetermined intervals, samples were retrieved from the solution and after removing the excess of PBS with filter papers their weight was determined. The swelling ratio was calculated using the following Equation (4):

$$Swelling\ ratio\ (Q) = \frac{W_t}{W_0} \quad (4)$$

where W_t and W_0 are the final and initial weight of the membranes, respectively.

2.2.4.5. *In vitro* analysis of membranes' biodegradation profile

The degradation profile of the produced membranes was monitored by immersing the samples in PBS (pH = 5.5), under stirring (40 rpm), at 37ºC. After 1, 3, and 7 days, samples were removed from the solutions, dried and weighted [210]. The degradation percentage at each time point was calculated through Equation (5):

$$Weight\ loss\ (\%) = \frac{W_i - W_t}{W_t} \times 100 \qquad (5)$$

where W_i corresponds to the initial weight of the sample and W_t to the weight of the sample at time t.

2.2.4.6. Contact angle determination

The PCL, PCL_GA and PCL_FA membranes water contact angles (WCA) were determined by using a Data Physics Contact Angle System OCAH 200 apparatus, operating in static mode [211]. For each sample, water drops (4 µL) were placed onto the surface of the membranes, at various locations, and then analyzed, at room temperature.

2.2.4.7. Water vapor transmission rate

The water vapor transmission rate (WVTR) through nanofibers was measured following the approach described elsewhere [181]. Briefly, PCL, PCL_GA and PCL_FA membranes were used to seal the opening of a glass test tube (1.77 cm^2) filled with 10 mL of ultrapure water. Parafilm tape was used to attach the membranes to the tubes and prevent water losses. Then the nanofibers were incubated at 37ºC. At specific time points, water evaporation from each test tube was determined by their weight and using the Equation (6):

$$WVTR = \frac{W_{loss}}{A} \qquad (6)$$

where W_{loss} is the daily weight loss of water and A is the area of the tube opening.

2.2.4.8. Antioxidant activity of the produced membranes

The antioxidant activity of PCL, PCL_GA and PCL_FA membranes was investigated using the 2,2-diphenly-1-picrylhydrazyl (DPPH) radical scavenging assay, which was performed according to a protocol previously described in literature [212]. Briefly, 5 mg of the electrospun mats were immersed in 3 mL of 100 µM DPPH solution in methanol. After 30 min of incubation in the dark, the absorbance of each sample was read at 517 nm using a microplate reader (Biorad xMark microplate spectrophotometer). The DPPH degradation was assessed using the following Equation (7):

$$DPPH\ scavenging\ (\%) = \frac{A_B - A_S}{A_S} \times 100 \qquad (7)$$

where A_B is the absorbance of the blank sample and A_S is the absorbance of the samples incubated with the membranes.

2.2.5. Characterization of the cytotoxic profile of the produced membranes

2.2.5.1. Evaluation of cell viability after cells being in contact with the produced membranes

Membranes biocompatibility was assessed through the MTS assay following ISO 10993-5 (Biological evaluation of medical devices-Part 5: Tests for *in vitro* cytotoxicity). Briefly, membranes (n=5) were placed in 96-well plates, occupying less than 10% of the well area, and sterilized under UV irradiation (254 nm, ~7Mw cm^{-2}) over 1 h. Then, cells were seeded at a density of 10 x 10^3 cells/well in the presence of the membranes and incubated at 37ºC, in a 5% CO_2 humidified atmosphere. Every two days the culture medium was changed until the end of the assay. At predetermined time points, the medium of each well was removed and replaced with a mixture of 100 µL of fresh culture medium and 20 µL of MTS/PMS (phenazine methosulfate) reagent solution. After 4 h of incubation (at 37ºC and 5% CO_2 atmosphere) the absorbance of each sample was measured at 492 nm using a microplate reader (Biorad xMark microplate spectrophotometer). Cells incubated without materials were used as a negative control (K^-), whereas cells incubated with EtOH (96%) were used as positive control (K^+).

2.2.5.2. dsDNA Quantification

DNA quantification was performed to analyse cell proliferation on the produced membranes, following a protocol reported elsewhere [213-215]. In brief, DNA content was measured using a Quant-iT PicoGreen dsDNA Assay kit on day 1, 3 and 7 after cell seeding. For each time point, cell lysis was induced by adding Triton X-100 to the cell-membrane complexes for 1 h. Then, membranes were transferred into a 1.5 mL Eppendorfs and exposed to a freeze-thaw cycle. Afterwards, the samples were sonicated and centrifuged at RT (14000 g) for 15 min. From the obtained supernatant, 100 µl was withdrawn and an equal volume of PicoGreen solution in TE buffer (1X) was added to the samples and incubated for 10-15 min in the dark. Finally, the fluorescence was measured with a microplate reader using excitation and emission wavelengths of 485 nm and 535 nm, respectively. A calibration curve was performed and the obtained fluorescence values were used to calculate the dsDNA concentrations of each sample.

2.2.5.3. Live-dead staining assay

Confocal laser scanning microscopy (CLSM, Zeiss, Oberkochen, Germany) was used to characterize cell viability at the surface of the produced membranes. To accomplish that, cells (10 x 10^3 cells/mL) were seeded on PCL, PCL_GA and PCL_FA nanofibers in µ-Slide 8 well Ibidi imaging plates (Ibidi GmbH, Germany). Cells seeded with only culture medium were used as negative control (K^-), whereas cells incubated with 200 µl of Triton X-100 for 15 min were used as positive control (K^+). After 1, 3 and 7 days, following the protocols described by the manufacturer, the samples were stained with Calcein (2 µM) and with Propidium iodine (PI), to evaluate the presence of live (green) and dead (red) cells, respectively. The image analysis was performed using Zeiss Zen 2010 software (Zeiss, Oberkochen, Germany).

2.2.5.4. Evaluation of cell adhesion and proliferation at the surface of the membranes

SEM analysis was also performed to characterize cell adhesion at the surface of the membranes. To perform the acquisition of the SEM images, the cells adhered at the surface of the electrospun membranes were fixed with 2.5% (v/v) glutaraldehyde for 30 min, and then freeze-dried for 3h. Afterwards, the samples were prepared for SEM analysis as previously described in section 2.2.4.1.

2.2.6. Evaluation of the antimicrobial properties of the produced membranes

2.2.6.1. Disc diffusion assay

The bactericidal activity of the PCL, PCL_GA and PCL_FA electrospun nanofibers was characterized by measuring the area of the inhibitory halo formed when the membranes were put in contact with *S.aureus* and *P.aeruginosa* [216]. To do that, 200 µL of bacteria medium containing 1 x 10^8 CFU/mL of *S.aureus* or *P.aeruginosa* were inoculated in agar plates. Then, circular samples of nanofiber membranes, with ~1 cm diameter, were gently placed on the inoculated agar plates and incubated during 24 h, at 37ºC. The inhibitory halo size around the samples was measured using ImageJ and the inhibition area was calculated using Equation (8):

$$Inhibition\ area\ (\%) = \frac{A_i - A_0}{A_0} \times 100 \qquad (8)$$

where, A_i and A_0 represents the inhibition area and the disc membranes area, respectively.

2.2.6.2. Optical density method

The optical density (OD) values were measured to evaluate the inhibitory effect of the PCL and MRPs-modified PCL nanofibrous mats against *S.aureus* and *P.aeruginosa*. Briefly, 50 mg of the produced membranes were sterilized under UV irradiation and immersed in 8 mL of bacterial solution (1 x10^5 CFU/mL). The mixtures were incubated at 37ºC for 24 h. The OD of the medium was measured at 600 nm, at different time points. The antibacterial efficiency of the membranes was then calculated from the following Equation (9):

$$Antibacterial\ efficiency\ (\%) = \frac{A_t - A_0}{A_0} \times 100 \qquad (9)$$

where A_0 represents the absorbance of the control, and A_t the absorbance of the samples with the membranes, at time t.

2.2.6.3. Evaluation of biofilm formation at the membrane's surface

The biofilm formation on the membrane's surface was characterized through SEM analysis. Samples of the produced membranes with ~1 cm diameter were placed on the surface of *S.aureus* or *P.aeruginosa* inoculated plates (1 x 10^8 CFU/ mL) and incubated at 37ºC. After 24 h, samples were gently removed from the plate, fixed with 2.5% (v/v) glutaraldehyde for 30 min, and then freeze-dried for 6h. Lastly, all samples were prepared for SEM analysis (see further details in section 2.2.4.1).

2.2.7. Statistical analysis

The statistical analysis of the obtained results was performed using one-way analysis of variance (ANOVA), with the Newman-Keuls post hoc test. A *p* value lower than 0.05 ($p<0.05$) was considered statistically significant.

Chapter III – Results and Discussion

3. Results and Discussion

3.1. Characterization of the produced MRPs

The MR is a non-enzymatic browning reaction involving the condensation between a carbonyl group of reducing sugars, aldehydes or ketones, and an amino group of amino acids, proteins or peptides, resulting in the production of MRPs, known as melanoidins [194, 195]. Herein, two sugars–amino acid model systems, glucose-arginine and fructose-arginine, were produced through this reaction.

ATR-FTIR spectra of the MRPs were acquired and their characteristic peaks, essentially composed of furans accompanied by carbonyl compounds, pyrroles, pyrazines and pyridines, are visualized in Figure 11 [217]. The spectra of GA and FA MRPs display the characteristics peaks of furans: CH stretching near 3000 cm^{-1} (band I); ring stretching between 1600 cm^{-1} and 1400 cm^{-1} (band II); CH in-plane deformation at 1300 - 1000 cm^{-1} (band III); and CH out-of-plane deformations and ring deformations bellow 1000 cm^{-1} (band IV) [218]. The presence of these peaks and the loss and/or decreased intensity of the characteristic peaks of the reagents, indicates a successful MRPs production.

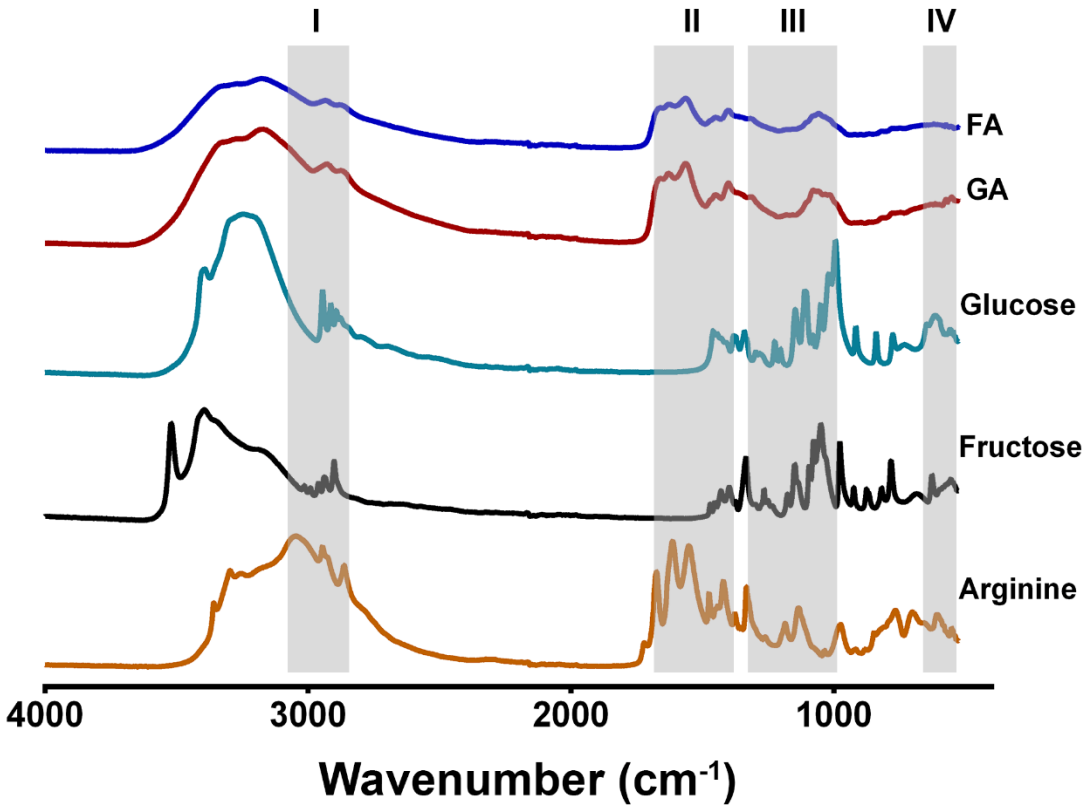

Figure 11. ATR-FTIR spectra of all the components used in the MR (glucose, fructose and arginine) and of GA/FA MRPs obtained at the end of the reaction.

3.2. Characterization of the produced membranes' morphology

The morphology and the diameter of the electrospun nanofibers were assessed through SEM analysis (Figure 12). The PCL membrane presents a highly porous 3D nanofiber network composed of fibers with average diameters of 399.6 ± 119.8 nm. This result is in agreement with the data previously reported by Zhang *et al.*, who also reported a similar diameter for PCL nanofibers [219]. In turn, the PCL_GA and PCL_FA membranes presented a mean diameter of 605.7 ± 322.9 nm and 458.3 ± 232.1 nm, respectively. These results reveal that the incorporation of the GA and FA MRPs into PCL nanofibers leads to the production of thicker fibers, that are responsible for lower fiber packing density as well as an enhanced porosity. Such morphologic characteristics avoid fluid accumulation, allow a higher moisture vapor transmission, and ultimately improve cell adhesion and proliferation [220].

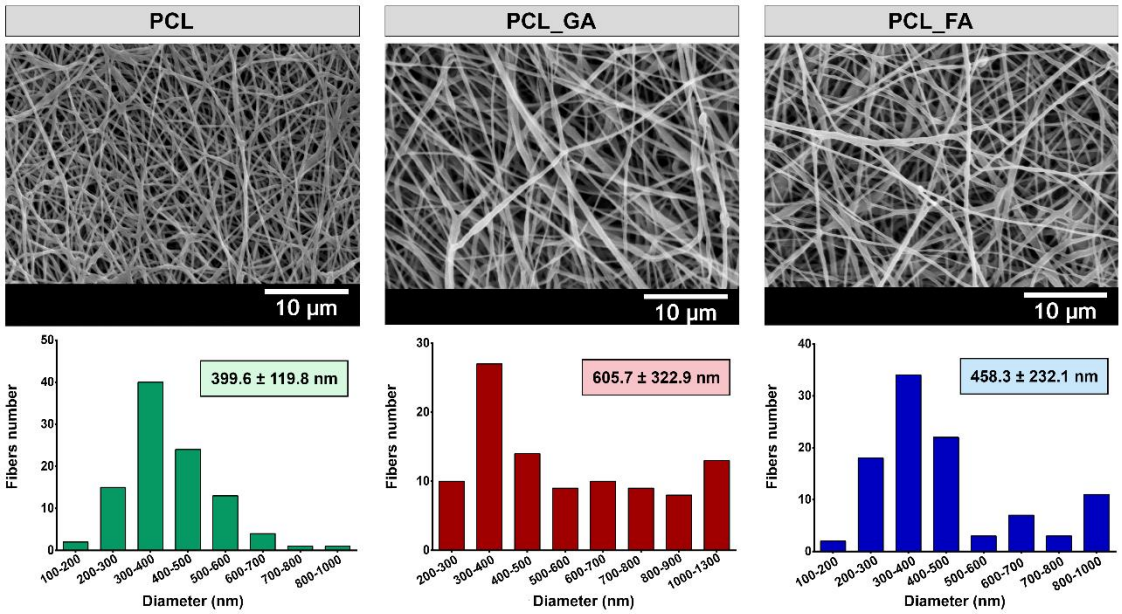

Figure 12. Characterization of the morphologic properties of the produced membranes. SEM images and fibers' diameters distribution of the PCL, PCL_GA and PCL_FA membranes.

3.3. Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopic analysis

The ATR-FTIR spectra of the PCL, PCL_GA and PCL_FA membranes are shown in Figure 13. All the acquired spectra present the characteristic peaks of PCL, at 2942 cm^{-1} (asymmetric CH_2 stretching) and 1723 cm^{-1} (carbonyl stretching) [221, 222]. Furthermore, the spectra of PCL_GA and PCL_FA present peaks near 3000 cm^{-1} (band I) and between 1600 cm^{-1} and 1400 cm^{-1} (band II) representing the CH stretching and ring stretching of the MRPs, respectively [218]. The presence of these peaks, demonstrates the successful blending of the MRPs with PCL polymer.

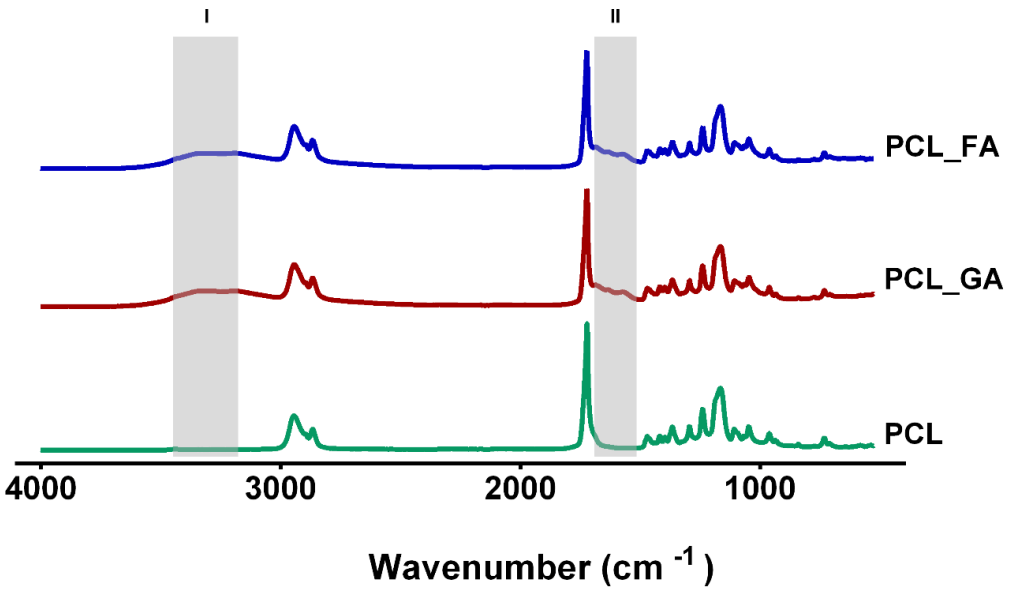

Figure 13. ATR-FTIR spectra of the produced membranes.

3.4. Characterization of the membranes' mechanical properties

In this study, Young's modulus, tensile strength and elongation at break of the produced nanofibrous membranes were characterized, both in wet and dry conditions [223].

The obtained results showed that the PCL membranes exhibited a Young Modulus of 21.32 ± 1.10 MPa in dry state, whereas in wet conditions a value of 24.19 ± 2.21 MPa was obtained. Such decreased in the elasticity was also observed for PCL_GA membranes (25.73 ± 4.03 MPa and 31.82 ± 2.72 MPa for dry and wet state, respectively). In contrast, PCL_FA membranes presented an increase of the elasticity, i.e. 22.03 ± 10.79 MPa and 15.41 ± 9.46 MPa for dry and wet conditions, respectively. In relation to the tensile strength, in dry conditions, PCL membranes presented a lower value in comparison with MRPs-modified PCL membranes, i.e., 3.43 ± 0.82 MPa, 13.16 ± 1.68 MPa and 13.55 ± 3.89 MPa for PCL, PCL_GA and PCL_FA, respectively. A similar trend was observed in wet conditions, where the PCL membranes displayed tensile strength values of 4.41± 0.89 MPa, whereas the PCL_GA and PCL_FA membranes exhibited values of 5.67 ± 0.01 MPa and 7.80 ± 2.98 MPa, respectively. On the other hand, the elongation at break assays revealed that PCL, PCL_GA and PCL_FA membranes can bear a strain of 16.23 ± 3.59%, 61.35 ± 3.48% and 86 ± 6.96% in dry state and 19.29 ± 2.15%, 101.95 ± 0.02%, 111.39 ± 3.09% in wet conditions, correspondingly.

The results presented in Table 5 show that MRPs-modified PCL membranes display better mechanical properties than non-modified PCL, i.e. they present a higher elasticity, tensile strength and elongation at break. In addition, PCL_GA and PCL_FA membranes exhibited mechanical properties, both in wet and dry state, that are similar to those displayed by the native skin (Young Modulus (4.6–20.0 MPa), Tensile strength (5.00–30.00 MPa) and Elongation at

break (35.00–115.00%)), allowing them to support cell proliferation, differentiation as well as new tissue formation [224].

Table 5. Mechanical properties exhibited by the produced membranes and the native human skin [208].

		Young Modulus (MPa)	Tensile Strength (MPa)	Elongation at break (%)
PCL	Dry	21.32 ± 1.10	3.43 ± 0.82	16.23 ± 3.59
	Wet	24.19 ± 2.21	4.41 ± 0.89	19.29 ± 2.15
PCL_GA	Dry	25.73 ± 4.03	13.16 ± 1.68	61.35 ± 3.48
	Wet	31.82 ± 2.72	5.67 ± 0.01	101.95 ± 0.02
PCL_FA	Dry	22.03 ± 10.79	13.55 ± 3.89	86 ± 6.96
	Wet	15.41 ± 9.46	7.80 ± 2.98	111.39 ± 3.09
Native skin		4.6-20	5-30	35-115

3.5. Evaluation of the membranes' porosity

In tissue regeneration the biomaterials' porosity has a direct impact on their performance [225]. The porous 3D architecture presented by the produced nanofibers meshes provide pathways for gases and fluids exchange at the wound site. In addition, the void spaces provide a substrate for cell accommodation, infiltration and proliferation, events that are essential for an effective healing process occurs [220, 226, 227].

The data presented in Figure 14A discloses that PCL membrane has the lowest porosity (86.1 ± 4.64%), whereas, PCL_FA membrane showed the highest porosity (100.14 ± 0.37%), which is in agreement with the higher number of void spaces available between nanofibers as previously depicted in SEM images. According to the data available in literature, materials with porosities above 90% are considered as ideal for promoting cell adhesion and proliferation, nutrient exchange as well as production of the new ECM [228-232].

3.6. Characterization of the membranes' swelling profile

During the healing process, the accumulation of exudate at the wound site occurs as a consequence of the body inflammatory response. However, when excessive amounts of exudate become accumulated at the wound area, tissue maceration, impaired cellular migration within the wound bed, defective tissue replacement and skin infection can occur [233, 234]. In this way, the swelling capacity of membranes aimed to be used as dressings must be characterized in order to check their capacity to absorb the wound exudate.

Herein, the swelling profile was analyzed by incubating the produced membranes in a PBS solution and at specific time points determine the samples weight variation (Figure 14B). The obtained results showed that the modified PCL membranes have a higher swelling capacity in comparison to non-modified membranes, which is essential for an improved wound exudate absorption occur. The higher water absorption capacity displayed by PCL_FA membranes is attributed to the hydrophilic nature of fructose (highly polar molecule with many hydroxyl groups) and arginine (polar hydrophilic charged amino acid) [189, 235, 236].

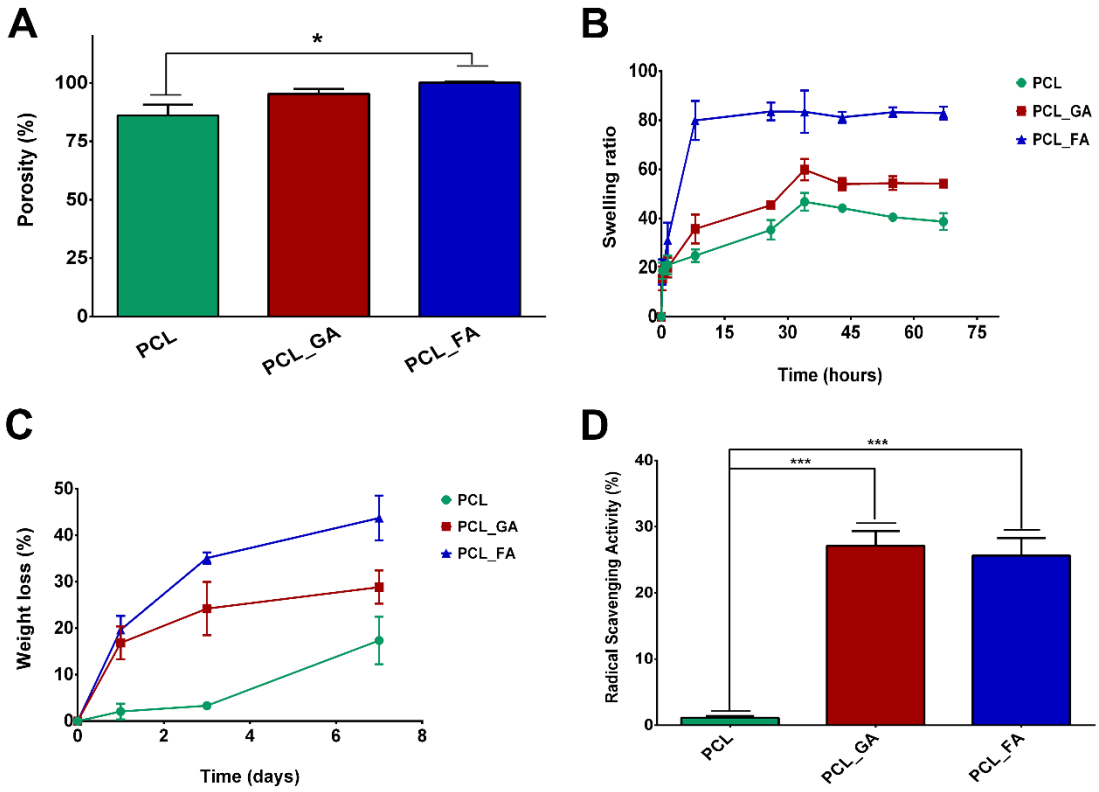

Figure 14. Characterization of the total porosity (A), swelling profile (B), weight loss (C), and antioxidant activity (D) of the produced membranes at different time points (each result is the mean ± standard deviation, n = 5, *p < 0.05 and ***p < 0.0001).

3.7. Characterization of the membranes' degradation profile

Despite of the progress that has been made in the area of tissue engineering, most of the commercially available wound dressings are non-degradable and need to be replaced/removed periodically from the wound, causing scar tissue formation and increasing the risk of bacterial contamination [237, 238]. To surpass such drawbacks, researchers are currently developing biodegradable dressings that protect the wound, allow cell migration and adhesion, and degrade into non-toxic products [239].

In this study, the degradation profile of PCL was adjusted by blending it with the MRPs (Figure 14C). After 7 days of incubation in PBS, PCL_GA and PCL_FA membranes presented a weight loss of 28.8 ± 3.56% and 43.7 ± 4.83%, respectively, while the PCL membrane only lost 17.4 ±

5.10% of the initial weight. The obtained results demonstrate that PCL degradation was greatly improved due to the hydrophilic nature of the MRPs, i.e. MRPs-modified PCL membranes degradation profile presented a weight loss three times greater in comparison to PCL membranes. A similar effect was reported by Ponjavic *et al.*, when PCL was blend to water soluble polyethylene oxide [240].

3.8. Assessment of the membranes' contact angle

Materials surface properties are a critical parameter that affects host response to the implanted material and cell adhesion, proliferation and differentiation, hence the wound healing process [241, 242]. According to literature, cell adhesion and proliferation are more favorable on moderate hydrophilic substrates (40º < WCA < 70º) than on hydrophobic (WCA > 90º) or very hydrophilic surfaces (WCA < 20º) [237, 243]. In this way, the WCA was determined to verify the wettability's surface of PCL, PCL_GA and PCL_FA membranes.

The PCL membrane exhibited a WCA value of 106.2 ± 7.06º, revealing a hydrophobic character, due to the presence of aliphatic polyester PCL [244, 245]. On the other hand, PCL_GA and PCL_FA membranes presented WCA values of 73.4 ± 6.91º and 54.5 ± 5.25º, respectively, thus revealing a moderate hydrophilic character. Such is explained by the hydrophilic character of glucose and fructose (rich in hydroxyl groups) and arginine (polar hydrophilic charged amino acid) [189, 235, 236]. Therefore, the MPRs-modified PCL membranes are able to provide a moist environment that supports cell attachment and proliferation, specifically PCL_FA membranes (see Section 3.11).

3.9. Water Vapor Transmission Rate

WVTR was performed to assess membranes' capacity to provide an appropriate moisture to the wound site. PCL membrane displays a WTVR of 1828.6 ± 162.31 mL/m^2/day, whereas PCL_GA and PCL_FA membranes present a WVTR of 2043.3 ± 160.23 mL/m^2/day and 2107.3 ± 72.20 mL/m^2/day, correspondingly. These results show that the incorporation of MRPs into the membranes enhanced their water vapor transmission rate. In literature, wound dressings with WVTR values between 2000 – 2500 mL/m^2/day are reported as the most efficient for maintaining a moist environment at the wound site, as well as allowing water vapor exchanges [246, 247]. Therefore, the WVTR values obtained for PCL_GA and PCL_FA membranes are within the range considered as optimal for being used as wound dressings.

3.10. Determination of the membranes' DPPH radical scavenging activity

During the inflammatory phase of the healing process, neutrophils and macrophages are attracted to the wound site to produce large amounts of ROS, which play an essential role in protecting the wound from invading bacteria [248]. Furthermore, ROS are also involved in angiogenesis [249], re-epithelization [250, 251] and in cell migration and proliferation [252]. However, a high concentration of ROS leads to the inhibition of keratinocytes migration and proliferation, and induce severe tissue damage [253]. Therefore, wound dressings with antioxidant activity can improve the wound healing process [254].

Herein, the produced membranes were incubated with a solution containing DPPH radicals and then their radical scavenging activity was determined (Figure 14D). PCL nanofibers exhibit a radical scavenging activity of 1.11 ± 0.29%, whereas PCL_GA and PCL_FA nanofibers reveal higher values (27.1 ± 2.24% and 25.6 ± 2.64%, respectively). These results are in agreement with data available in literature reporting the high scavenging activity displayed by GA/FA MRPs [255, 256].

3.11. Evaluation of the cytotoxic profile of the produced membranes

Wound healing is a dynamic process that involves the integrated action of different cell types [257]. Fibroblasts have a critical role in wound healing since they are involved in key processes such as, breaking down the fibrin clot, re-epithelization, production of new ECM components, as well as contraction of the wound [257-259]. In this study, the cytotoxic profile of the produced membranes was evaluated through Optic microscopy, dsDNA quantification, SEM analysis, MTS and Live/Dead assays. Optical microscopic images of NHDF cells in contact with the membranes after 1, 3 and 7 days were acquired (Figure 15). The data obtained reveals that NHDF cells did not suffer any morphological variations when in contact with the produced membranes. Additionally, the results obtained in the MTS assay show that the produced membranes did not induce any cytotoxic effect on NHDF cells, over 7 days, confirming that the incorporation of MRPs in the PCL nanofibers did not affect their biocompatibility (Figure 16A). Moreover, no significant differences were noticed in DNA content of cells incubated with the produced membranes and those of the control group (Figure 16B). The SEM images show that after 7 days, cells adhered to the surface of all the produced membranes (Figure 16C).

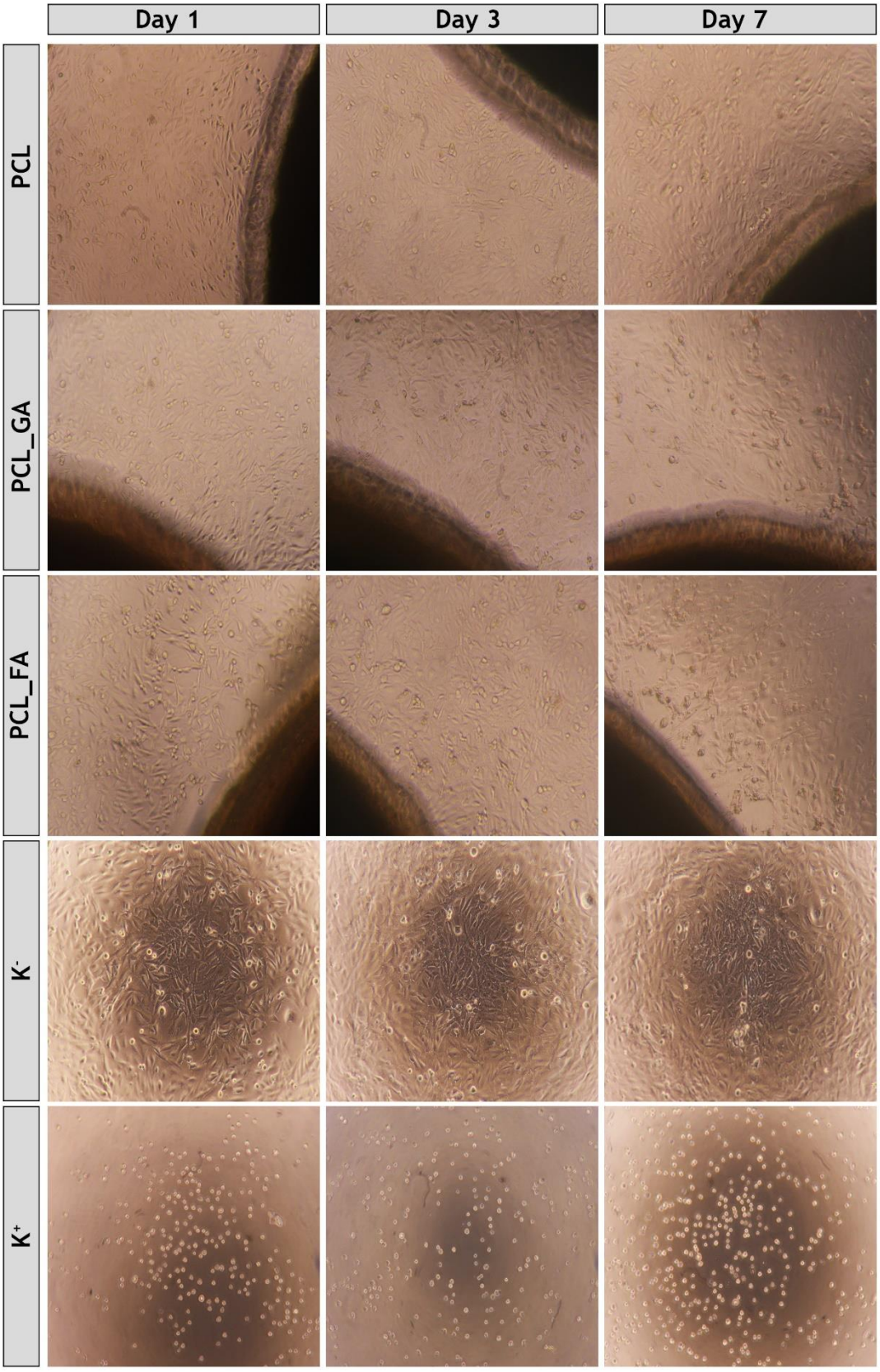

Figure 15. Optical microscopy images of NHDF in contact with the produced membranes after 1, 3 and 7 days of incubation; K^- (negative control); K^+ (positive control).

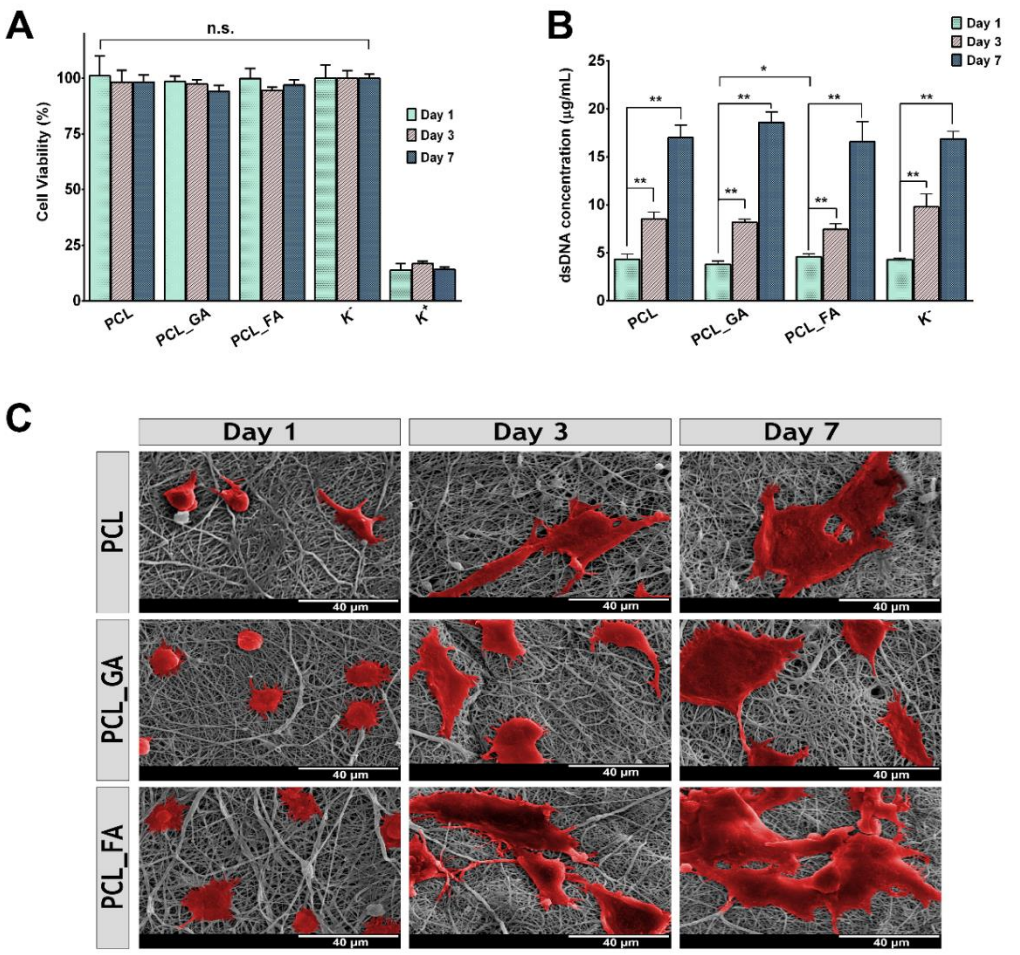

Figure 16. Characterization of the biological performance of the produced membranes. NHDF cell viability (A) and dsDNA content (B) was evaluated after 1, 3 and 7 days of cells incubation (each result is the mean ± standard deviation, n = 5, *p < 0.05 and **p < 0.001). SEM images of NHDF cells morphology at the surface of the electrospun membranes after 1, 3, and 7 days are presented in (C).

The Live/Dead assay allows the visualization of cell survival onto the membranes surface, by stained live and dead cells with Calcein and Propidium iodine, respectively. Calcein is a non-fluorescent dye that is converted to a green-fluorescent calcein by intracellular esterases, present in live cells. In turn, Propidium iodine enters to cells with damaged membranes, and binds to nucleic acids producing a bright red fluorescence. In this way, the viable NHDFs (green labeled) and dead NHDFs (red labeled) were visualized by fluorescence microscopy analysis. As shown in Figure 17, at all culture times, the viable fibroblasts cells (stained green) present a uniform cell distribution throughout the produced membranes. Specifically, after 7 days, the results show that the membranes did not have any cytotoxic effect on cells' viability, thus confirming the results obtained in the MTS assay.

The PCL_FA membrane revealed to be the most effective formulation, as NHDF cells proliferated and reached a confluent monolayer faster than those cells in contact with PCL and PCL_GA membranes, thus confirming the results obtained in SEM images. These results can be explained by the hydrophilic character displayed by this membrane (see section 3.8) which improves cell attachment and subsequent proliferation [260].

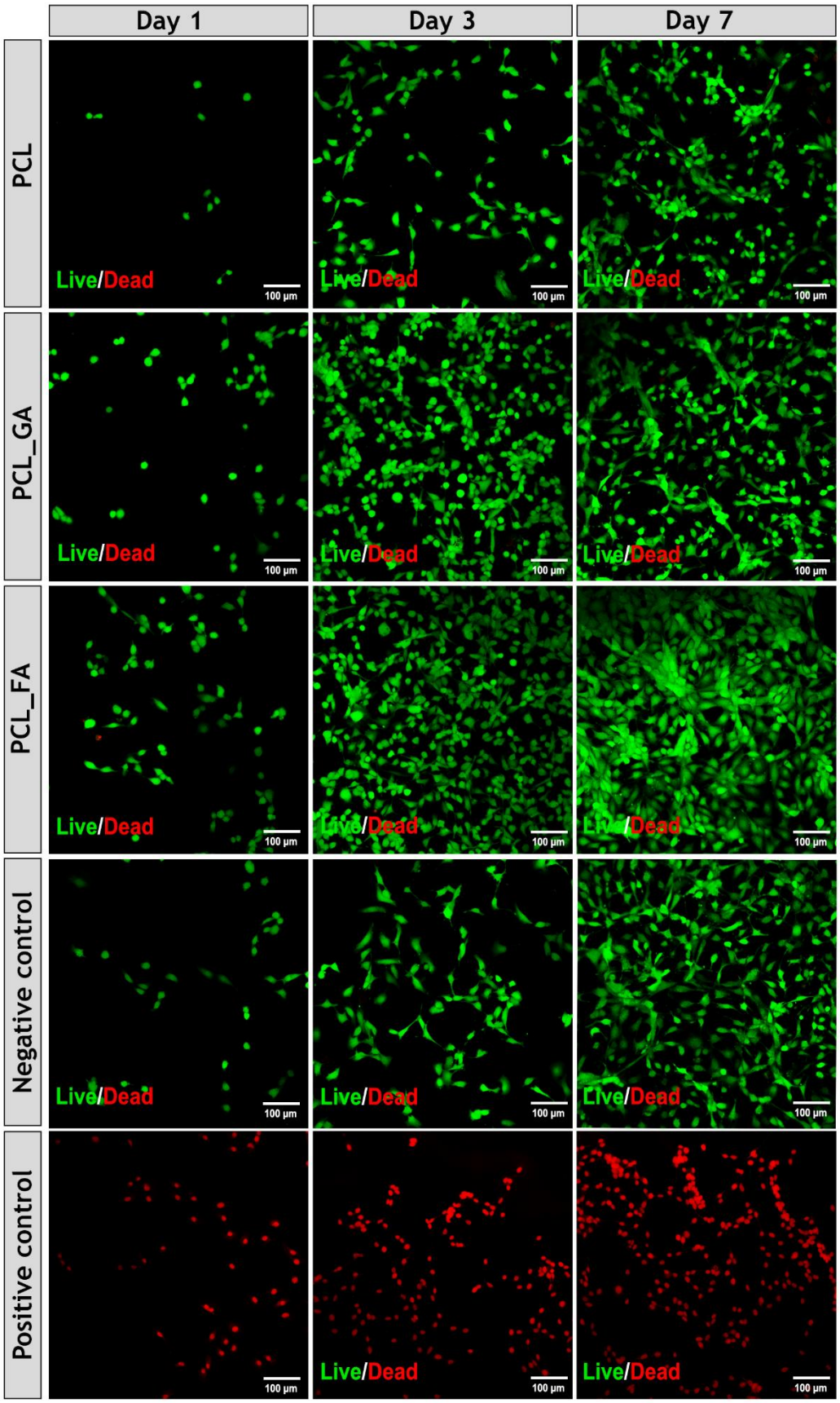

Figure 17. Fluorescence microscopy images of fibroblast cells cultured on the surface of the produced membranes after, 1, 3 and 7 days. Green channel: viable cells labeled with Calcein; red channel: dead cells stained with Propidium iodine.

3.12. Characterization of the membranes' antimicrobial properties

Bacterial contamination of skin wounds impairs the healing process, leading to high rates of morbidity and mortality. Once bacteria become adhered to the wound surface, a cascade of events leads to the formation of biofilms [261]. Hence, to prevent biofilm formation and consequently avoid wound infection it is crucial that new wound dressings displaying antimicrobial activity are developed. So far, different strategies, including materials' surface functionalization or antimicrobial agents incorporation (e.g. antibiotics, nanoparticles and natural products), have been used to confer bactericidal activity to the dressings [262]. In the present study, the antibacterial properties of the produced membranes were evaluated using *S.aureus* and *P.aeruginosa* as model bacteria for gram-positive and gram-negative bacteria, respectively. These bacterial strains were considered appropriate for this assay, since they are the most common pathogens found in skin infections [263].

In Figure 18A and 18B are presented the inhibitory areas exhibited by the nanofibers against *S.aureus* and *P.aeruginosa*, respectively. The attained results show that MRPs-modified PCL membranes have a higher bactericidal activity than the unmodified PCL membrane. PCL_GA and PCL_FA membranes present an inhibitory effect of ~13% and ~24% for *S.aureus* and ~15% and ~28% for *P.aeruginosa*, respectively. Furthermore, the SEM images (Figure 18C) show that no biofilm formation occurred at the surface of the PCL_GA and PCL_FA membranes. In contrast, a great number of bacterial colonies were observed at the PCL surface.

Additionally, the capacity of the membranes to inhibit bacterial growth and provide an aseptic environment at the wound site was also evaluated. All the produced membranes were able to decrease the bacterial growth along time (see Figure 19A and 19B). Among them, the PCL_FA membrane displayed the highest activity, i.e. it reduces the growth of *S.aureus* and *P.aeruginosa* in ~50%.

All the results obtained in the antibacterial assays highlight the potential of MRPs, especially those membranes containing FA MRPs, to be used in skin regeneration. Up to now, several mechanisms have been proposed to explain the antibacterial effect of the MRPs [200]. The most accepted mechanism proposes that MRPs antimicrobial activity, specifically melanoidins, results from its capacity to bind to iron ions [264]. Several studies, have described that MRPs, which act as anionic hydrophilic compounds, are prone to bind and form stable complexes with ferric ion (Fe^{3+}) [205]. This ability, disturbs the bioavailability of iron extracellular medium, which has a direct impact on cellular processes, namely ATP generation, DNA replication, cell growth, proliferation and respiration in bacteria [206].

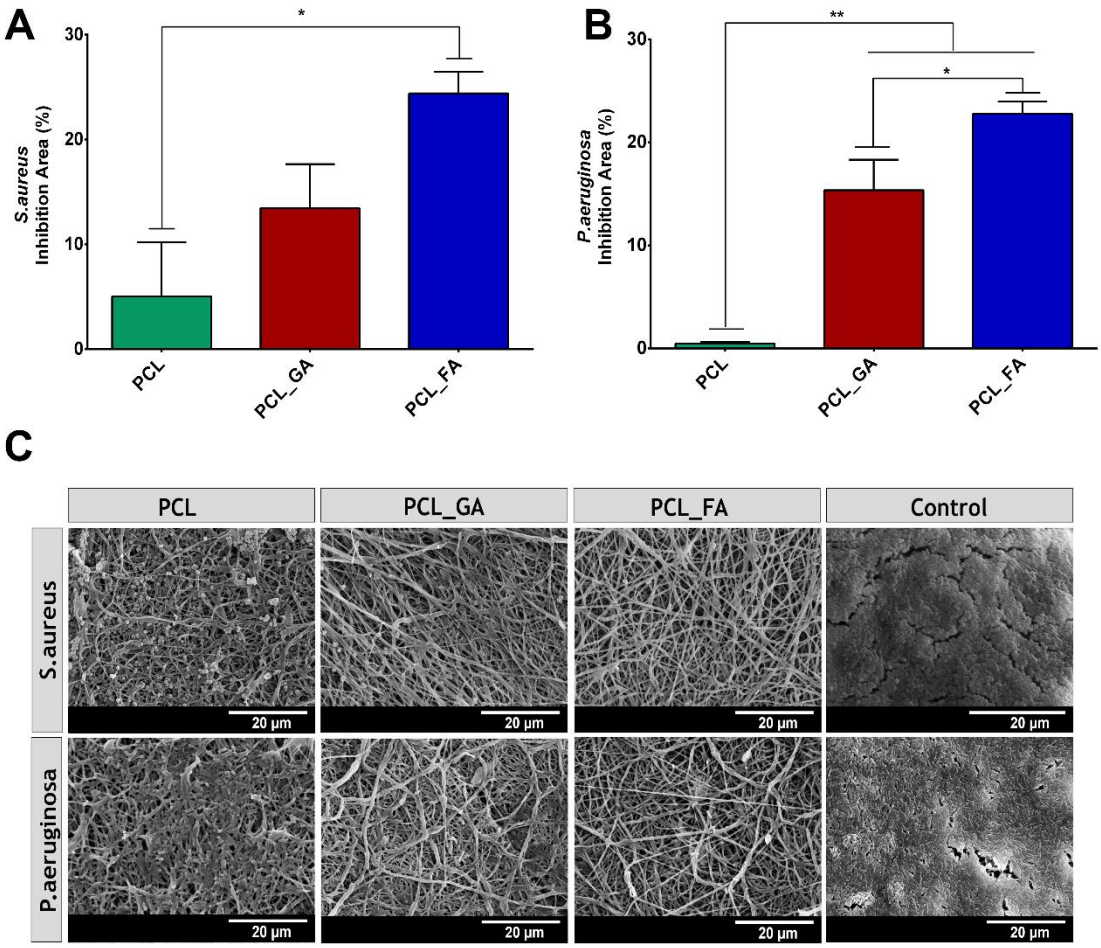

Figure 18. Characterization of the bactericidal activity of the produced membranes. The bactericidal activity of the PCL, PCL_GA and PCL_FA membranes against *S.aureus* and *P.aeruginosa* are shown in (A) and (B), respectively (each result is the mean ± standard deviation, n = 5, *p < 0.05 and **p < 0,001); SEM images to characterize bacteria growth at the surface of the produced membranes are presented in (C).

Furthermore, MRPs can also interfere with cell integrity and permeate cell membranes by removing magnesium cations from the cell membrane [200]. In turn, hydrogen peroxide, produced during the MR, can act as an important contributor to the antimicrobial activity of the MRPs, i.e. it reacts and disrupt cell wall, lipids, proteins and nucleic acids in bacteria [169, 170, 207]. In Figure 19C, are illustrated the strategies that bacteria use to obtain iron (C_1) and all the mechanisms by which MRPs performed their bactericidal effect (C_2).

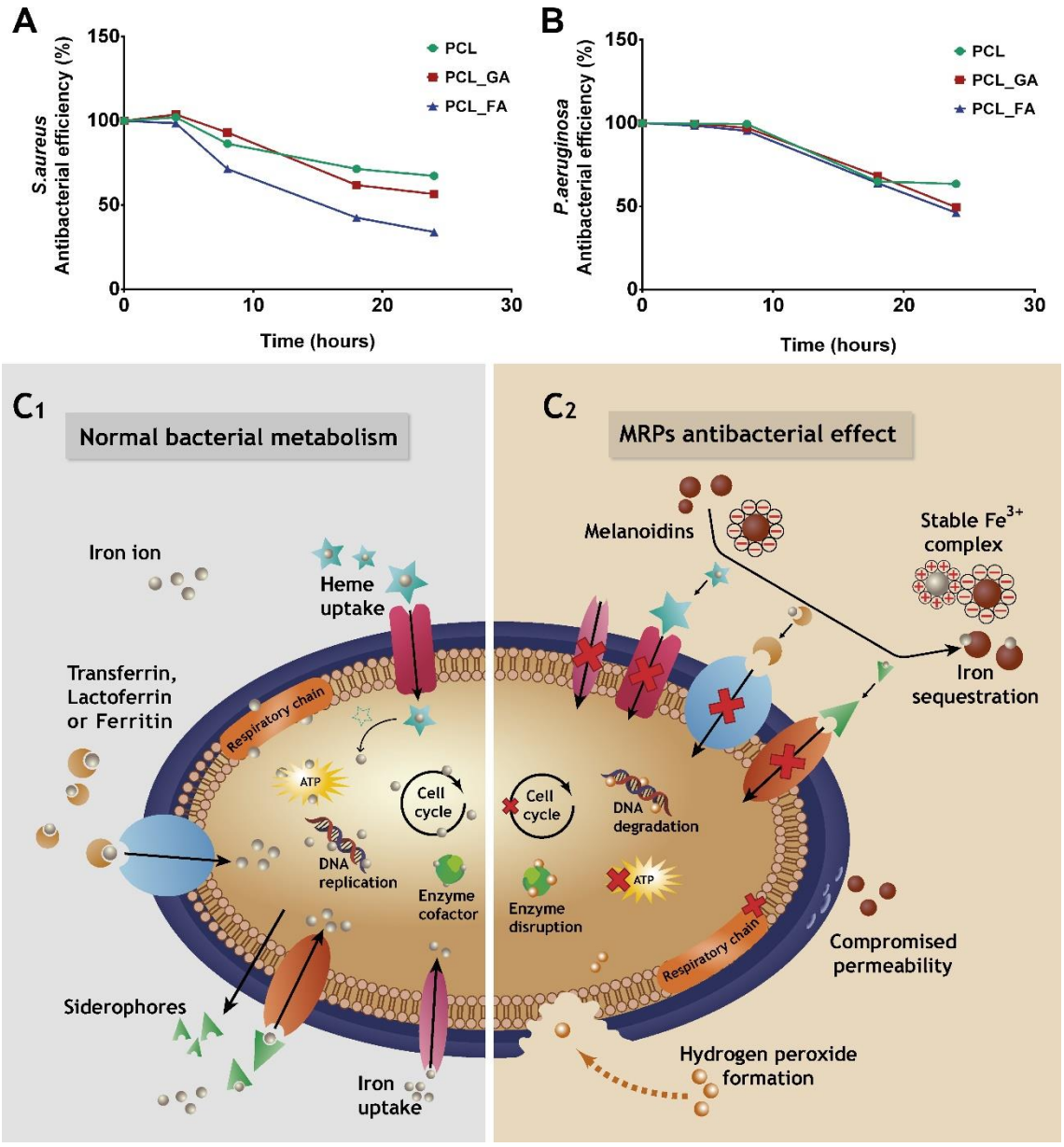

Figure 19. Evaluation of the antibacterial activity of the produced membranes against *S.aureus* (A) and *P.aeruginosa* (B) as function of time. Representation of the bacterial metabolism under normal conditions (C_1) and in the presence of MRPs (C_2).

Chapter IV - Conclusion

4. Conclusion

Nowadays, nearly everyone has already suffered an open skin wound as a consequence of a cut, burn, diseases (e.g. diabetes) or surgical interventions. In some circumstances those wounds can easily be contaminated by different pathogens found in the surrounding environment, endogenous microbes living in the mucous membranes, or by the microflora available on the adjacent skin. Gram-positive bacteria, like *E.coli* and *P.aeruginosa*, and gram-negative bacteria, such as *S.aureus*, are the predominant pathogens responsible for skin contamination and subsequent infections. Wound dressings displaying antimicrobial activity started to be produced in order to avoid this health problem. In this thesis, electrospun PCL membranes modified with MRPs were produced and then their suitability for being applied as wound dressings was assessed. The incorporation of MRPs into PCL nanofibers allowed the production of membranes with the required mechanical features, wettability and porosity. Such properties allow them to absorb wound exudate, keep a moist environment as well as perform nutrients and gas exchange. Furthermore, MRPs-modified PCL membranes were successfully able to inhibit *S.aureus* and *P.aeruginosa* growth, without compromising fibroblast morphology or viability. The PCL_FA membrane revealed to be the most effective formulation.

In a near future, the incorporation of bioactive molecules (e.g. growth factors, proteins, enzymes and other biomolecules) and cells (e.g. fibroblasts, keratinocytes, endothelial and stem cells) may improve even further the healing capacity of these PCL based dressings.

Chapter V - Bibliography

5. Bibliography

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Appendix

1. Simões, D., Miguel, S. P., Ribeiro, M. P., Coutinho, P., Mendonça, A. G., & Correia, I. J. (2018). Recent advances on antimicrobial wound dressing: A review. *European Journal of Pharmaceutics and Biopharmaceutics*. *127*, 130-141.

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Review article

Recent advances on antimicrobial wound dressing: A review

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ABSTRACT

Skin and soft tissue infections (SSTIs) have high rates of morbidity and mortality associated. Despite the successful treatment of some SSTIs, those affecting the subcutaneous tissue, fascia, or muscle delay the healing process and can lead to life-threatening conditions. Therefore, more effective treatments are required to deal with such pathological situations. Recently, wound dressings loaded with antimicrobial agents emerged as viable options to reduce wound bacterial colonization and infection, in order to improve the healing process. In this review, an overview of the most prominent antibacterial agents incorporated in wound dressings along with their mode of action is provided. Furthermore, the recent advances in the therapeutic approaches used in the clinic and some future perspectives regarding antibacterial wound dressings are also discussed.

1. Introduction

Skin is the largest and outermost organ that covers the entire body. Therefore, above all, skiń s primary function is to protect underlying muscles, bones, ligaments and internal organs from external biological, chemical, mechanical and physical agents [1,2]. Furthermore, skin is also involved in sensation, temperature regulation, immunological surveillance, prevention of water loss (dehydration) and synthesis of vitamin D3 [3]. However, the structure and functions performed by this organ can be affected by cuts, burns, surgical incisions or illnesses, such as diabetes [4]. After skin structure be compromised, its structure and functions must be re-established, as soon as possible to ensure the body homeostasis. To accomplish that, the wound healing process begins almost immediately after a skin injury occurs, in order to avoid the risk of bacterial contamination [5]. Non-healing wounds usually appear after this type of contamination occur [4].

Skin and soft tissue infections (SSTIs) are the most common types of infections and they affect approximately 14 million people every year in the United States [6,7]. Depending on the etiology and severity of the microbial invasion, SSTIs can range from minor superficial to life-threatening infections [8]. In the initial stage of the infectious process, gram-positive organisms such as *Staphylococcus aureus* (*S. aureus*) and *Streptococcus pyogenes* (*S. pyogenes*) are the dominant organisms involved, while gram-negative organisms like *Escherichia coli (E. coli)* and *Pseudomonas aeruginosa (P. aeruginosa)* are only found in later stages of the process, i.e. when a chronic wound is developed [7].

In a healthy human being, infection is avoided, by activating the immune system for abolishing the invading pathogens. In this process, macrophages initiate the migration to the wound site and subsequently perform phagocytosis of the pathogens (which are destroyed in a phagolysosome or by nitric oxide production). In a later stage of infection, the immune response is performed by the activation of lymphocytes T helper which secrete interferon-γ and CD40 ligand to coordinate the immune adaptive and humoral response to kill and remove the invading

Abbreviations: A. iwoffii, *Acinetobacter iwoffii*; AMPS-Na$^+$, 2-acrylamido-2-methylpropane sulfonic acid sodium salt; *B. cereus*, *Bacillus cereus*; *B. subtilis*, *Bacillus subtilis*; BC, Bacterial cellulose; CA, Cellulose Acetate; *C. freundii*, Citrobacter freundii; CMCS, Carboxymethyl Chitosan; CMGG, Carboxymethyl Guar Gum; CS, Chitosan; DHBA, 2,3-dihydroxybenzoic acid; *E.aerogenes*, *Enterobacter aerogenes*; *E.coli*, *Escherichia coli*; EDA, Ethylenediamine; *E. faecalis*, *Enterococcusi faecalis*; GMs, Gelatin Microspheres; HNTs, Halloysite Nanotubes; HA, Hyaluronic acid; *K. pneumoniae*, *Klebsiella pneumoniae*; MMSA, Methicillin susceptible *Staphylococcus aureus*; MRSA, Methicillin resistant *Staphylococcus aureus*; nAg, nano silver; NIPAAm, N-isopropyl acrylamide; OAlg, Oxidized Alginate; *P. aeruginosa*, *Pseudomonas aeruginosa*; PCD, β-cyclodextrin polymer; PCL, Polycaprolactone; PEI, Polyethyleneimine; PEO, Polyethylene oxide; PHEA, Poly(2-hydroxyethylacrylate); PLA, Poly(lactic acid); PLGA, Polylactic-co-glycolic acid; Plur, Pluronic F127; *P. mendocina*, *Pseudomonas mendocina*; PP, Polypropylene; PRP, Platelet rich-plasma; PSSA-MA, Poly(styrene sulfonic acid-co-maleic acid); PU, Polyurethane; PVA, Polyvinyl alcohol; PVP, Polyvinylpyrrolidone; *P. vulgaris*, *Proteus vulgaris*; SA, Sodium Alginate; *S. aureus*, *Staphylococcus aureus*; *S. epidermidis*, *Staphylococcus epidermidis*; *S. haemolyticus*, *Staphylococcus haemolyticus*; *S. pyogenes*, *Streptococcus pyogenes*; *S. typhi*, *Salmonella typhi*; *S. typhimurium*, *Salmonella typhimurium*; SF, Silk Fibroin; *V. vulnificus*, *Vibrio vulnificus*; ZN, Zein

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bacteria [9]. However, if the immune system is not able to remove the pathogen, infection occurs and causes the deterioration of granulation tissue, growth factors and extracellular matrix components (collagen, elastin and fibrin), thus compromising the normal wound healing process [10,11]. Therefore, it is fundamental to develop wound dressings that are capable of preventing bacteria penetration into the wound or avoid microorganisms' growth. To accomplish that, different approaches involving materials with intrinsic bactericidal activity, modified surface or incorporating antimicrobial agents, are being used to produce wound dressings displaying bactericidal activity [12].

Herein, an overview of the most prominent antibacterial agents incorporated in wound dressings along with their mode of action is provided. Furthermore, the recent advances in the therapeutic approaches used in the clinic and some future perspectives regarding antibacterial wound dressings are also discussed. Due to the higher prevalence of bacterial infections, this review does not provide any data concerning SSTIs caused by viral, fungal or parasites (protozoa, helminths, and ectoparasites).

2. Wound pathophysiology and the wound healing process

Wounds occur when a tissue is disrupted or the cellular integrity is compromised due to mechanical, physical or metabolism-related issues [13]. According to the duration and nature of the healing process, skin wounds can be classified as acute or chronic. An acute wound occurs suddenly, as a consequence of abrasions, avulsions, burns, incisions, lacerations and punctures, and have associated an healing time that is dependent on the size and number of layers of skin that have been affected [12,14]. Under normal physiological conditions, the restoration of the epidermal structure is highly efficient, however when a chronic wound occur, it is characterized by displaying a defective healing process, that do not allow skin to be repaired in an orderly and timely manner [15]. Based on etiology, the Wound Healing Society classifies chronic wounds into four categories: pressure, diabetic, venous and arterial insufficiency ulcers [16]. Bacterial colonization usually occurs in chronic wounds and it is considered as a primary cause of chronic inflammation [17].

The healing process comprises a cascade of precisely synchronized events in which are involved both resident and migratory cell populations, extracellular matrix components and soluble mediators [18]. This process includes five distinct phases: hemostasis, inflammation, migration, proliferation and remodeling [19]. In the first phase, hemostasis, a fibrin clot is formed to prevent blood loss through vasoconstriction as well as to avoid microbial contamination [20]. The inflammatory phase begins almost simultaneously with hemostasis and it involves the recruitment of neutrophils (that engulf bacteria and decontaminate the wound through proteases and antimicrobial peptides secretion and by producing reactive oxygen intermediates), monocytes/macrophages (monocytes differentiate into macrophages to remove apoptotic neutrophils and other cells and secrete cytokines and multiple growth factors), and lymphocytes that exert a specific response against microbes (B–lymphocytes produce antibodies, while T–lymphocytes secrete cytokines involved in cytolytic activity) [20–22]. The migration and proliferative phases begin with fibroblast migration to the wound site and differentiation into myofibroblasts to produce extracellular matrix components like fibronectin, hyaluronic acid, collagen and proteoglycan, that are involved in the production of extracellular matrix (ECM), new blood vessels and re-epithelization [20]. Maturation, or remodeling, is the last stage of the wound healing process and in this phase all processes that were activated after injury are ceased [19].

In order to ensure an effective wound healing process, it is fundamental to maintain a controlled set conditions at the wound site (i.e. oxygenation, temperature and high availability of vitamins, minerals, and trace elements) that sustain the complex cellular activity during this process [23]. However, chronic wounds, burns, diabetic ulcers and post-surgical wounds have extended healing times and, in some cases, even fail. For example, burn wounds usually display high levels of exudate, which provides a moist and nutrient-rich environment that promotes bacterial growth, namely *Pseudomonas* species [24]. These bacteria produce virulence factors that mediate a number of processes like adhesion, nutrient acquisition, leucocyte killing and bloodstream invasion. Furthermore, these microorganisms are also able to produce endotoxins that promote pro-inflammatory cytokines expression, such as interleukin-1 and tumor necrosis factor-α, that ultimately lead to wound inflammation [25–27]. Wounds exhibiting an extended inflammation, show a high content of metalloproteinases (MMPs) that are involved in the degradation of ECM components, thus avoiding the formation of the granulation tissue and consequently delaying the healing [11,28].

On the other hand, patients suffering from Diabetes mellitus (DM) have an impaired protective sensation and altered pain response, which makes them vulnerable to trauma and extrinsic forces. Diabetic wounds are characterized by their dry and keratinized aspect, that usually crack or suffer fissures more easily, leading to an extended healing time [29]. Therefore, patients with DM are predisposed to cutaneous infections occur, namely those caused by *S. pyogenes* and *S. aureus* [30].

3. Wound dressings displaying antimicrobial activity

In 1987, Gristina came up with the expression "race for the surface" to describe the competition that occurs between cells and bacteria for colonize a surface. Bacteria are inherently favored in this event, due to is natural ability to colonize both biological and non-biological surfaces [31]. An open wound is a favorable niche for microbial colonization [32]. Generally, the majority of infected wounds present polymicrobial and are usually contaminated by pathogens found in the surrounding environment, i.e. endogenous microbes living in the mucous membranes, and by the microflora available on the adjacent skin [33]. In the initial stages of chronic wound formation, gram-positive organisms, specifically *S. aureus*, are predominant. In the later stages, gram-negative *E. coli* and *Pseudomonas* species are observed and tend to invade deeper layers of skin causing significant tissue damage. Furthermore, *Staphylococci* and *Streptococci* species are also found in 50% of chronic wounds [7].

Nowadays, bacterial contamination of skin wounds are responsible for the high rates of morbidity and mortality [34]. To address this health issue, different labs around the world started to develop antimicrobial wound dressings to prevent wound contamination [35]. The wound dressings develop up to now have been produced with different materials (synthetic or natural) and with various physical forms (sponges, hydrogels, hydrocolloids, films, membranes). These different formulations have distinct properties that make them suitable for the treatment of a particular type of wound. For example, sponges exhibit a huge porosity, provide thermal insulation and sustain a moist environment at the wound site. Nonetheless, the sponges are mechanically weak, may provoke skin maceration and are unsuitable for the treatment of third-degree burns or wounds with dry eschar [36,37]. On the other hand, hydrogels are characterized by their capacity to store high amounts of water within their 3D polymeric network, which allow them to provide a moist environment to the wound. However, hydrogels display weak mechanical properties, thus demanding a secondary dressing [38,39]. Furthermore, hydrocolloids are easily removed by saline or sterilized water, non-adherent, present high density and are painless dressings. Nevertheless, hydrocolloids display some disadvantages that may limit their use, i.e. they may be cytotoxic, display an unpleasant odor, present a low mechanically stability and maintain an acid pH at the wound site [40,41]. Films used as wound dressings are impermeable to bacteria, allow healing monitorization and are painless. However, this type of dressing are hard to handle, adhere to the wound bed and cause exudate accumulation [14,40]. In turn, membranes (specially electrospun membranes) are known to act as physical barriers

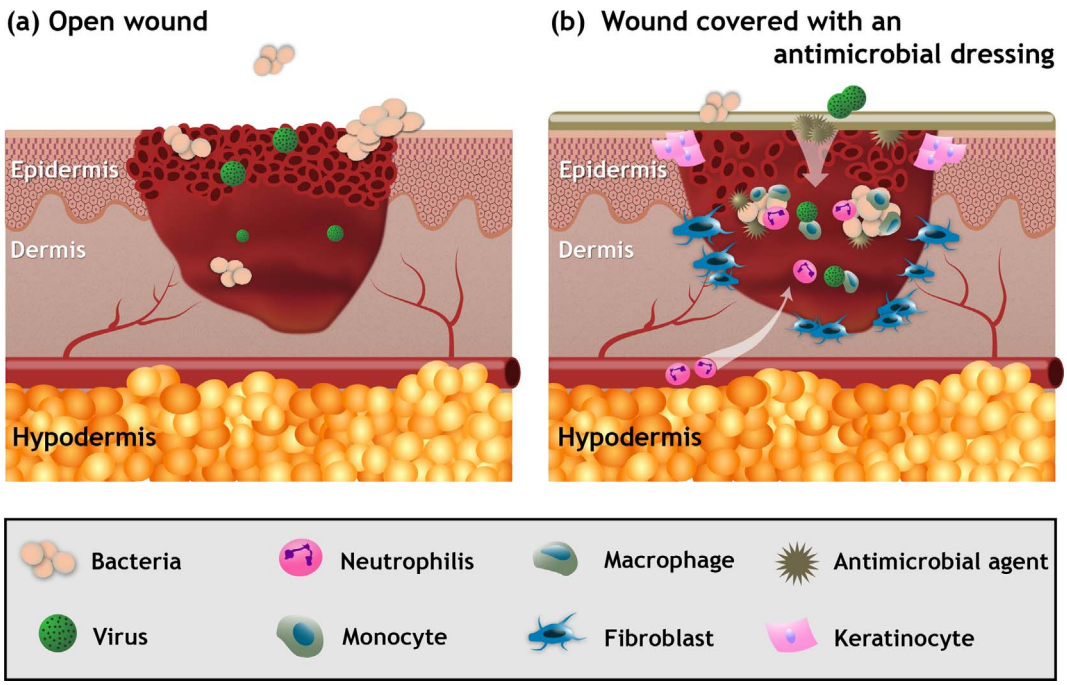

Fig. 1. Representation of the healing process in an open (a) and antimicrobial wound dressing covered (b) wound: The open wound is vulnerable to bacterial contamination, leading to an extended inflammatory phase and an increased expression of metalloproteinases that are involved in the degradation of ECM components and also inhibit the formation of new granulation tissue. When the antimicrobial dressing was used to cover the wound bed, it acts as a physical barrier to prevent pathogens entrance into the wound or to kill the invading microorganisms. In addition, the antimicrobial dressing supports the healing process by stimulating the immune system and fibroblast/keratinocyte migration.

as well as to reproduce the 3D architecture of native ECM. Moreover, their high surface-to-volume ratio and interconnected pores are crucial to assure cell proliferation, gas exchange, nutrient supply, and to control fluid loss. The main drawbacks associated with membrane use are originated by the materials and solvents used in their production [42,43].

So far, to improve dressing antimicrobial properties different agents have been incorporated within their structure. Those antimicrobial agents comprise essentially antibiotics (e.g. tetracycline, ciprofloxacin, gentamicin and sulfadiazine), nanoparticles (e.g. silver nanoparticles) and natural products (e.g. honey, essential oils and chitosan) [33]. A schematic representation of a polymeric antimicrobial dressing, designed to act as a physical barrier that protects the wound from microbial invasion and supports fibroblasts migration and differentiation, is presented in Fig. 1.

4. Antibacterial agents used to functionalize wound dressings

4.1. Antibiotics

The discovery of natural compounds that exhibit antimicrobial activity was a major breakthrough in the treatment of infectious diseases, like SSTIs [44]. Although thousands of antibiotics are known, less than 1% is currently in use in the clinic, due to toxicity related issues or lack of uptake by the host cells [45]. Up to now, only aminoglycosides [46], beta-lactams [47], glycopeptides [48–50], quinolones [51], sulphonamides [52,53] and tetracyclines [54,55] have been used to produce wound dressings displaying antimicrobial activity. The incorporated antibiotics can interfere with a function/feature of the bacteria structure or on their metabolic pathways through one of the following four mechanisms (as represented in Fig. 2):

1. Inhibition of bacterial cell wall synthesis: β-lactams and glycopeptides are among the classes of antibiotics that interfere specifically with the cell wall biosynthesis [56]. For example, β-lactams (including penicillins, carbapenems and cephalosporins) block the crosslinking of peptidoglycan units, by inhibiting the peptide bond formation reaction catalysed by Penicillin Binding Proteins (PBPs). Contrariwise, glycopeptides antibiotics (i.e. vancomycin) inhibit peptidoglycan synthesis, by binding peptidoglycan units and by blocking transglycosylase and PBPs activity [57]. Such events will impact on the shape of the bacteria and eventually lead to their lysis due to the high internal osmotic pressure [58].

2. Blockage of key metabolic pathways: The presence of folate pathway in many pathogenic microorganisms and its absence in mammals, has made this pathway an attractive target for antimicrobial drugs [59]. Some antibiotics mimic the folic acid structure (e.g. sulphonamides) and allow their competitive binding to bacterial enzymes. Such interferes with the production of DNA, RNA and proteins, leading to the disruption of bacteria proliferation [60].

3. Interference on protein synthesis: Antibiotics that interfere with protein synthesis can be divided into two subclasses: the 50S and the 30S inhibitors. According to the data available in literature, only the 30S inhibitors have been used to treat skin infections. Aminoglycosides (i.e. streptomycin) and tetracyclines are antibiotics that act as 30S ribosome-inhibitors, by obstructing the access of aminoacyl-tRNAs to the ribosome [60,61].

4. Inhibition of nucleic acids synthesis: Some antibiotics have the capacity to interfere with nucleic acid synthesis, by inhibiting the replication or transcription processes. Antibiotics that inhibit nucleic acid synthesis usually target topoisomerase II and topoisomerase IV of bacteria and RNA polymerase activity, thereby preventing the production of mRNA [56,60]. The quinolone group of synthetic antimicrobial drugs acts by converting their targets (DNA gyrase and topoisomerase IV), into enzymes that fragment the bacterial chromosome [62].

Among the different antibiotics incorporated so far in wound dressings, tetracycline [63], ciprofloxacin (CIP) [64,65], gentamicin [66] and sulfadiazine [52,67] have been the most used. García et al. developed films based on chitosan (CS), at concentration of 1% (w/v), and modified-hybrids CS-weisocyanate as functional carriers of CIP. The obtained results revealed that these films loaded with antibiotic were able to inhibit *S.aureus* and *P. aeruginosa* growth, and the films' inhibitory activity was proportional to the antibiotic content loaded

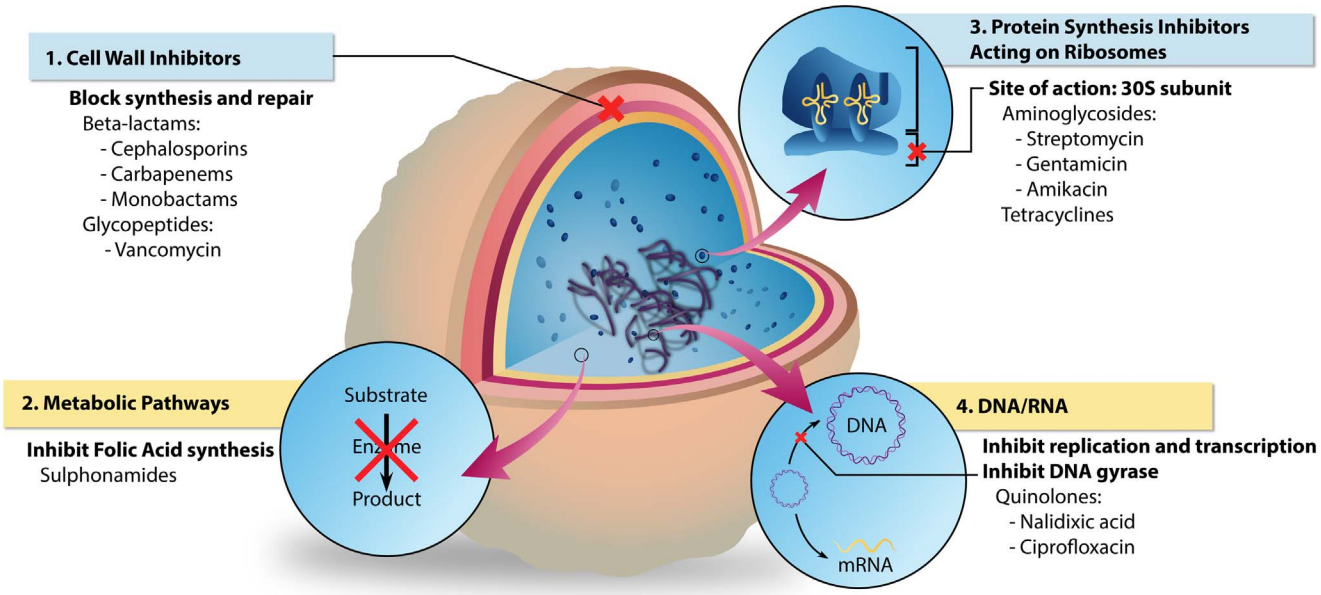

Fig. 2. Representation of the different targets of antibiotic agents within bacteria.

within their structure [68]. Moreover, Heyu Li and co-workers produced electrospun fibers with thermoresponsive polymers, (poly(N-isopropylacrylamide) (PNIPAAm) and poly (l-lactic acid-co-ε-caprolactone) (PLCL)) at different ratios with a total concentration of polymer of 10% (w/v) and loaded them with CIP to confer antibacterial activity to the fibers. Their results showed CIP-loaded fibers displayed similar inhibitory growth effect against *E. coli* and *S. aureus* [69].

On the other hand, Wei Shao and colleagues developed a tetracycline hydrochloride-loaded bacterial cellulose (BC) composite membrane. The antibacterial activity of the produced composite was investigated by both disc diffusion method and plate count method against *E. coli*, *S. aureus* and *Bacillus subtilis* (*B. subtilis*) [55]. The composite membrane presented a higher inhibitory effect against *E. coli* (45.7 mm) than for *S. aureus* (38.5 mm) and *B. subtilis* (34 mm). Regarding the plate counting method, the wound dressing reduce *E. coli* growth by 99.98%, while for *S. aureus* and *B. subtilis* the membrane was able to completely inhibit their growth [55]. Furthermore, Chen et al. fabricated an alginate-CS hydrogel dressing loaded with gelatin microspheres containing tetracycline hydrochloride to be used as a skin substitute. The bactericidal activity assays showed that the composite gel dressing was able to inhibit *E. coli* and *S. aureus* growth [63]. Recently, a semisynthetic derivative of tetracycline (glycylcyclines) was approved for the treatment of SSTIs. Tigecycline was the first member of this new class of antibiotics and presents a similar mechanism of action to that exhibited by tetracycline (30S ribosome-inhibitors), however it was designed to avoid the bacteria efflux-mediated resistance mechanisms [70,71]. Dhanalakshmi and colleagues encapsulated tigecycline within chitosan nanoparticles (CNPs), and used them for treating infected chronic wounds [72]. Subsequently, Nimal et al. incorporated tigecycline loaded chitosan nanoparticles into a chitosan-platelet-rich plasma (PRP) hydrogel. The produced system showed an improved antibacterial activity against *S. aureus* [73].

Monteiro et al. produced a gentamicin-loaded liposome immobilized at the surface of CS nanofibers mesh (NFM), to confer this electrospun membrane antibacterial activity. The obtained results showed that the produced mesh was able to inhibit *E. coli*, *P. aeruginosa* and *S. aureus* growth [66]. Fajardo et al. incorporated silver sulfadiazine (AgSD) into CS/chondroitin sulfate (CHI) film to improve their applicability as a wound dressing. The antibacterial activity of the CHI/CS/AgSD was evaluated through the determination of their capacity to inhibit *P. aeruginosa* and *S. aureus* growth. The produced film presented an inhibitory growth effect against both bacteria, especially against *P. aeruginosa* [74].

Table 1 summarizes different studies where various antibiotics have been incorporated in wound dressings to improve their bactericidal activity.

Despite of several antibiotics be available to treat skin infections, their recurrent use can trigger bacterial resistance [87]. In literature, various studies report that an improper use of antibiotics leads to the development of new resistance mechanisms by bacteria, thus causing their global dissemination [88]. More than 70% of the bacteria that are responsible for wound infections display resistance to at least one of the antibiotics used in the clinic [89]. Methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin-resistant enterococci are two multi-resistant bacteria that are involved in skin infections [88]. Such type of infections are a major health issue, since vancomycin belongs to the latest generation of antibiotics and it is assumed that it is the most effective agent against *S. aureus* [89].

The number of multidrug resistant bacteria is increasing at an alarming rate, i.e. bacteria are gaining resistance to all known classes of natural and synthetic antibiotics leading to an urgent need for new therapeutic alternatives [90]. Nanomedicine tools, particularly nanoparticles, constitute a different approach for the development of new antimicrobial agents [91].

4.2. Nanoparticles as potential antimicrobial agents

Based on the data available in literature, nanoparticles (NPs) are regarded as promising alternatives to conventional antibiotics, since they display bactericidal activity against a large number of strains and are able to minimize the undesirable side effects of drugs and do not trigger microbial resistance [92,93]. Due to the intrinsic properties displayed by NPs, they have been used in different therapeutic approaches that aim to circumvent the problems associated with the acquisition of resistance to antibiotics by bacteria.

NPs alone can perform their bactericidal effect through direct contact with the bacterial cell wall, through the release of toxic metal ions or by the generation of Reactive Oxygen Species (ROS) [94]. When NPs are in contact with bacterial cells walls, the positively-charged NPs are attracted by the negatively-charged groups found in bacteria surfaces (lipopolysaccharides in gram-negative and teichoic acid/peptidoglycan in gram-positive). Then, van der Waals forces, receptor-ligand, and

Table 1
Wound dressings functionalized with antibacterial agents.

	Antibiotic	Wound dressing	Materials	Tested bacteria	Ref.
Beta-lactams	Ceftadizime	Electrospun membrane	SF/Gelatin	*P. aeruginosa*	[75]
		Film	Collagen/CMGG/EDA	*S. aureus*	[76]
				P. aeruginosa	
	Ampicillin	Electrospun membrane	PCL	*S. aureus*	[77]
				K. pneumoniae	
		Hydrogel	PVA/SA	*E. coli*	[78]
	Cefazolin	Electrospun membrane	Gelatin	*S. aureus*	[79]
Aminoglycosides	Streptomycin	Electrospun membrane	PU/CA/ZN	*E. coli*	[80]
				S. aureus	
				S. typhimurium	
				V. vulnificus	
				B. subtilis	
		Hydrogel	PVA/Cellulose	*S. aureus*	[81]
				E. coli	
	Gentamicin	Electrospun membrane	CS	*P. aeruginosa*	[66]
				S. aureus	
				E. coli	
	Neomycin	Electrospun membrane	PSSA-MA/PVA	*S. aureus*	[82]
				E. coli	
Quinolones	Ciprofloxacin	Electrospun membrane	PVP;	*E. coli*	[64,65]
			PU/Dextran	*B. subtilis*	
				S. aureus	
				S. typhimurium	
				V. vulnificus	
	Levofloxacin	Sponge	CS/PHEA	MSSA	[83]
				MRSA	
				P. aeruginosa	
	Norfloxacin	Film	CS	*S. aureus*	[84]
				B. cereus	
				E. coli	
				K. pneumoniae	
	Moxifloxacin	Electrospun membrane	PVA/SA	*P. aeruginosa*	[85]
				S. aureus	
Sulphonamides	Sulfadiazine	Film	BC/SA; CS/CHI	*E. coli*	[53,74]
				S. aureus	
		Sponge	CS	*E. coli*	[67]
				S. aureus	
				B. subtilis	
		Electrospun membrane	PCL/PVA	*S. aureus*	[52]
	Sulfanilamide	Fiber	Alginate	*E. coli*	[86]
				S. aureus	
Tetracyclines	Doxycycline	Film	Collagen/DHBA/GMs	*P. aeruginosa*	[54]
	Tetracycline hydrochloride	Membrane	BC	*E. coli*	[55]
				S. aureus	
				B. subtilis	
		Hydrogel	OAlg/CMCS/GMs	*E. coli*	[63]
				S. aureus	
Glycopeptides	Vancomycin	Hydrogel	SF/GMs	*E. coli*	[50]
				S. aureus	
		Film	Alginate/HNTs/Gelatin	*S. epidermidis*	[49]
				S. aureus	
				S. haemolyticus	
				S. pneumoniae	
				S. pyogenes	
				E. faecalis	

hydrophobic interactions are established and the cell wall permeability is altered through the formation of “pores” at bacteria surface, leading to its disruption and consequent loss of intracellular components [95]. At the same time, NPs can also cross the cell wall and affect metabolic pathways, or even target mitochondria and cause its disruption and, consequently, induce ROS production [96]. In addition, NPs can also affect proton efflux pumps resulting in a serious pH deregulation and on the variation of membrane’s surface charge [94]. Furthermore, NPs can likewise interact with DNA, lysosomes, ribosomes, and enzymes, leading to oxidative stress, electrolyte imbalance, enzyme inhibition, protein deactivation and variations in the gene expression profile [97]. Fig. 3 presents the main bacterial targets of NPs and also some examples of NPs that have been used in the treatment of skin infections.

Among the available NPs, silver nanoparticles (AgNPs) have gained considerable attention owing to their broad inhibitory activity towards nearly 650 species of microbes, and more importantly, against antibiotic resistant bacteria [98]. With the advancement of nanotechnology, the scientific community was able to enhance the antimicrobial properties of silver, and consequently decrease silver NPs minimum inhibitory concentration (MIC), as well as reduce the possible interference of AgNPs in the wound healing process [99]. Such developments trigger the use of AgNPs in the production of wound dressings and their subsequent introduction in the market [100]. Acticoat®, Aquacel Ag® and Silvasorb® are examples of AgNPs containing dressings [101].

In 2013, Jian Wu and collaborators developed a new method to produce a BC hybrid gel-membranes containing AgNPs. The antibacterial activity of AgNP-BC membranes was investigated against gram-negative (*E. coli* and *P. aeruginosa*) and gram-positive (*S. aureus*) bacteria. For comparative purposes, they also used a commercial silver-

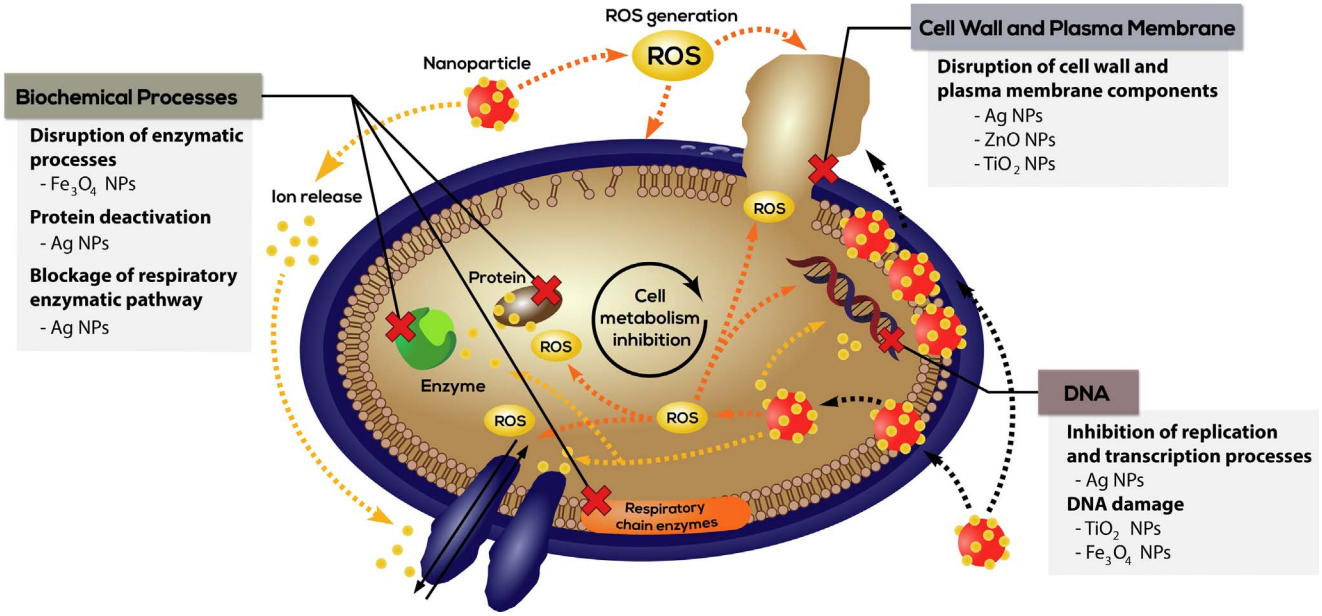

Fig. 3. Representation of NPs targets in the bacteria and some examples of NPs that have been used as antimicrobial agents.

containing dressing (Coloplast®Ag non-adhesive foam dressing). The obtained results revealed that the composite membranes loaded with AgNPs inhibited the growth of all tested bacteria. No inhibitory effect was observed for the pure BC membrane (control), thus demonstrating the crucial role of AgNPs in conferring antimicrobial properties to the produced dressings. In comparison to results obtained for a commercial dressing, no significant difference was observed, meaning that the antimicrobial dressing produced in this study has potential to be used as an effective wound dressing [102]. In another study, Augustine et al. produced a polycaprolactone (PCL) electrospun membranes loaded with AgNPs to be used as wound dressings. The fabricated membranes showed excellent antibacterial activity against both *S. aureus* and *E. coli* [103].

In recent studies, the antimicrobial properties of metallic nanocomposites were combined with natural products, in order to increased their antibacterial effect and biocompatibility [104]. Anisha and their co-workers developed an antimicrobial sponge composed by CS, Hyaluronic acid (HA) and nano silver (nAg) to treat diabetic foot ulcers. The produced sponges showed antimicrobial effect against *E. coli*, *S. aureus*, *P. aeruginosa* and *K. pneumonia*. Further, those sponges loaded with higher nAg (0.005%, 0.01% and 0.02%) concentrations were the most effective in reducing the *in vitro* growth of MRSA [105].

Other studies where AgNPs and other metal nanoparticles have been used to confer antimicrobial properties to the wound dressings are summarized in Table 2.

Despite of the bactericidal activity presented by NPs, some studies report that the same properties that make NPs so unique (small size, large surface area, chemical composition, solubility and geometry) could also be hazard to the human health, i.e. NPs due to their size can easily enter the human body and cross various biological barriers, reaching the most sensitive organs and disrupt the cell normal biochemical pathways [96,117]. Costa et al., demonstrated that AgNPs decrease the activity of mitochondrial respiratory chain complexes I, II, III, and IV from Wistar rats tissues that were analyzed (brain, skeletal muscle, heart and liver) [118]. Furthermore, Botelho et al. demonstrated that TiO_2 NPs induce tumor-like phenotypes in human gastric epithelial cells [119].

Regardless of the several studies available in literature, the cytotoxic profile of a particular NP must be characterized in deeper detail for a particular therapeutic purpose [120]. One strategy that is currently being followed to reduce the possible toxicity associated with NPs use, is based on the obtainment of these carriers from natural sources, namely plant extracts, to be incorporated in wound dressings.

Table 2
Examples of nanoparticles with bactericidal activity that have been incorporated in wound dressings.

Type of nanoparticle	Wound dressing	Materials	Tested bacteria	Ref.
Iron oxide (Fe_3O_4) nanoparticles	Electrospun membrane	CS/Gelatin	*E. coli* *S. aureus*	[106]
Titanium dioxide ($Ti0_2$) nanoparticles	Composite	CS/human ECM sheet; CS/PVP	*E. coli* *S. aureus* *B. subtilis* *P. aeruginosa*	[107,108]
	Electrospun membrane	PVA/Plur/ PEI	*E. coli* *S. aureus* *S. typhi*	[109]
Zinc oxide (ZnO) nanoparticles	Hydrogel	CS; SA/gum acacia	*E. coli* *S. aureus* *B. cereus*	[110,111]
	Composite	BC	*E. coli* *S. aureus* *P. aeruginosa* *C. freundii*	[112]
	Hydrogel	Alginate	*E. coli* *S. aureus* *MRSA*	[113]
Silver (Ag) nanoparticles	Sponge	CS/HA SF/ CMCS	*K. pneumoniae* *MRSA* *E. coli* *S. aureus* *P. aeruginosa*	[105,114]
	Hydrogel	AMPS; PVP	*S. aureus* *P. aeruginosa* *S. epidermidis* *MRSA* *E. coli* *A. iwoffii* *B. cereus* *S. pyogenes*	[115,116]

4.3. Natural products

Nowadays, an increasing number of wound dressings have been functionalized with compounds obtained from natural sources, to

Table 3
Wound dressings containing natural antibacterial agents isolated from plants.

Natural products	Wound dressing	Materials	Tested bacteria	Ref.
Henna (*Lawsonia inermis*)	Electrospun membrane	CS/PEO; gelatin/ oxidized starch	*E. coli* *S. aureus*	[123,124]
St John's-wort EO (*Hypericum perforatum*)	Film	CS	*E. coli* *S. aureus*	[125]
	Electrospun membrane	PCL	*E. coli* *S. aureus*	[126]
Curcumin	Composite	PVA	*E. coli* *S. aureus* *B. subtilis* *P. vulgaris* *E. faecalis* *S. epidermidis* *K. pneumoniae* *E. aerogenes* *P. mendocina*	[127]
	Electrospun membrane	CA/PVP	*S. aureus*	[128]
Aloe vera	Electrospun membrane	PLGA	*S. epidermidis* *S. aureus*	[129]
Thymol	Electrospun membrane	PCL/PLA	*E. coli* *S. aureus*	[130]

increase their antimicrobial activity [121,122]. Table 3 summarizes some studies where natural products displaying bactericidal activity were incorporated into wound dressings.

4.3.1. Honey

Honey has been regarded since the ancient times as a natural healing agent. Due to its antimicrobial activity and capacity to perform the topical nutrition to the wound, debriding activity, minimize inflammation and stimulate angiogenesis, granulation, wound contraction and epithelialization, honey has been incorporated into wound dressings [131,132]. Different honey-impregnated dressings are already available in the market, like MediHoney®, Activon Tulle®, Algivon® and Actilite® [132].

Honey's antimicrobial activity has been attributed to its acidity, low water content, and presence of antimicrobial substances such as hydrogen peroxide, antimicrobial peptide bee defensin-1, flavonoids, and phenolic acids [133–136]. The acidic character exhibited by honey results from the presence of gluconic acid and some authors believe that the acidic pH of honey may aid macrophages to kill bacteria and prevent microbial biofilm formation [137,138]. In turn, the low water content (< 20%) provides an unfavorable environment for microorganism survival and growth. High osmolarity inhibits microbial growth, since water molecules are chemically tied to the sugar molecules, leading to an inappropriate environment for organisms survival [139,140]. Lastly, the production of hydrogen peroxide by honey is responsible for the bacterial growth inhibition, i.e. hydrogen peroxide is able to react with the cell wall, lipids, proteins and nucleic acids available in bacteria [141,142]. In Fig. 4 are illustrated the bacterial activities exhibited by honey.

Although, honey in the presence of catalase (an enzyme that degrades hydrogen peroxide) displays a decreased antimicrobial activity [131]. To surpass this drawback, Manuka Honey (MH), which is obtained from the manuka tree (Leptospermum scoparium), unlike other honeys, contains a non-peroxide component, that is not degraded by catalase, is able to sustain its antibacterial activity in biologic fluids [131,132]. MH inhibits the growth of a broad range of microorganisms (including gram-positive strains such as MRSA and *S. pyogenes*, as well as gram-negative strains like *E. coli*, *Proteus mirabilis (P. mirabilis)*, *Enterobacter cloacae*, and *P. aeruginosa*) and avoids biofilm formation at the wound site [143]. Furthermore, Packer et al. have described that methylglyoxal (MGO), a component of MH, interferes with ribosome and its translational capacity (see further details in Fig. 4) [131].

Bulman et al. incorporated MGO as a functional antibacterial agent into polyvinyl alcohol fibers. The obtained results confirmed that the fibers containing MGO exhibited bactericidal activity against *E. coli* and *S. aureus* [144]. Moreover, Yang et al., incorporated MH in an electrospun membrane produced with silk fibroin (SF), for being used as an antimicrobial wound dressing. The SF/MH fibrous matrices presented antibacterial activity against both gram-positive (MRSA and *S. aureus*) and gram-negative (*E. coli* and *P. aeruginosa*) bacteria [145].

4.3.2. Essential oils

In addition to honey, essential oils (EOs) have been incorporated as antibacterial agents in bioactive wound dressings. Essential oils, also called volatile natural mixtures, are plant secondary metabolites that exhibit antioxidant, antiviral, anticancer, insecticidal, anti-inflammatory, anti-allergic and antimicrobial properties [146].

Different authors stated that the antimicrobial activity of EOs that are usually incorporated in wound dressings is attributed to phenolic compounds, specifically to thymol and carvacrol [131,132,143]. Kavoosi et al., reported that EOs attack the phospholipids present in the cell membranes and the lipids available on the cell wall of the bacteria, leading to an increased permeability and ultimately to cell lysis. Such results in the cytoplasm leakage, pH decrease, and loss of cellular processes, like ATP biosynthesis, DNA transcription and protein synthesis. Moreover, Altiok et al. described that EOs disturbs the function of the cytoplasmic membrane, by disrupting the active transport of nutrients through the cell membrane, and coagulation of bacteria cell contents [147]. In Fig. 5 are illustrated different mechanisms through which EOs exert their antimicrobial activity.

Amongst the different EOs components, cinnamaldehyde, geraniol, thymol analogues, menthol and carvacrol (a major ingredient of Zataria multiflora EO) are the most used for antibacterial purposes. Liakos et al., incorporated 1% and 5% of EOs (cinnamon, lemongrass and peppermint) in cellulose-based fibrous dressings and they noticed that the fibrous dressings were able to inhibit the growth of *E. coli*, even when small amounts of EOs were used [148]. In another study, Liakos et al. prepared polymeric composite films with sodium alginate (NaAlg) incorporating different EOs (chamomile blue, cinnamon, lavender, tea tree, peppermint, eucalyptus, lemongrass and lemon oils) and three different concentrations of EOs were tested (16%, 50% and 66%). They produced dressings able to inhibit *E. coli* growth [149].

4.3.3. Chitosan

Chitosan (CS) and its derivatives display a high antimicrobial activity against fungi, bacteria, algae and viruses [150]. In the literature there are at least three mechanisms proposed for explaining the antibacterial activity of CS (as depicted in Fig. 6) [151]. The most accepted mechanism proposes that CS antimicrobial activity results from the electrostatic interactions occurring between the positively-charged groups of chitosan (amine groups of glucosamines) and the negatively-charged groups available on the bacterial cell wall (surface components like peptidoglycans) [152]. This electrostatic interaction can affect the permeability of the cell wall, thus causing internal osmotic imbalances and consequently inhibit the growth of microorganisms. On the other hand, the electrostatic interactions can induce the hydrolysis of the peptidoglycans of the microorganisms' cell wall, prompting the leakage of intracellular electrolytes [151]. The second proposed mechanism involves the formation of a polymeric envelope around bacteria, leading to the inhibition of cell exchanges and nutrients absorption [152]. The last mechanism includes the chelation of trace metals and oligo-elements that are essential for bacterial growth, i.e. the amino groups of CS might interact with essential trace metals and thereby inhibit the production of toxins and microbial growth [151,152]. These specific properties triggered its use in the production of wound dressings available in the market, such as HidroKi®, Patch®, Chitopack®,

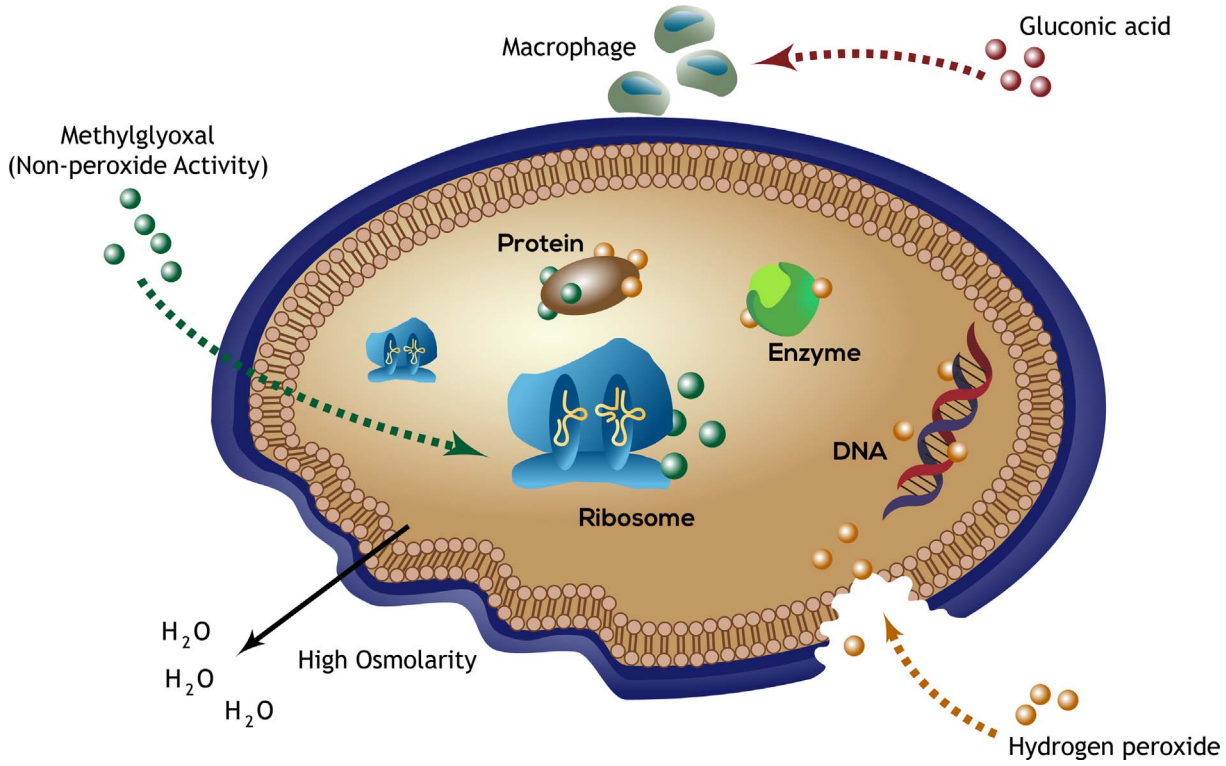

Fig. 4. Representation of the mechanisms proposed to explain the bactericidal activity exhibited by Honey and MH.

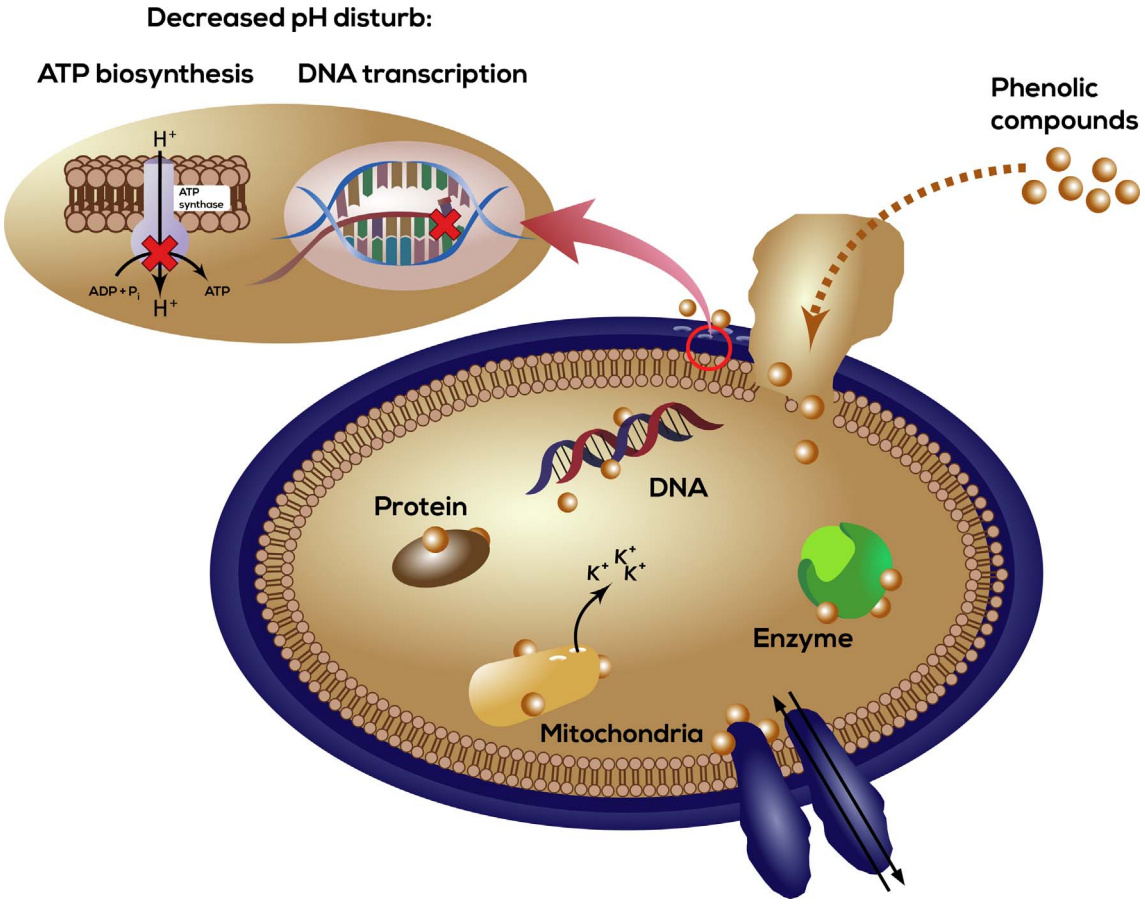

Fig. 5. Representation of phenol (active component of essential oils) targets in bacteria.

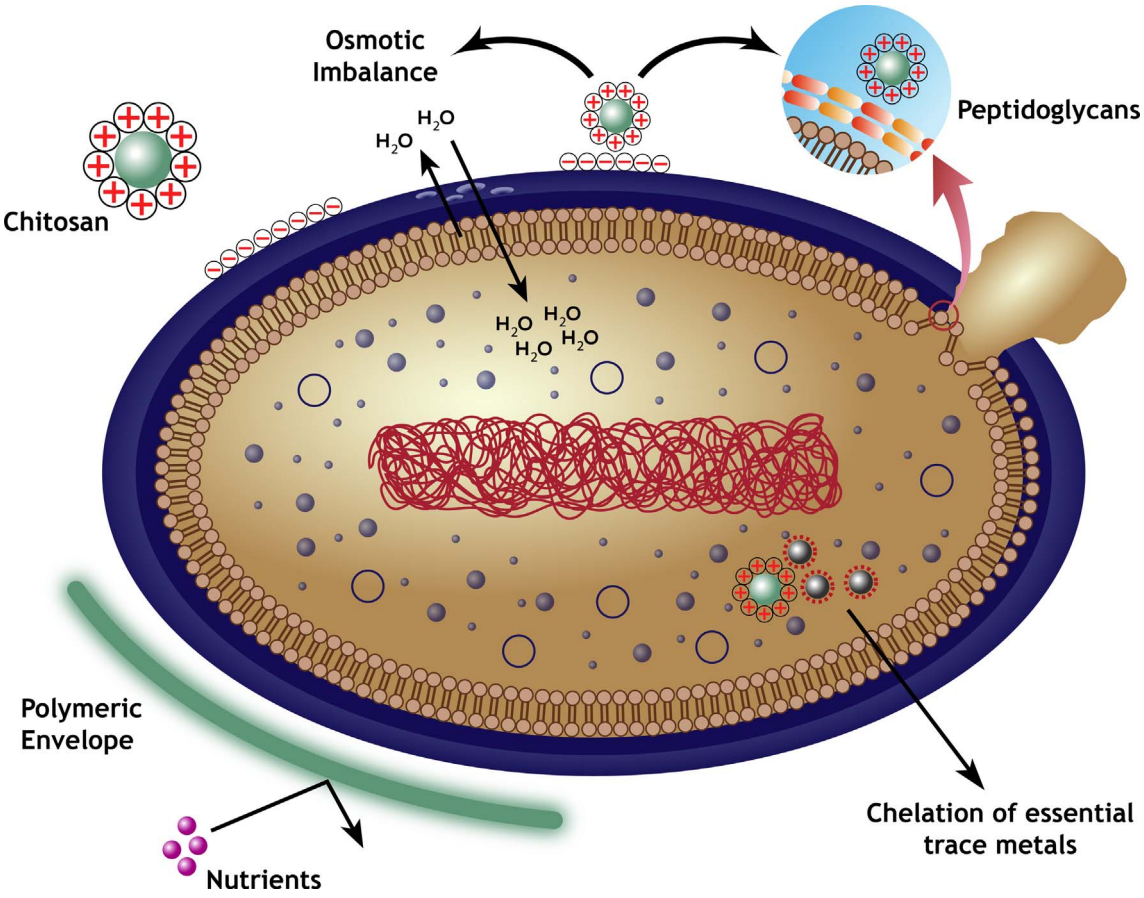

Fig. 6. Representation of the mechanisms proposed to explain the antibacterial activity of Chitosan.

Table 4
Chitosan-based wound dressings for skin regeneration applications.

Chitosan-based wound dressing	Materials	Tested bacteria	Ref.
Membrane	CS	*P. aeruginosa* *S. aureus*	[156]
	CS/BC	*E. coli* *S. aureus*	[157]
	CS/PP/NIPAAm/CG	*S. aureus*	[158]
	CS/SF	*E. coli* *S. aureus*	[159]
	CS/sericin	*E. coli* *B. subtilis*	[160]
	CS/CS-glucan	*E. coli* *K. pneumoniae* *B. subtilis* *S. aureus*	[161]
	CS/Aloe vera	*E. coli* *S. aureus*	[153]
Hydrogel	CS/Agarose	*E. coli* *S. aureus*	[162]
Sponge	CS/PCD	*S. aureus*	[163]
	CS/PVA	*E. coli*	[164]
Film	CS/PVP/ nanocellulose	*S. aureus* *P. aeruginosa*	[165]
	CS/Hyaluronan	*E. coli* *S. aureus*	[166]

Tegasorb® and KytoCel® [153].

Antunes et al. produced an electrospun membrane comprised by deacetylated/arginine modified chitosan (CS-A) to be used as a wound dressing. In this study, CS was modified with arginine to increase the number of positively charged groups available on material's surface, to enhance its electrostatic interaction with bacteria cell wall. The obtained results highlight the importance of coupling arginine residues to CS, since the modified CS exhibit a much higher antimicrobial activity [154]. Furthermore, Yuan et al. prepared a CS and polyethylene oxide (PEO) nanofibrous meshes for wound healing application. The electrospun mesh was able to significantly reduce the bacterial colonies attached to the membrane surface. In addition, those materials with a higher concentration of chitosan present a greater bactericidal effect [155]. Other studies where CS has also been used for the production of antimicrobial wound dressings are summarized in Table 4.

5. Concluding remarks and future prospects

Nowadays, nearly everyone has already suffered an open skin wound as a consequence of a cut, burn, diseases (e.g. diabetes) or surgical interventions. In some circumstances those wounds can easily be contaminated by different pathogens found in the surrounding environment, endogenous microbes living in the mucous membranes, or by the microflora available on the adjacent skin. Gram-positive bacteria, like *E. coli* and *P. aeruginosa*, and gram-negative bacteria, such as *S. aureus*, are the predominant pathogens responsible for skin contamination and subsequent infections. Wound dressings displaying antimicrobial activity started to be produced in order to surpass this health problem. Different strategies, comprising materials' surface functionalization with different groups or antimicrobial agents incorporation (antibiotics, nanoparticles and natural products), have been used to confer bactericidal activity to dressings. However, despite of developments attained so far, further improvements of these type of dressings is demanded. In a near future it is expected that the co-administration of antibacterial agents leads to an increased therapeutic outcome. Furthermore, the development of new NPs or the loading of antimicrobial agents into nanodevices may open new avenues to treat

infected wounds. In addition, dressings containing sensors and therapeutic molecules may also be produced in order to simultaneously perform the monitoring and treatment of an infected wound.

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Electrospun polymeric nanofibres as wound dressings: A review

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ABSTRACT

Skin wounds have significant morbidity and mortality rates associated. This is explained by the limited effectiveness of the currently available treatments, which in some cases do not allow the reestablishment of the structure and functions of the damaged skin, leading to wound infection and dehydration. These drawbacks may have an impact on the healing process and ultimately prompt patients' death. For this reason, researchers are currently developing new wound dressings that enhance skin regeneration. Among them, electrospun polymeric nanofibres have been regarded as promising tools for improving skin regeneration due to their structural similarity with the extracellular matrix of normal skin, capacity to promote cell growth and proliferation and bactericidal activity as well as suitability to deliver bioactive molecules to the wound site. In this review, an overview of the recent studies concerning the production and evaluation of electrospun polymeric nanofibrous membranes for skin regenerative purposes is provided. Moreover, the current challenges and future perspectives of electrospun nanofibrous membranes suitable for this biomedical application are highlighted.

Contents

1. Introduction

Electrospun nanofibres have been applied in the biomedical field, as drug delivery systems [1–3] as well as 3D constructs for tissue regeneration of cartilage [4], bone [5], heart valves [6,7], muscle [8,9], neural tissue [10] and skin [11,12] (see Fig. 1 for further details).

When a skin injury occurs, it is extremely important to re-establish, as quickly as possible, the skin's structure and functions

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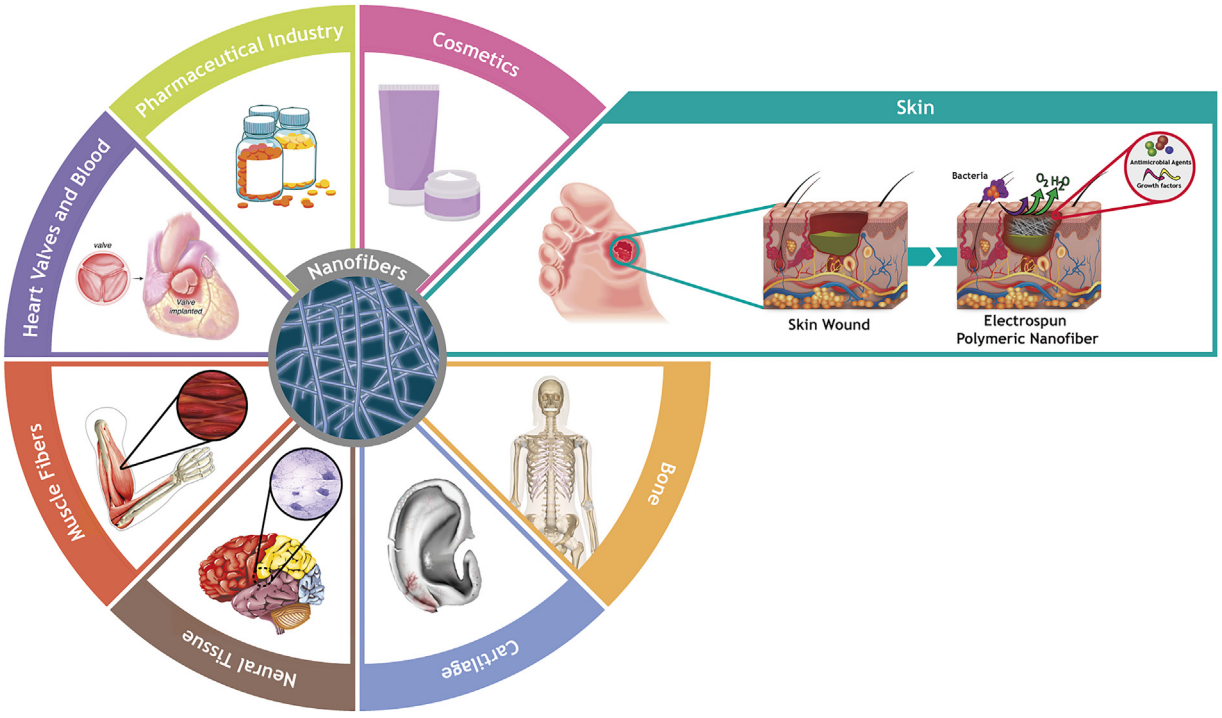

Fig. 1. Illustration of nanofibrous meshes applications in different fields of Biomedicine.

to assure the maintenance of the body's homeostasis. Although the skin exhibits self-regenerative capacity, some types of wounds do not heal as a consequence of extensive lesions and/or chronic wounds [13].

To overcome such drawbacks, new bioactive dressings have been developed or are under optimization to mimic the skin's native structure and are compatible with cell loading (keratinocytes, fibroblasts and stem cells). Depending on their capability to replace epidermis, dermis or both skin layers, they are grouped respectively as epidermal, dermal and epidermal-dermal substitutes [14]. Nonetheless, the associated production costs are high and the bioactive dressings are unable to fully re-establish all native skin features.

Different techniques, including self-assembly, phase separation and electrospinning have been used to produce micro to nano scale meshes aimed to be used as wound dressings [15]. Among them, electrospinning has captured the attention of researchers due to its simplicity, cost-effectiveness and versatility to produce nanofibrous membranes that are capable of mimicking the morphological characteristics of the skin's extracellular matrix (ECM). Moreover, these nanofibrous meshes are also able to support cell adhesion, migration, growth and differentiation as well as angiogenesis, which are vital events for the occurrence of an effective wound healing process [16–20]. In addition, bioactive molecules have also been incorporated into the electrospun nanofibres to improve the biologic performance of these membranes [21].

In the following sections of this review, an overview is provided of the recent studies concerning the production, surface functionalisation and evaluation of electrospun polymeric nanofibrous membranes performance in skin regeneration.

2. Electrospinning set-up used to produce electrospun nanofibrous membranes for the regeneration of skin

An electrospinning apparatus usually is comprised of a syringe pump, a capillary needle (the spinneret), a high-voltage power supply and a metal collector (see Fig. 2 for further details) [14]. During the electrospinning process, a high voltage is generated to produce an electrically charged jet of the polymeric solution that is directed to a collector by the electrostatic forces, resulting in the production of an interconnected fibrous membrane [14,22]. The features of the electrospun membranes are dependent on the properties of the precursor solution (e.g. conductivity, surface tension, viscosity and solvent selection), processing variables (e.g. flow rate, voltage, and the distance between the capillary and the collector) and environmental conditions (e.g. temperature and humidity) [16]. The control of these particular parameters has a direct impact on the mean diameter and arrangement of the produced nanofibres [23]. Moreover, the type of collector used has an impact on the arrangement and packing of the produced fibres, thus determining the morphological and mechanical properties of the electrospun fibres [24]. When the nanofibres are collected in a stationary collector, the membranes produced show a highly porous and randomly-orientated structure that mimics the 3D architecture of collagen fibres found within the ECM of normal skin [25,26]. To the contrary, nanofibrous membranes to be used for muscle and nervous tissue regeneration must display a specific orientation. To obtain meshes with these structural features, researchers are using rotating collectors, where the rotation speed of the collector has a direct effect on the diameter and the alignment of the produced fibres [24,27].

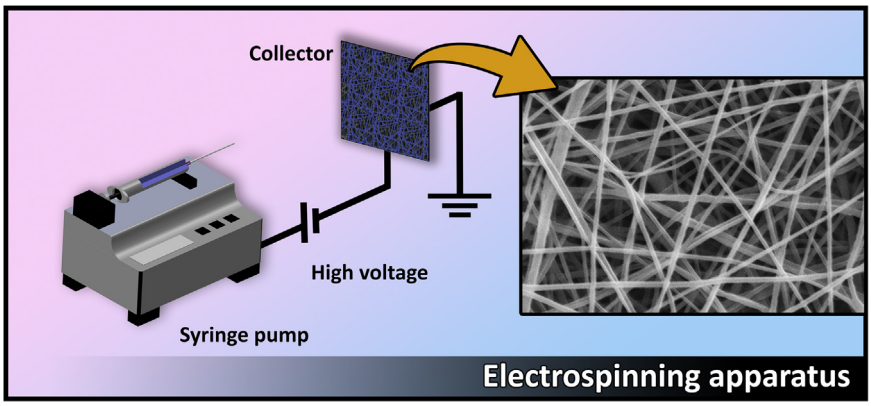

Fig. 2. Representation of the electrospinning setup, that is usually used to produce nanofibrous meshes.

Through the optimisation of the experimental set-up of this technique, researchers have been producing electrospun membranes with particular characteristics that allow them to confer protection to the wound against external contaminants and also display a 3D fibre mesh architecture that mimics skin ECM (as represented in Fig. 3). Furthermore, the high surface-to-volume ratio promotes cell attachment and the microscale interconnected pores are compatible with gas exchange, nutrient supply and control of fluid loss [28–30]. Such properties are vital for assuring the maintenance of a moist environment at the wound site to prevent wound dehydration and enhance angiogenesis and collagen synthesis [31]. Moreover, dressings composed of nanofibres can reduce scar formation, since the biodegradation of fibres provides a suitable roadmap for tissue healing [32,33].

3. Polymers used to produce electrospun nanofibres membranes

3.1. Synthetic polymers

Synthetic polymers have been used to produce electrospun nanofibres membranes. This type of polymers can be tailored to exhibit excellent mechanical properties, thermal stability and an appropriated degradation profile. In 2003, for the first time, Khil et al. used Polyurethane (PU) to produce nanofibrous membranes to be applied as skin substitutes [34]. Their results revealed that the PU membranes were able to control the water vapor transmission rate, displayed an excellent oxygen permeability, and presented fluid drainage ability. The assessment of the biologic performance of the membranes demonstrated their biocompatibility as well as their capacity to avoid exogenous microorganisms penetration into the wound. Moreover, *in vivo* data showed that, after 15 days of treatment, animals treated with PU-electrospun membranes displayed a well-organized dermis and granulation tissue [34]. Kumbar et al. produced Polylactide-polyglycolide (PLGA) nanofibres that were then seeded on the surface with human skin fibroblasts [35]. The results obtained showed that cells were able to spread, adhere and form multiple layers, after 28 days in culture.

However, in other studies, the hydrophobic character of the synthetic polymers used (e.g. Polycaprolactone (PCL) and Poly(glycolic acid) (PGA)), and the absence of peptide sequences on the materials' surface impaired cell adhesion and/or proliferation [36].

3.2. Natural polymers

To circumvent some of the limitations presented by synthetic materials, natural polymers became a viable option because of the availability of peptide sequences at their surfaces that can be recognized by cell surface receptors and, subsequently, trigger cell adhesion and proliferation [21,37].

In 2006, Rho et al. produced a Collagen nanofibrous matrix to be used as wound dressing, for the first time. Their results showed that this matrix exhibited a good tensile strength (≈7 MPa), high porosity and high surface area-to-volume ratio as well as features required for cell adhesion, growth and proliferation. Moreover, *in vivo* assays also demonstrated that collagen nanofibrous membranes were able to improve the healing process [38].

Hyung et al. used Silk Fibroin (SF) to produce nanofibrous membranes and evaluated their biologic performance in *in vivo* assays. The acquired data revealed that the produced SF nanomatrices were able to induce a higher rate of epithelialization and collagen production than commercially available dressings (e.g. Mediofoam® and medical gauze). Furthermore, the SF membranes produced were also able to modulate the concentration of inflammatory cytokines involved in wound healing (IL-10 and TGF-β1). This emphasizes the suitability of SF nanomatrices for the treatment of injured skin, i.e. they were able to decrease injury inflammation and reduce the wound healing period as well as scar formation [39].

Lin et al. produced a biocompatible nanofibrous membrane using Zein (ZN) and Collagen and then evaluated its capacity to be used in the treatment of full-thickness skin wounds induced in mice [40]. Nevertheless, the biodegradation rate and mechanical properties displayed by these natural materials was found to be restrictive, so their application may be avoided in the biomedical field [41].

3.3. Synthetic/Natural blend polymers

The blending of synthetic and natural polymers was also seen as a promising strategy to overcome the limitations of both synthetic and natural polymers. This approach combines the strength and durability of a synthetic polymer with the biocompatibility and bioactivity of natural polymers [42–44].

In 2004, Venugopal et al. evaluated the performance of electrospun matrices prepared with PCL and Collagen blends. These membranes were able to promote cell adhesion and proliferation where their PCL nanofibres counterparts could not. This result can be explained by the excellent intrinsic biological properties displayed by collagen [45]. Nonetheless, the collagen used in tissue engineering applications is usually obtained from animal sources, which is associated to the risk of disease transmission. Therefore, alternative materials have been considered, like gelatin which, despite being a cheaper collagen derivative (obtained through collagen denaturation), exhibits all the required biological properties. Duan et al. investigated the suitability of PCL/Gelatin electrospun membranes to be used as engineered epidermal skin grafts.

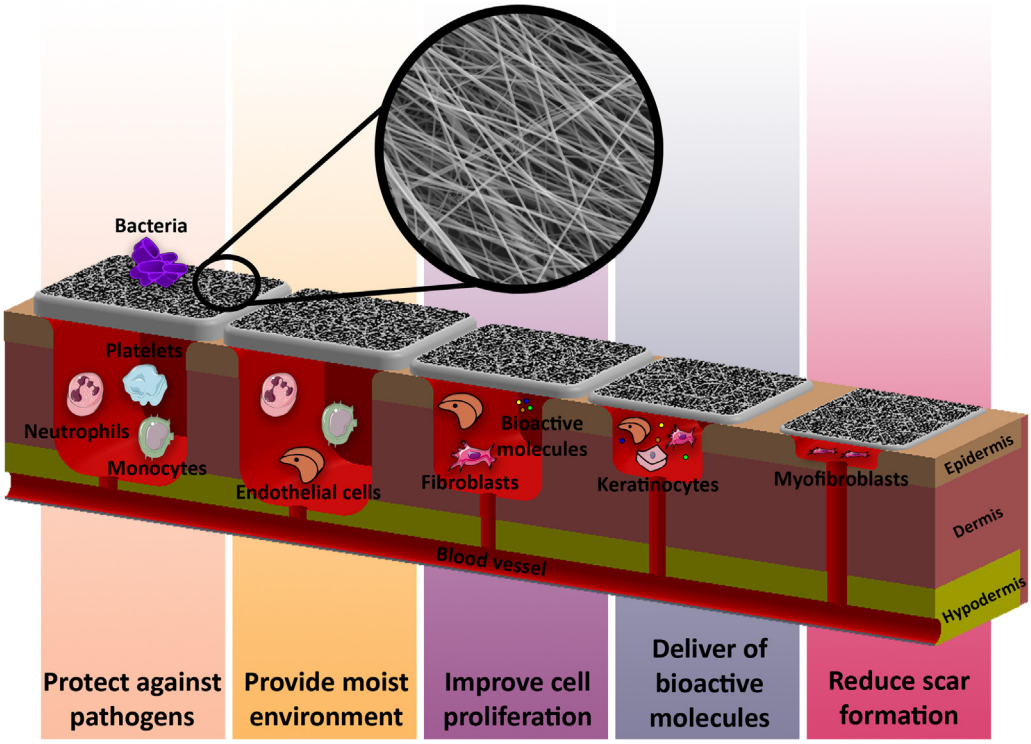

Fig. 3. Representation of the properties that electrospun membranes must display to be used as wound dressings.

Their results showed that HaCaT cells (a human keratinocyte cell line) were able to adhere and spread on the membranes' surface for at least 7 days. Furthermore, the *in vivo* assays demonstrated that the groups treated with PCL/Gelatin membranes exhibited an improved healing process, since the wound closure occurred in a shorter period of time [46]. In order to overcome the drawbacks associated with PCL (e.g. its hydrophobic character as wells as its low degradation profile), other researchers tested alternative materials to produce nanofibrous meshes, like Polyethylene oxide (PEO), Polyvinyl alcohol (PVA), Polylactic acid (PLA) and PLGA.

Zhou et al. produced a water-soluble carboxyethyl Chitosan (CS)/PVA blend to improve chitosan electro-spinnability. Subsequently, mouse fibroblast cells were seeded at the surface of the membranes produced and, after 48 h in culture cells, these were able to adhere and proliferate [47].

Gu et al. used a mixture of PLA and gelatin to produce electrospun meshes. These membranes were able to reduce water loss in wounds, which is essential for avoiding dehydration. Moreover, dermal fibroblasts remained viable, for at least 4 days, after seeded on the top of those membranes [48].

In another study, PLGA was combined with collagen to produce nanofibrous wound dressings that reproduce the native structure and biological function of the ECM of skin. Both *in vitro* and *in vivo* assays showed that PLGA/collagen membranes improve the healing process [49].

Suganya et al. combined poly(L-lactic acid)-co −poly(ϵ −caprolactone) (PLACL) with Aloe Vera (AV) and SF to produce electrospun meshes that promote dermal regeneration. Their results showed that the SF provides a favorable environment for cell adhesion and migration, while the AV components, mannose 6-phosphate and acemannan are known to promote epithelialization and the synthesis of collagen, processes that are essential for an effective healing process [50].

Besides the data available in the above published articles, there are also some patents focused on the application of electrospun membranes as wound dressings. Kataphinan et al. patented their production of a skin mask through the direct deposit of electrospun fibres onto the skin surface [51]. Smith et al. patented their production of electrospun fibres containing a pH-adjusting compound that prevents wound contamination and promotes the healing process [52]. Yarin et al. patented the use of the electrospinning technique for the production of a biodegradable plant-based wound dressing (composed of a biopolymers extracted from plants and synthetic polymers) [53].

Table 1 presents further examples of different electrospun membranes produced with natural, synthetic and blends of natural and synthetic polymers to be applied as skin substitutes.

4. Modification of the surface of electrospun polymeric nanofibres

The modification of a material's surface can be done by changing the topography or the functional groups available [63]. In order to improve the performance of electrospun polymeric nanofibres performance in skin regeneration, the surfaces have been chemically and physically modified with bioactive molecules and cell-recognizable ligands [64]. In the following sections, the most common surface modification techniques used for this purpose are presented.

4.1. Techniques used to functionalise a material's surface

As previously described, the use of synthetic polymers presents some limitations, due to their hydrophobic character and inability to encourage cell adhesion and proliferation [36]. As a possible solution, surface functionalisation techniques, which are represented in Fig. 4, like the wet chemical method, plasma treatment and graft polymerisation are usually applied [64].

The wet chemical method is based on the reaction under acidic or basic conditions, between the mesh and liquid reagents in order to add new chemical groups to the polymeric backbone [65]. Khorsand-Ghayeni et al. produced electrospun PLGA nanofibrous matrices and modified them by adding carboxyl and hydroxyl groups on their surface through alkaline hydrolysis, followed by a

Table 1
Nanofibrous meshes produced through electrospinning that are aimed to be used as wound dressings.

Polymers	Solvent	Cell line used in biocompatibility assays	Main findings	Ref
Carboxyethyl chitosan/PVA	Deionized water	Mouse fibroblasts (L929)	Biocompatible nanofibres were prepared using water soluble chitosan. *In vitro assays* showed that fibrous mats promote cell adhesion and proliferation.	[47]
Chitosan/arginine-chitosan	TFA:DCM	Human dermal fibroblasts	The modification of chitosan with L-arginine allowed the production of nanofibrous meshes able to improve the healing process and increased bactericidal activity.	[54]
CS/SF	HFIP:TFA	Mouse fibroblasts (L929)	Blended CS and silk fibroin nanofibrous membranes promoted cell attachment and proliferation.	[55]
CS/PVA	Deionized water for PVA;HOBt, TPP and EDTA for CS	Human foreskin fibroblast	CS/PVA membranes induce a reduction in wound size, during the first week after tissue damage.	[56]
Collagen	HFIP	Normal human oral keratinocytes	The electrospun collagen nanofibrous membranes were produced and characterized, for the first time, aimed to be used as wound dressings.	[38]
Collagen/ZN	AA	L9229 fibroblast cells	The ZN improved the electrospinnability of the blend and fibre tensile strength, while the collagen enhanced cell adhesion.	[40]
Gelatin/PU	HFIP	NIH3T3 fibroblast	The gelatin improved cell adhesion and proliferation, whereas PU allowed the production of elastic nanofibres.	[57]
Gelatin/PLLA	Acetic acid; DCM	WI-38 fibroblast	The electrospun gelatin/PLLA membranes showed controlled water loss, displayed fluid drainage ability, and an excellent biocompatibility.	[48]
Gelatin/PCL	TFE: acetic acid	HaCaT keratinocytes	Membranes exhibited good mechanical properties and did not elicit any toxic effect on cells.	[46]
PCL/SF/HA	HFP; Formic acid:HFP	FEK4 derived from a newborn foreskin explants	The incorporation of hyaluronic acid into nanofibrous scaffolds enhanced cell infiltration both *in vitro* and *in vivo*.	[58]
PCL/SF	THF and DMF	NIH3T3 fibroblasts	PCL/SF nanofibrous matrix provided favourable spatial cues, surface topography and chemistry for cell infiltration.	[59]
PCL/collagen	TFE	Human dermal fibroblast	Core-shell composite nanofibres improved cell-scaffold interactions.	[60]
PCL/ZnO	Acetone	Human dermal fibroblast	PCL/ZnO membranes showed excellent fibroblast cell attachment and good antimicrobial activity.	[61]
PLGA/collagen	HFIP	Human dermal fibroblasts	PLGA/collagen nanofibrous meshes improve the wound-healing process in an early stage.	[49]
PLGA/gelatin	Chloroform: acetone	Postnatal human fibroblasts	Hybrid scaffolds presented the desired bioactivity, hemostasis, and are also capable of encapsulate and perform a controlled release of EGF. Such properties highlight their potential to be applied in skin tissue engineering.	[21]
PCL_HA/CS_ZN	TFE; DMF; AA and EtOH	Human dermal fibroblasts	The produced bilayered electrospun membrane protect the wound as well as enhanced the wound healing process.	[11]
PLACL/SF/AV	DCM: DMF	Human dermal fibroblasts	The synergistic effect of AV and SF resulted in the production of the nanofibrous scaffolds with excellent properties to be used in skin tissue regeneration.	[50]
PCL/AV_CS	TFE; AA and water	Human dermal fibroblasts	The asymmetric membrane containing AV showed enhanced biological properties.	[12]
PLA/MWCNTs/REC	DCM:DMF	L9229 fibroblast cells	The incorporation of inorganic materials improved the thermal stability of the composite nanofibrous membranes. Moreover, these membranes exhibited a biocompatible profile.	[62]
SF	Formic acid	Oral keratinocytes, epidermal keratinocytes	SF nanofibres exhibited a pore size distribution, porosity and surface area-to-volume ratio favourable for cell attachment, growth and proliferation.	[28]

AA: Acetic acid; AV: Aloe Vera; CS: Chitosan; DCM: Dichloromethane; DMF: Dimethylformamide; EDTA: Ethylenediaminetetraacetic acid; EGF: Epidermal growth factor; EtOH: Ethanol; HA: Hyaluronic acid; HFP: Hexafluoro-2-propanol; HOBt: Hydroxybenzotriazole; MWCNTs: multi-walled carbon nanotubes; PCL: Polycaprolactone; PLGA: Poly Lactic-co-Glycolic Acid; PLA: Polylactic acid; PU: Polyurethane; PVA: Polyvinyl alcohol; REC: rectorite; SF: Silk fibroin; TFA: Trifluoroacetic acid; TFE: 2,2,2-Trifluoroethanol; THF: Tetrahydrofuran; TPP: Tripolyphosphate; ZN: Zein; ZnO: Zinc oxide.

collagen coating. Their results demonstrated that the surface functionalisation decreased the PLGA hydrophobicity, which is essential for this material to exhibit a wettability suitable for cell adhesion [66].

The plasma treatment of electrospun polymeric nanofibres has been commonly employed to tailor surface adhesion and optimise wetting properties by changing the surface chemical composition [67]. Depending on the type of plasma used (e.g. oxygen, ammonia, argon), diverse functional groups can be added to the polymer surface in order to improve the biocompatibility of a material [64]. Besides introducing functional groups, plasma treatments can also be used to control surface roughness and induce processes like crosslink formation, graft polymerisation and thin film coating of the polymeric surface [64,68]. Jeong et al. prepared electrospun SF nanofibres and then treated them with oxygen plasma to increase their hydrophilicity. The results obtained revealed a higher cell adhesion and proliferation, for both normal human epidermal keratinocytes (NHEK) and fibroblasts (NHEF), on the surface of functionalised nanofibrous membranes [69].

Graft polymerisation involves the covalent immobilisation of bioactive molecules at the nanofibre's surface to enhance cell adhesion, proliferation and differentiation [70]. In 2013, Gautam et al. fabricated a tri-polymer PCL/gelatin/collagen type I, by grafting collagen type I on electrospun PCL/gelatin mesh to improve fibroblast and keratinocyte cells adhesion and proliferation [71].

Graft polymerisation may require the use of initiators to start the grafting of a monomer on the membrane surface, like when using UV irradiation [72]. Plasma treatment is able, by itself, to

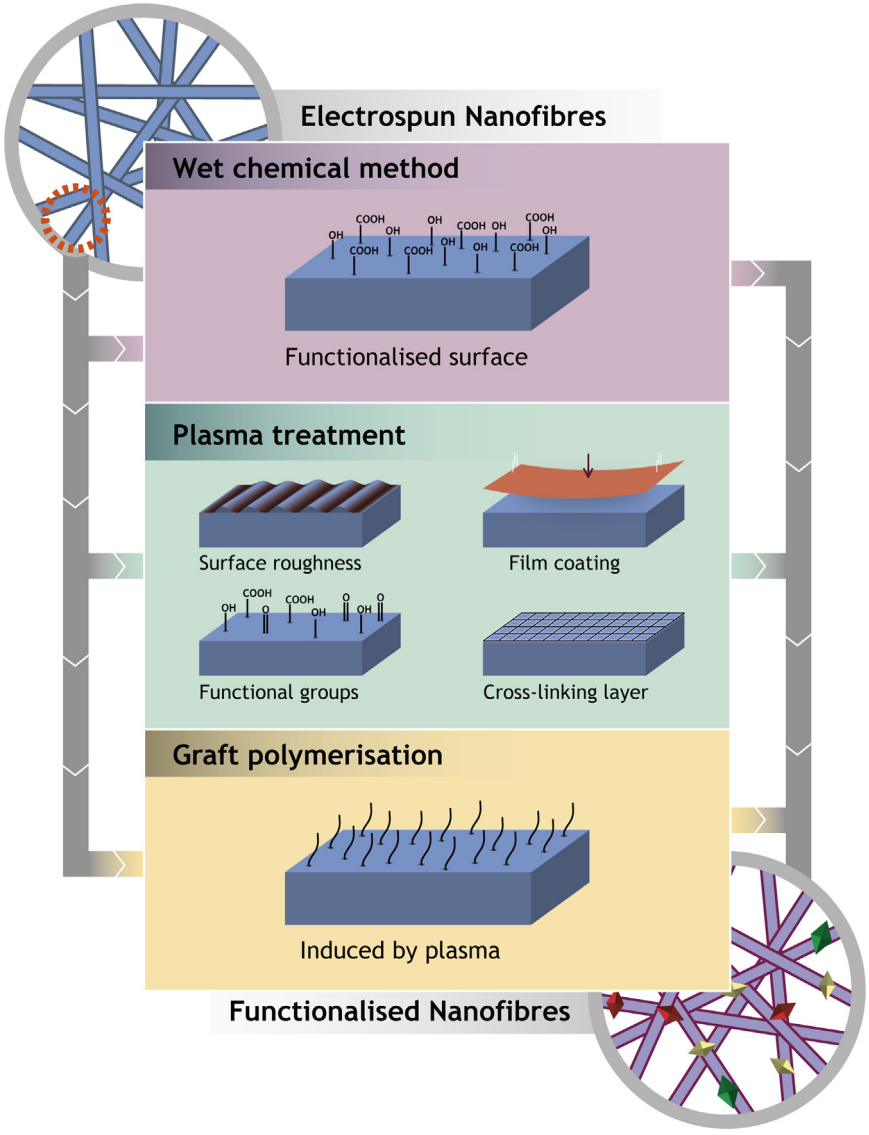

Fig. 4. Representation of the main surface modification techniques used up to now to improve the surface nanofibres properties: wet chemical method, plasma treatment and graft polymerisation.

generate free radicals on the polymeric matrix and therefore initiate the polymerisation reaction. Park et al. produced electrospun biodegradable nanofibrous meshes with PGA, PLLA and PLGA. These nanofibrous surfaces were chemically modified by *in situ* graft polymerisation of acrylic acid using oxygen plasma treatment. The results obtained revealed that the surface-modified membranes showed a significant improvement in cell attachment and proliferation, due to the incorporation of the hydrophilic functional groups [73].

4.2. Surface functionalised nanofibrous meshes to be used as drug delivery systems

A number of nanomaterials provide an excellent platform for local delivery of therapeutic agents due to their functionality and inherent nanoscale morphological characteristics [74,75]. The electrospun nanofibres display a high surface-to-volume ratio that can enhance the solubility of the drug and, consequently, improve its therapeutic effectiveness [76]. Multiple agents (antimicrobial agents, growth factors (GFs), etc.) have been incorporated into nanofibrous meshes by using blend, co-axial and emulsion electrospinning [64,77]. Other, post-electrospinning surface modifications techniques, like physical adsorption, layer-by-layer assembly and chemical immobilisation, have also been used. An overview of the techniques used for incorporating bioactive agents in nanofibrous meshes is provided in Fig. 5 and in the following sections.

4.2.1. Pre-electrospinning surface modification techniques

The blend electrospinning method involves the encapsulation of bioactive molecules that are dissolved or dispersed in the polymeric solution. Then, hybrid fibres are produced when this mixture is electrospun [78]. The encapsulation of the biomolecules within the fibres, assures their sustained release and also avoids the occurrence of an early burst release [79]. Li et al. mixed PVA with sodium alginate and organic rectorite (OREC) (which is recognized as a bacterial inhibitor) and then produced an electrospun nanofibrous mesh. The *in vitro* data showed that the OREC improved the bactericidal activity of the nanofibres [80]. Chouhan et al. developed non-mulberry SF based (NMSF) electrospun mats functionalised with EGF and ciprofloxacin to be used as wound dressings. The results obtained showed that NMS-based mats are biocompatible,

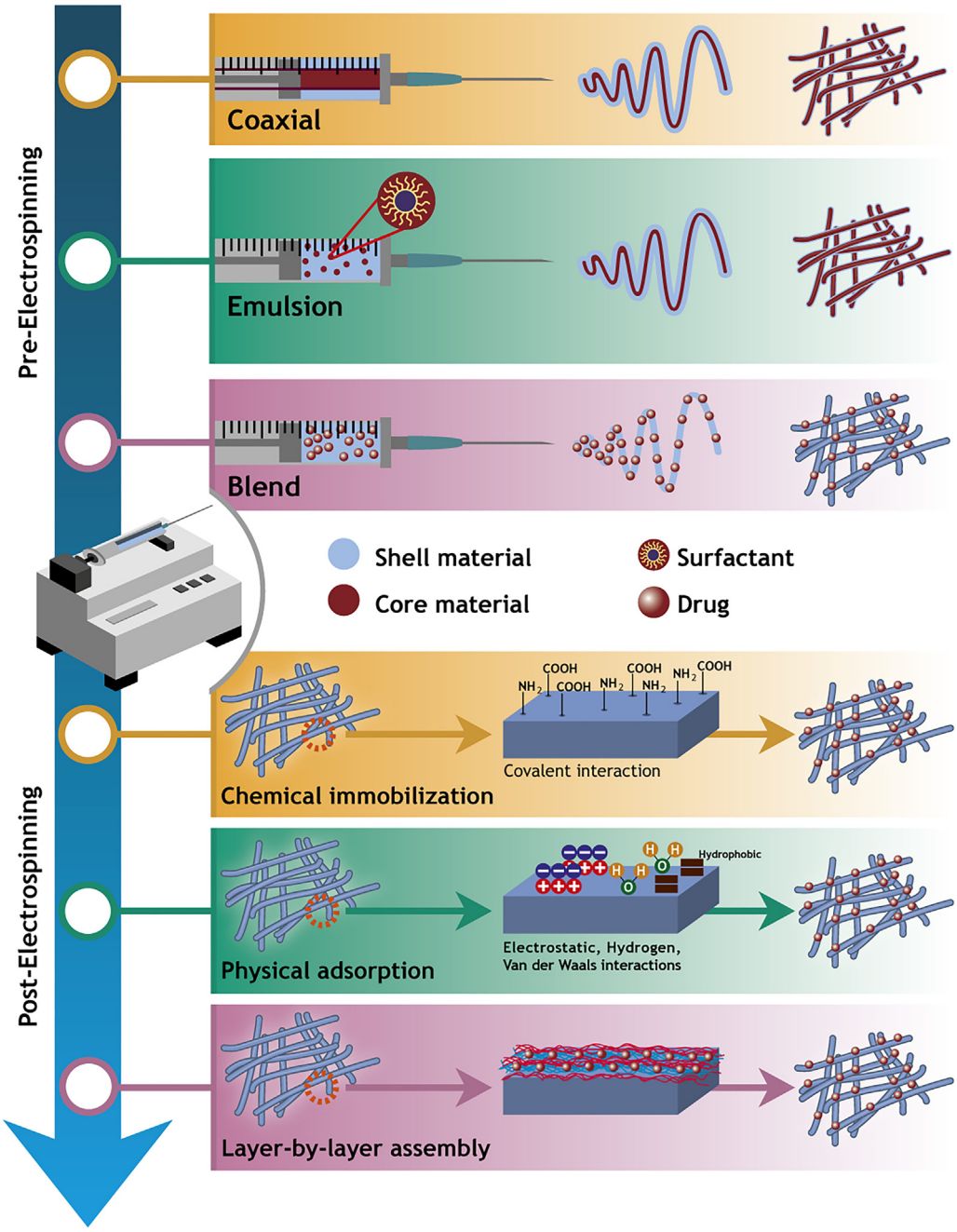

Fig. 5. Illustration of the surface modification techniques used to produce a carrier-based drug delivery nanofibres: pre-electrospinning (blend, co-axial and emulsion electrospinning) and post-electrospinning (physical adsorption, layer-by-layer assembly and chemical immobilization).

display antimicrobial activity and can perform a controlled drug release [81].

Despite the versatility of the blend electrospinning, one major disadvantage of the fibres fabricated with this methodology is the loss of function and activity of the incorporated biomolecules, which compromises the therapeutic effectiveness of the system [79]. To overcome this shortcoming, core-shell fibres have been produced through co-axial and emulsion electrospinning. This type of nanofibres displays an enhanced encapsulation efficiency of drug/bioactive molecules and avoids the direct contact of the biomolecules with the external environment, which is fundamental for unstable biological agents to maintain their biologic activity [82,83].

In co-axial electrospinning, different syringe pumps and coaxial needles are used to produce the core, where the bioactive components are usually loaded, and the shell that provides protection and assures a sustained release of the encapsulated molecules [82]. Maleki et al. compared the capacity of tetracycline hydrochloride-loaded PLGA core-shell nanofibres (produced with a co-axial electrospinning system) with blend fibres (prepared with the same materials) for a sustained drug release [84]. The core shell nanostructures revealed better results.

Emulsion electrospinning can also be used to manufacture core-shell nanofibres without requiring a specific needle setup. This method relies on a chemical separation through the creation of an emulsion within a single solution and the subsequent organisation of the emulsified droplets into two distinct phases, as the solvent evaporates from the electrospun fibres [85]. Wang et al. produced and EGF-loaded PCL/hyaluronan nanofibrous mesh, using an emulsion electrospinning technique. Their findings showed that the

resulting modified hyaluronan-based membranes can encapsulate and release EGF, which is fundamental for skin tissue engineering [86].

4.2.2. Post-electrospinning surface modifications techniques

A number of approaches have been employed to immobilize biomolecules or small cell recognition motifs onto the surfaces of electrospun polymeric nanofibres. Drug immobilization on the nanofibre's surface that is stronger and offers a more stable linkage can be achieved by physical immobilization through simple physical adsorption, layer-by-layer assembly or by chemical immobilization techniques [64].

Physical surface adsorption is the simplest approach for the preparation of surfaces with well-defined properties, and does not rely on chemical processing [87]. Generally, weak nonspecific intermolecular interactions (like those found in electrostatic interactions, hydrogen bonding, hydrophobic interactions and Van der Waals forces) are established between the surface and peptide sequences [88]. One strategy used for a successful physical immobilisation on the surface is based on the use of materials with a high surface area-to-volume ratio, as presented by electrospun polymeric nanofibres. The typical porous structure of these matrices results in a higher drug loading capacity per unit mass than for any other morphologies [64]. Casper et al. produced electrospun PEG nanofibres functionalised with low molecular weight heparin, a highly sulfated glycosaminoglycan that binds GFs, for applications in drug delivery and wound repair. The analysis of their results suggests the conclusion that this functionalisation method enables the binding of the basic fibroblast growth factor (bFGF) on the surface of PEG nanofibres [89].

Over the past few decades, layer-by-layer (LbL) assembly has attracted research attention due to its ability to exert nanometer control over film thickness and provide a simple, useful and versatile methodology for material surface modification [90]. Generally, LbL assembly is a cyclical process embracing the alternated depositing of polymers exhibiting opposite charges at their surface to formulate a coating of polyelectrolyte multilayers (PEMs) or free-standing film. The depositing process can be repeated until a multilayer film of the desired thickness is assembled [64,90,91]. In the assembly process, electrostatic attraction is the main driving force, although, hydrogen bonding, hydrophobic, covalent and biological interactions can also play a vital role [64]. This build-up can precisely control the composition, morphology and structure of the film [91].

In recent studies, LbL assembly has been used in a wide range of applications in the biomedical field, including wound healing. Huang et al. produced cellulose acetate nanofibrous mats that were used as a substrate for LbL films composed of positively charged chitosan derivative, HTCC (lysozyme (antibacterial agent)- N-[(2-hydroxy-3-trimethyl-ammonium) propyl]), and negatively charged sodium alginate. The results obtained showed that the average diameter of fibres increased with the number of coating bilayers applied. Moreover, the produced fibres exhibited antimicrobial activity [92]. Similarly, Xin et al. developed novel cellulose nanofibrous mats coated with SF (negatively charged) and lysozyme (positively charged). The *in vitro* and *in vivo* assays demonstrated that the mats can promote both healing in wounds and avoidance of wound infection [93]. Huang et al. reported the production of biomimetic nanofibrous matrices that were coated using LbL assembly of chitosan (positively charged) and Type I collagen (negatively charged). The LbL structured nanofibrous membranes displayed enhanced *in vitro* cell migration and promoted *in vivo* skin re-epithelialization and vascularisation. These results demonstrate the potential of LbL structured nanofibrous matrices to restore the structural and functional properties of skin [94].

Although the LbL assembly process is simple and mild, it can be affected by many factors such as the concentration and ionic strength of the polyelectrolyte solution as well as the pH, temperature, assembly time and molecular weight of the polymers used [91]. In addition, as the driving force is a result of electrostatic interactions, they are easily leached out, when incubated over an extended period, from the surface of the modified nanofibres. Therefore, chemical immobilisation of bioactive molecules is favoured over physical immobilisation [64].

Table 2
Electrospun membranes loaded with antimicrobial agents to be used as wound dressings.

Antimicrobial agents	Polymers	Incorporation technique	Ref.
Ampicillin	PCL; PMMA/nylon;	Blend; Co-axial	[109,110]
Amoxicillin	nano-HA/PLGA; PLGA; PCL	Blend; Nanoparticle incorporation;	[111–114]
Berberine	Collagen/ZN	Blend	[40]
Cefazolin	PLGA; Gel:CS/PEO	Blend; Nanoparticle incorporation	[115–117]
Cefoxitin	PLGA	Blend	[118,119]
Chitosan	PLA; PEO; SF; PCL; AV/PEO; sericin; pectin/organic rectorite	Blend; Layer-by-layer	[12,55,108,120,121]
Cinnamaldehyde	CS/PEO; PLA/β-CD;	Blend; β-cyclodextrin incorporation	[122,123]
Ciprofloxacin	Dextran/PU; PU; coPLA/PEG; PDEGMA/PLACL	Blend	[101,124,125]
Fusidic acid	PLGA	Blend	[126–128]
Gentamycin	PCL; CS	Co-axial; liposomes adsorption	[129,130]
Lysostaphin	Cellulose/CS;	Chemical immobilisation	[131]
Lysozyme	Cellulose acetate	Physical surface adosprtion	[132]
Mefoxin	PLGA; PDLA/PLA	Blend	[119,133]
Mupirocin	PLA	Blend	[134]
Plant extracts	PCL/PVP; PCL; PVA; PLA/HPG	Blend	[135–138]
Rifampicin	PCL; PLGA	Blend	[126,139]
Salicylic Acid	CS/ZN	Blend	[11]
Silver nanoparticles	Gel; CS/PVA; PVA/MTT; PMMA; PLLCL; CS; SF; PCL;PU	Adsorption; Chemical immobilisation; Blend	[98,99,140–142]
Streptomycin	PU/AC/Zein	Blend	[96]
Tetracycline	PEUU/PLGA; PVA/CS	Blend	[100,143,144]
Titania	PVAc; PU; PMMA; Nylon;	Blend; Chemical immobilisation	[145–148]
Zinc	SA/PVA	Blend	[149]

AC: Cellulose acetate; AV: Aloe vera; coPLA: Poly(l-lactide-co-d,l-lactide); CS: Chitosan; Gel: Gelatin; HPG: Hyperbranched polyglycerol; MTT: Montmorillonite; Nano-HA: Nano-hydroxyapatite; PCL: Polycaprolactone; PDEGMA: poly-(2-(2ethoxy)- ethoxy)methoxy methatcrylate; PDLA: Poly-D-lactide; PEG: Poly(ethylene glycol); PEO: Poly (ethylene oxide); PEUU: Poly(ester urethane) urea; PLA: Poly(lactic acid); PLACL: Poly(l-lactic acid-co-ε-caprolactone); PLGA: Poly Lactic-co-Glycolic Acid; PLLCL: Poly(l-lactic acid)-b-poly(ε-caprolactone); PMMA: Poly(methyl methacrylate); PU: Polyurethane; PVA: Polyvinyl alcohol; PVAc: Polyvinyl acetate; PVP: Polyvinylpyrrolidone; SA: Sodium alginate; SF: Silk fibroin; ZN: Zein; β-CD: β- Ciclodextrins.

In order to fabricate biomimetic materials that can withstand long-term survival, a stable covalent binding of functional biomolecules is required to maintain their bioactivity [87]. To accomplish that, chemical immobilisation of primary amine and carboxylate groups have been extensively employed to immobilise bioactive molecules onto the surface of nanofibres for wound healing [64]. In addition, hydrophilic linkers have also been used to bind bioactive molecules, which can be recognized by cells. For this purpose, Choi et al. produced PCL/PEG electrospun nanofibres functionalised with amine groups on their surfaces, using PEG linkers. Then, in order to use these nanofibrous mats in the treatment of diabetic foot ulcers, EGF was chemically bound to the surface of the meshes. The results obtained showed that the EGF functionalised nanofibres exerted a superior therapeutic effect on wound healing in comparison to control groups. In addition, EGF-receptor was highly expressed in keratinocytes, due to the stimulation exerted by the EGF nanofibre group, showing their suitability to be used as skin substitute [95].

5. Bioactive molecules incorporated into electrospun nanofibrous membranes

5.1. Antimicrobial agents

In wound care management, wound infections are a major concern since they delay the healing process, leading to disfigurement or even patient death [96]. To decrease the probability of a wound becoming infected, researchers are currently producing electrospun nanofibres functionalised with antimicrobial agents, such as nanoparticles, antibiotics and plant extracts (see information presented in Table 2). Silver nanoparticles (AgNPs) are the most common antimicrobial agent incorporated into this type of membranes. The antimicrobial activity exhibited by AgNPs against bacteria and fungi is attributed to the release of Ag^+ ions. These ions bind to thiol groups available on enzymes and cell surface proteins, causing bacteria membranes and cellular walls destabilisation or disruption. In 2008, Rujitanaroj et al. produced electrospun gelatin fibres mats loaded with AgNPs that displayed antibacterial activity against *Pseudomonas aeruginosa* (*P.aeruginosa*), *Staphylococcus aureus* (*S.aureus*), *Escherichia coli* (*E.coli*), and methicillin-resistant *S. aureus* (MRSA) [97]. Furthermore, Wang et al. functionalised PU/keratin nanofibrous mats surfaces with AgNPs and then evaluated their bactericidal activity using *E.coli* and *S.aureus*, as bacteria model. The results showed antimicrobial activity against both bacteria [98].

In 2012, Uttayarat et al. functionalised SF mats with AgNPs and tested their bactericidal activity against *S.aureus* and *P.aeruginosa* [99]. Their results demonstrated that the SF mats coated with low concentrations of AgNPs ($\leq$4 mM) exhibited similar antibacterial properties to those exhibited by commercially available wound dressings, which contain higher concentrations of ionic silver (Tegarderm™Ag and Aquacel®Ag).

Electrospun membranes may also exhibit antimicrobial activity by incorporating antibiotics within nanofibres structures. Liao et al. incorporated tetracycline hydrochloride within PCL/cellulose/dextran electrospun nanofibrous mats. The bactericidal activity of the membranes produced was evaluated against *S.aureus* and *E.coli*. The results revealed that only the samples loaded with the antibiotic displayed bactericidal activity [100].

Heyu Li et al. prepared poly (di(ethylene glycol) methyl ether methacrylate) (PDEGMA) and PLACL thermoresponsive electrospun fibre mats loaded with ciprofloxacin (commonly used for the treatment of skin infections). The *in vitro* assays demonstrated that the produced mats were able to inhibit *E.coli* and *S.aureus* growth. In turn, the *in vivo* assays highlighted that ciprofloxacin-loaded fibres resulted in better wound healing than commercially available gauzes [101].

Over the past decades, due to the limited number of antibiotics available, researchers have evaluated other materials that can be used as antimicrobial agents. Among them, CS, due to its intrinsic antimicrobial activity, emerged as a viable option [3,102–104]. CS antibacterial activity is attributed to the interactions established between the protonated amino groups of CS and the electronegative residues available on the surface of bacteria (lipopolysaccharides in gram-negative and teichoic acid/peptidoglycan in gram-positive) [105]. These interactions lead to the loss of membrane permeability and cell leakage and, ultimately, to the death of the cell [106]. Ignatova et al. produced CS/PLA electrospun membranes and reported that these membranes inhibit *S.aureus* and *E.coli* growth [107]. Zhao et al. produced an electrospun membrane composed of CS and sericin that did not display any cytotoxic effect for fibroblasts cells, while exhibiting an excellent antibacterial activity against *E.coli* and *Bacillus subtilis* [108].

Table 3
Examples of produced electrospun membranes loaded with growth factors to be used in skin regeneration.

Growth Factors	Polymers	Incorporation technique	Ref.
EGF	PLGA/AV	Emulsion	[160]
	PVA/SF	Blend	[81]
	PLGA	Blend	[161]
	PLGA/Gel	Emulsion	[21]
	PCL/Collagen	Chemical immobilisation	[162]
	PCL/Gel	Chemical immobilisation	[163]
	PVA/carbon nanotubes	Blend	[164]
EGF (BSA, insulin, T3)	PLGA/Collagen	Blend and emulsion	[165]
EGF (insulin, hydrocortisone, retinoic acid)	PLACL	Blend and co-axial	[157]
VEGF (BSA)	PLGA	Emulsion	[166]
VEGF, PDGF, bFGF,EGF	Collagen/HA/gelatin nanoparticles	Blend:EGF and bFGF; Gelatin nanoparticles: PDGF and VEGF	[158]
VEGF and PDGF	CS/PEO and PLGA nanoparticles	Blend: VEGF and PDGF:PLGA nanoparticles	[167]
VEGF and TGF-β3	PLGA	Adsorption	[168]
PDGF	EUP3/gelatin	Blend	[144]
bFGF	Polyplexes of PEI/PELA	Emulsion	[169]
	PELA	Emulsion	[29]
PRP (PDGF, TGF-β, VEGF, IGF, HGF)	CS/PEO	Blend	[159]

AV: Aloe vera; bFGF: Basic fibroblast growth factor; BSA: Bovine serum albumin; CS: Chitosan EGF: Epidermal growth factor; EUP3: Platelet-derived growth factor-BB binding polysaccharide; Gel: Gelatin; HA: Hyaluronic acid; HGF: Hepatocyte growth factor; IGF: Insulin-like growth factor; PCL: Polycaprolactone; PDGF: Platelet-derived growth factor; PEI: Polyethylenimine; PELA: Polyethylene oxide (PEO)/polylactic acid (PLA) block copolymers; PEO: Poly (ethylene oxide); PLA: Poly(lactic acid); PLACL: Poly(l-lactic acid-co-ε-caprolactone); PLGA: Poly Lactic-co-Glycolic Acid; PRP: Platelet-rich plasma; PVA: Polyvinyl alcohol; SF: Silk fibroin; TGF-β3: Transforming Growth Factor-β3; T3: Thyroid hormone triiodothyronine; VEGF: Vascular endothelial growth factor.

In 2015, Antunes et al. used deacetylated/arginine-modified CS to produce electrospun membranes. The membranes produced displayed an enhanced bactericidal activity against *E.coli* and *S.aureus*, due to the surface charge presented by arginine-modified CS. This property allowed these membranes to improve the regeneration of full-thickness wounds in comparison to electrospun membranes manufactured with non-modified CS [54].

5.2. Growth factors

The incorporation of GFs within the structure of electrospun nanofibrous membranes is another strategy that has been used to increase the performance of this type of membranes in the wound healing process (see Table 3). GFs stimulate angiogenesis, cell proliferation, differentiation, and the production of ECM components, events that are fundamental for skin regeneration [150]. Nonetheless, the free administration of GFs is not effective, since they rapidly disperse from the target site and suffer enzymatic degradation or deactivation. Therefore, the encapsulation of GFs within drug delivery systems can provide them with protection against *in vivo* degradation and maintain the required GFs concentration at the target site for extended periods, leading to improved skin tissue regeneration [151,152].

Electrospun nanofibres can be easily functionalised with GFs and, depending on the demands of the healing process, EGF, bFGF, VEGF and PDGF can be incorporated within the nanofibrous meshes [153–155]. Table 3 provides an overview of the GFs that have been added to electrospun nanofibrous membranes. Schneider et al. functionalised SF mats with EGF, which promoted cell proliferation and synthesis of ECM molecules, as well as angiogenesis and granulation tissue formation. The mats produced were able to increase the rate of wound closure by more than 3.5-fold in comparison to non-functionalised silk dressings [156]. In 2015, a core-sheath structure composed of PLGA/gelatin loaded with EGF was produced using emulsion electrospinning. The membranes produced were able to sustain the release of the GF for 9 days, which is essential for improving the healing process. Furthermore, the histological data obtained by the authors confirmed that the collagen synthesis was higher in the group treated with PLGA/gelatin/EGF electrospun membranes in comparison to the group treated with PLGA membranes. These results confirmed that the hybrid membranes displayed the bioactivity and hemostasis required for skin tissue engineering [21].

In another study, Jin et al. incorporated multiple epidermal induction factors (EIF) such as EGF, insulin, hydrocortisone and retinoic acid into a gelatin and PLACL solution. Then, they produced nanofibrous meshes using two different approaches: blend and core-shell spinning. Unlike blend fibres, the core-shell nanofibres produced were able to sustain the release of EIF, which contributed to increase the percentage of differentiated adipose-derived stem cells (ADSCs), which are known to reduce the wound size and enhance the re-epithelialization process [157].

Yang et al. produced nanofibrous membranes loaded with bFGF through emulsion electrospinning that were able to gradually release GF over the course of 4 weeks. A complete re-epithelization was achieved when the bFGF-loaded fibrous mats were applied on the dorsal wounds induced on diabetic rats [29].

Lai et al. manufactured a collagen/HA stacking nanofibrous skin equivalent loaded with multiple angiogenic GFs (VEGF, PDGF, bFGF and EGF), that were either directly embedded in the nanofibres or encapsulated within gelatin nanoparticles. Their results showed that bFGF and EGF (loaded into nanofibres) were released according to the demands of the initial phases of the wound healing process (hemostasis and inflammation phases), whereas VEGF and PDGF (encapsulated within gelatin nanoparticles and posteriorly within nanofibres) were released during the proliferation and remodelling phases of the healing process. This data suggested the authors' proposal for the future application of these membranes in the treatment of wounds [158].

Bertoncelj et al. incorporated Platelet-rich plasma (PRP) into CS/PEO electrospun nanofibres and evaluated its performance in the treatment of chronic wounds. Their *in vitro* results showed that CS/PEO nanofibres exhibited suitable properties to support the release of PRP at a rate that promotes keratinocyte and fibroblast cell growth [159].

6. Recent advances in the production of electrospun membranes for skin regeneration

Despite tremendous advancements, electrospun membranes still present some limitations for wound management, since they are unable to fully reproduce the structural features of native skin. In the next section are described two of the most recent approaches used to improve the wound healing process: production of asymmetric membranes and seeding of stem cells on nanofibrous meshes.

6.1. Electrospun asymmetric membranes

Recently, researchers have begun producing asymmetric membranes to reproduce skin anatomy and to further enhance the healing process. Usually, this type of membranes displays a dense and/or hydrophobic microporous top layer that prevents bacteria penetration as well as a macroporous bottom layer that allows the exudate absorption, gaseous exchange and cell migration/proliferation [170,171]. Wu et al. reported the production of nanofibrous asymmetric membranes, using polymeric self-assembly and electrospinning. Their results showed that the upper and hydrophobic layer (composed of hydrophobic β-glucan butyrate) was waterproof and breathable as well as capable of preventing bacterial penetration and controlling moisture evaporation, while the bottom layer (comprised of hydrophilic β-glucan acetate) exhibited good aqueous stability and swelling ratio and was capable of promoting the wound healing process [172]. Figueira et al. produced a bilayered electrospun membrane with a top layer made of PCL and HA. This layer displayed adequate mechanical properties, porosity and wettability that enabled it acts as a physical barrier against external threats to the wound site. ZN, CS and salicylic acid were also combined to produce the bottom layer. Due to their properties, this layer was able to promote human fibroblast adhesion, spreading and proliferation, while also avoiding the growth of microorganisms, reinforcing its suitability for wound healing [11].

Recently, Miguel et al. produced asymmetric membranes with PCL, CS and AV using an electrospinning apparatus. The top layer of the membrane was produced with PCL (in order to mimic the epidermis) while the bottom layer was produced with CS and AV. The results obtained revealed that the top layer has low porosity and excellent mechanical properties. The porous bottom layer, which reproduces the dermis layer structure, promoted fibroblast cell adhesion and proliferation, which are involved in the production of ECM components and play a pivotal role in the healing process. While the dense top layer avoids bacterial infiltration, the bottom layer, due to its composition, inhibits the growth of *S.aureus* and *E.coli* at the wound site [12].

6.2. Electrospun nanofibres membranes and cell engineering

Due to the crucial role played by cells in the healing process, recent studies report the use of different cell lineages (e.g. fibroblasts, keratinocytes, endothelial and stem cells) for the treatment of cutaneous wounds [173–177]. Among these, mesenchymal stem

cells (MSCs) have been widely used, since they are involved in almost all phases of wound healing and are able to stimulate new blood vessel formation, modulate the inflammatory response, promote the migration of keratinocytes and improving ECM production [178,179]. Furthermore, the use of stem cells derived from adipose tissue (ASCs) for regenerative purposes has also been explored by researchers, who find that these cells, which display a high potential for multilineage differentiation, can be obtained using minimally invasive procedures [180].

When stem cells are directly applied at the wound site, rapid cell death/clearance occur. To overcome this shortcoming, stem cells have been seeded on the surface of electrospun nanofibres surface. The ultrafine fibres of electrospun membranes that mimic the ECM topography promote stem cells survival and proliferation. Moreover, alignment of these fibres can control cellular arrangement and differentiation [181–184].

In 2011, Jin et al. produced electrospun nanofibrous using collagen and then seeded MSCs on the surface of the membranes. The results obtained confirmed that nanofibrous meshes promoted the differentiation of MSCs into epidermal cells [185]. The potential use of biomimetic nanofibre scaffolds, functionalised with bone-marrow-derived mesenchymal stem cells (BM-MSCs) for the treatment of acute full-thickness skin wounds, has also been evaluated by Ma et al. Their results demonstrated that enhanced healing was achieved with local delivery of BM-MSCs since these cells become differentiated into epidermal cells [186].

Additionally, Bayati et al. evaluated the effect of electrospun PCL fibres on ASCs differentiation into keratinocyte and on the healing process. The results obtained revealed an increased cell proliferation and an overexpression of the keratinocyte markers (such as cytokeratin 14, filaggrin and involucrin) [187].

Despite these promising results, cell infiltration into electrospun membranes continues to be limited since cells remain at the surface of the electrospun membranes. Currently, to surpass this limitation researchers are currently investigating the results of cell grafting, cell coaxial electrospinning and using layer-by-layer approaches [176,188].

7. Concluding remarks and future perspectives

In the last decades, tremendous progress has been achieved in the development of therapeutic approaches to be used in the treatment of wounds. Among the types wound dressings developed, electrospun membranes are regarded as one of the most efficient wound dressing materials, since they show morphological similarities with skin ECM, *i.e.* they display a high surface area to volume ratio as well as a porous structure that enhances homeostasis, exudate absorption, gas permeability and cell adhesion, migration and proliferation. Herein, insights concerning the recent advances attained in the production of polymeric electrospun nanofibres meshes to be used as wound dressings were provided. The functionalisation methods used to improve the surface properties of nanofibres or to produce nanofibres for carrier-based drug delivery, such as plasma treatment, LbL and grafting have also been described.

However, despite the recent achievements, further developments of nanofibrous meshes are required to improve the healing process. New asymmetric dressings and electrospun nanofibres loaded with stem cells are currently under development for this purpose. In a near future, other techniques, like 3D printing, may be combined with electrospinning to obtain 3D constructs that reproduce in further detail the structure and properties of the ECM of native skin. The incorporation of sensors into electrospun membranes may also impact the diagnostic and theranostic applications of these membranes. Finally, the combination of electrospun membranes with electrical stimulation, mechanical stress or pulsed magnetic field may also contribute toward improving the healing process.

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