DEVELOPMENT OF AN ANTIMICROBIAL WOUND DRESSING BY CO-ELECTROSPINNING BACTERIOCINS OF LACTIC ACID BACTERIA INTO POLYMERIC NANOFIBERS

by

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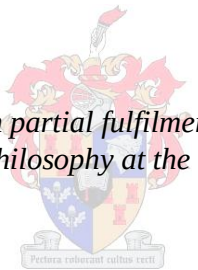

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DECLARATION

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SUMMARY

Skin is the largest organ in the human body and serves as a barrier that protects the underlying tissue of the host from infection. Injury, however, destroys this protective barrier and provides a perfect opportunity for microorganisms to invade the host and cause infection, thereby affecting the normal wound healing processes. Furthermore, the ability of microbial pathogens to rapidly develop resistance towards a variety of antimicrobial compounds hampers the effective treatment and control of infections. Antimicrobial-resistant pathogens are increasingly being isolated from patients, placing a huge burden on the health care sector. The search for new and novel antimicrobial agents and treatments is thus of utmost importance and will continue to play an integral role in medical research.

Antimicrobial peptides (AMPs) may serve as possible alternatives to antibiotics, or may be used in combination with antibiotics to reduce the risk of antimicrobial resistance. AMPs play a role in innate defence and are produced by a variety of mammals, plants, reptiles, amphibians, birds, fish and insects. The AMPs of bacteria (bacteriocins), especially those of lactic acid bacteria (LAB), are receiving increased attention as antimicrobial agents to treat bacterial infections.

Electrospun nanofibers have characteristics that make them suitable as wound dressings, i.e. high oxygen permeability, variable pore size, high surface area to volume ratio and nanofibers are morphologically similar to the extracellular matrix. The ability to incorporate of a variety of biologically active compounds into nanofibers increases their potential as wound dressings. A novel approach would be to incorporate bacteriocins from LAB into nanofiber scaffolds to generate antimicrobial wound dressings.

In this study, the feasibility of co-electrospinning bacteriocins from LAB into nanofibers was investigated. Plantaricin 423, produced by *Lactobacillus plantarum* 423, was successfully co-electrospun into poly(ethylene oxide) (PEO) nanofibers. Plantaricin 423 retained activity after the electrospinning process and continued to inhibit the growth of *Lactobacillus sakei* DSM 20017^{T} and *Enterococcus faecium* HKLHS. Viable cells of *L. plantarum* 423 were also successfully co-electrospun into PEO nanofibers, albeit with a slight reduction in viability. A nanofiber drug delivery system was developed for plantaricin 423 and bacteriocin ST4SA, produced by *Enterococcus mundtii* ST4SA, by blending PEO and poly(D,L-lactide) (PDLLA) in a suitable solvent before electrospinning. Nanofibers were

produced that released the bacteriocins over an extended time period. The PEO:PDLLA (50:50) nanofiber scaffold retained its structure the best upon incubation at 37 °C and released active plantaricin 423 and bacteriocin ST4SA. Nisin A was also successfully co-electrospun into a PEO:PDLLA (50:50) nanofiber scaffold and nisin A, released from the nanofibers, inhibited the growth of *Staphylococcus aureus in vitro*. Nisin A-containing nanofiber scaffolds significantly reduced viable *S. aureus* cells in infected skin wounds and promoted wound healing in non-infected wounds. As far as we could determine we are the first to show that bacteriocin-eluting nanofiber scaffolds can be used to treat skin infections and influence wound healing.

OPSOMMING

Vel is die grootse orgaan in die menslike liggaam en dien as buitelaag wat die gasheer se onderliggende weefsel teen infeksie beskerm. Beskadigde vel verloor egter hierdie beskermende eienskap en gee mikroörganismes die geleentheid om die liggaam binne te dring, infeksie te veroorsaak en die normale prosesse geassosieer met wondgenesing te beïnvloed. Die suksesvolle behandeling en beheer van infeksies word gedemp deur die vermoë van mikroörganismes om vinnig weerstand teen antimikrobiese middels te ontwikkel. Mikroörganismes met antimikrobiese weerstand word geredelik van pasiënte geïsoleer en dit plaas enorme druk op die gesondheidssektor. Die soeke na nuwe antimikrobiese middels en behandelings is dus van uiterste belang en sal altyd ‘n integrale rol in geneeskunde navorsing speel.

Antimikrobiese peptiede (AMPe) kan moontlik as alternatief tot antibiotika dien, of kan in kombinasie daarmee gebruik word om die ontwikkeling van antimikrobiese-weerstandbiedenheid te verhoed. AMPe speel ‘n rol in ingebore beskerming en word deur soogdiere, plante, reptiele, voëls, visse en insekte geproduseer. AMPe van bakterieë (bakteriosiene), veral die van melksuurbakterieë (MSB), wek toenemende belangstelling as antimikrobiese middels vir die behandeling van bakteriële infeksies.

Nanovesels, wat deur middel van ‘n elektrospin proses geproduseer word, het eienskappe wat hul aanloklik maak as wondbedekking, naamlik hoë suurstof deurlaatbaarheid, verskeie porie grottes, ‘n hoë oppervlakte tot volume verhouding, sowel as ‘n morfologiese struktuur wat die ekstrasellulêre matriks naboots. Die vermoë om ‘n verskeidenheid biologies aktiewe komponente in nanovesels te inkorporeer verhoog hul potensiaal as wondbedekkingsmateriaal. ‘n Unieke benadering is die inkorporasie van bakteriosiene van MSB in nanovesels om ‘n antimikrobiese wondbedekking te ontwikkel.

In hierdie studie is die vermoë om bakteriosiene van MSB in nanovesels te inkorporeer, deur middel van ‘n mede-elektrospin proses, ondersoek. Plantarisien 423, geproduseer deur *Lactobacillus plantarum* 423, was suksesvol deur die mede-elektrospin proses in poliëtileen oksied (PEO) nanovesels geinkorporeer. Plantarisien 423 het na die elektrospin proses steeds sy antimikrobiese aktiwiteit behou en het die groei van *Lactobacillus sakei* DSM 20017^{T} en *Enterococcus faecium* HKLHS geïnhibeer. Lewende selle van *L. plantarum* 423 was ook suksesvol deur die mede-elektrospin proses in PEO

nanovesels geinkorporeer, alhoewel die lewensvatbaarheid van die selle effens afgeneem het. ‘n Nanovesel matriks is ontwikkel om die vrystelling van plantarisien 423 en bakteriosien ST4SA, geproduseer deur *Enterococcus mundtii* ST4SA, te beheer deur PEO en poli(D,L-melksuur) (PDLMS) in ‘n geskikte oplosmiddel te vermeng voor die elektrospin proses. Nanovesels is geproduseer wat die bakteriosiene oor ‘n verlengde tydperk kon vrystel. ‘n PEO:PDLMS (50:50) nanovesel matriks het sy stuktuur die beste behou tydens inkubasie by 37 °C en het aktiewe plantarisien 423 en bakteriosien ST4SA vrygestel. Nisien A was met dieselfde tegniek in PEO:PDLMS (50:50) geinkorporeer en nisien A, wat deur die nanovesels vrygestel was, het die groei van *Staphylococcus aureus in vitro* geïnhibeer. Die nisien A-bevattende nanovesel matriks het die aantal lewende selle van *S. aureus* noemenswaardig verminder in geïnfekteerde wonde en kon die genesing van wonde, wat nie geïnfekteer was, stimuleer. Sover ons kon vastel is hierdie die eerste gepubliseerde navorsing wat toon dat bakteriosiene, geinkorporeer in nanovesels, gebruik kan word om vel infeksies te beheer en wondgenesing te stimuleer.

BIOGRAPHICAL SKETCH

Tiaan de Jager Heunis was born in Port Elizabeth on the 5th of August 1986. He matriculated at Outeniqua High School, George, in 2004. He enrolled for a B.Sc. degree in Molecular Biology and Biotechnology at Stellenbosch University in 2005 and obtained the degree in 2007, majoring in Microbiology, Genetics, Biochemistry and Biotechnology. In 2008 he obtained his B.Sc. (Hons) in Wine Biotechnology at the Institute for Wine Biotechnology, Department of Viticulture and Oenology, at Stellenbosch University. In 2009 he enrolled as M.Sc. student in Microbiology at the Department of Microbiology, Stellenbosch University. The degree was upgraded to a Ph.D in November 2010.

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LIST OF SCIENTIFIC PUBLICATIONS

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PATENT FILED FROM THIS WORK

A product for delivering antimicrobially active substances to an infected site. WIPO Patent Application WO/2011/045764.

CONTENTS

CHAPTER 1

INTRODUCTION

Severe damage disrupts the protective skin barrier and exposes underlying tissue to microbial invasion (Altoparlak *et al.*, 2004). *S. aureus* is a major pathogen implicated in skin and soft-tissue infections and is frequently isolated from patients (Daum, 2007). Antibiotic-resistant strains of *S. aureus* have also emerged which cause major problems in hospitals, as well as the community environment (Chang *et al.*, 2003, Kim, 2009, Tsiodras *et al.*, 2001). Antibiotic resistance in bacterial pathogens is becoming problematic and it has now been described as the "end of the antibiotic era" (Alanis, 2005, Hancock, 2007, Neu, 1992). This is emphasized by the fact that no new classes of antibiotics were introduced between 1962 and 2000, followed by only three new classes up to 2010 (Fischbach and Walsh, 2009). There is thus a need to investigate new antimicrobial agents to pave the way forward in infection control.

Bacteria produce a variety of ribosomally synthesized antimicrobial peptides (bacteriocins) that are usually active against closely related species. Some bacteriocins, however, show a broader spectrum of activity (Jack *et al.*, 1995, Kleanhammer, 1993). Bacteriocins can be divided into different classes, which include Class I (post-translationally modified bacteriocins), class II (unmodified bacteriocins) and the bacteriolysins, formerly class III bacteriocins (Cotter *et al.*, 2005). Bacteriocins from LAB have attracted increased attention as antimicrobial agents and may have various biomedical applications (Dicks *et al.*, 2011). Some bacteriocins are active against antibiotic-resistant pathogens including methicillin-resistant *S. aureus*, vancomycin-resistant enterococci and heterogeneous vancomycin-intermediate *S. aureus* (Piper *et al.*, 2011) and can play an integral role as future antimicrobial agents. Bacteriocins also have potential to be used in conjunction with antibiotics to treat infections and prevent the development of resistant microorganisms (Diep *et al.*, 2007).

Nanofibers are polymeric fibers with diameters in the nanometer range and can be produced by various techniques, including electrospinning (Huang *et al.*, 2003). Electrospinning is a process where an electric charge is applied to a polymer solution to form thin fibers at a collector plate (Huang *et al.*, 2003). Electrospun nanofibers have been extensively investigated for the incorporation and release of various molecules, including antibiotics, growth factors, proteins, antifungals, nucleic acids, anti-inflammatories, silver particles as well as bacteria, viruses and mammalian cells (Agarwal *et al.*, 2008). Nanofibers have also been extensively investigated for tissue regeneration and to stimulate wound healing

(Agarwal *et al.*, 2008, Zahedi *et al.*, 2010). The ideal wound dressing should facilitate healing by forming a barrier to prevent microbial colonization and at the same time be able to treat infection, absorb wound exudate, allow gas exchange, be non-immunogenic and non-toxic and must be easily removable without causing damage (Zahedi *et al.*, 2010). Nanofibers fulfil some of the abovementioned roles by having high oxygen permeability, variable pore size, a high surface area to volume ratio and show morphological similarity to the extracellular matrix (Smith and Ma, 2004, Zahedi *et al.*, 2010, Zhou *et al.*, 2008). The ability to co-electrospin a variety of bioactive molecules into nanofibers can increase this potential to develop a wound dressing that fulfils all of these properties.

A novel approach to wound treatment would be to incorporate bacteriocins from LAB into nanofibers to develop an antimicrobial wound dressing. The challenge, however, is to develop a wound dressing that would release the bacteriocins in a localized and controlled manner to inhibit the growth of bacteria in the wound and at the same time promote the healing process.

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CHAPTER 2

LITERATURE REVIEW

1. Skin Infection: Issues and Concerns Regarding *Staphylococcus aureus*

1.1 The wound healing process

Skin protects the host from infection and plays a role in thermoregulation and maintaining of homeostasis, it has immunological, neurosensory and metabolic functions. Skin is divided into the epidermis and the dermis, which form layers above the subcutaneous fat (Church *et al.*, 2000). The epidermis is the outermost layer of skin and consists of epidermal cells, whereas the dermis consists mainly of collagen. Severe skin damage exposes underlying tissue to microbial invasion (Altoparlak *et al.*, 2004, Vindenes and Bjerknes, 1995). If preventative measures are not taken, pathogens can develop biofilms, rendering wounds even more difficult to treat (Ceri *et al.*, 1999, Costerton *et al.*, 1999, de Beer *et al.*, 1994, Edwards and Harding, 2004). Biofilms consist of a complex community of microorganisms aggregated in an extracellular polysaccharide matrix (Costerton *et al.*, 1999). These organisms undergo a phenotypic change, channels are formed in the biofilms to transport nutrients and waste products, and they respond to environmental stimuli as a unit (de Beer *et al.*, 1994, Edwards and Harding, 2004).

Infection prevents successful wound healing, which can be divided into different, but overlapping stages, including homeostasis, inflammation, new tissue formation, remodeling and maturation (Gurtner *et al.*, 2008, Nishio *et al.*, 2008). Components of the coagulation cascade, inflammatory pathways and immune system are involved immediately after injury and play an important role in preventing further blood and fluid loss, removing dead and dying tissue, as well as preventing infection (Gurtner *et al.*, 2008). Homeostasis is rapidly established directly after injury with vascular constrictions and formation of a platelet plug (consisting of platelet, red- and white blood cells), followed by a fibrin-fibronectin matrix (Guo and DiPietro, 2010, Rodero and Khosrotehrani, 2010, Valenick *et al.*, 2005). The fibrin matrix forms a scaffold for cells infiltrating the wound. The platelet plug and surrounding tissue release pro-inflammatory cytokines and growth factors, including transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), as well as epidermal growth factor (EGF) (Guo and DiPietro, 2010).

The inflammatory phase, or cellular phase, is characterized by infiltration of polymorphonuclear neutrophils (PMN's), one hour after injury, which plays a role in degrading bacterial products, prevention of bacterial invasion and the removal of debris (Grose and Werner, 2004, Guo and DiPietro, 2010). They produce proteases and reactive

oxygen species, which facilitate this process, but may also damage host tissue (Greenhalgh, 1998, Guo and DiPietro, 2010). PMN's are attracted to the wound by growth factors and inflammatory cytokines produced by endothelial cells as well as by degradation products from pathogens (Rodero and Khosrotehrani, 2010). They are the dominant cells in the wound during the initial stages and reach a peak 24 h after injury (Kim *et al.*, 2008). PMN's undergo apoptosis once they have completed their task in the wound healing process and release pro-inflammatory cytokines [such as interleukins 1 alpha and beta (IL-α and β) and tumor necrosis factor alpha (TNF-α)], which activate local fibroblasts and keratinocytes and play an important role in macrophage recruitment (Hübner *et al.*, 1996, Martin and Leibovich, 2005, Rodero and Khosrotehrani, 2010). Monocytes infiltrate the wound and differentiate into macrophages 2-3 days after injury (Gurtner *et al.*, 2008, Nishio *et al.*, 2008). They play an important role in wound healing and replace PMN's as the dominant cell type. Macrophages engulf (phagocytise) and degrade bacteria, apoptotic PMN's, as well as damaged tissue (Guo and DiPietro, 2010). They debride tissue by releasing proteases and will release cytokines and growth factors, which stimulate keratinocytes and fibroblasts to promote tissue regeneration ending the inflammatory phase (Guo and DiPietro, 2010, Meszaros *et al.*, 2000, Mosser and Edwards, 2008).

The new tissue formation phase, or proliferative phase, usually overlaps with the inflammatory phase and occurs 2-10 days after injury. It is characterized by cellular proliferation and re-epithelialization of the wound (Guo and DiPietro, 2010, Gurtner *et al.*, 2008). Keratinocytes, from the wound edges, will initially migrate over the injured dermis and migration is facilitated by the production of specific proteases, such as collagenase, to degrade the extracellular matrix (ECM) (Gurtner *et al.*, 2008, Pilcher *et al.*, 1997). Fibroblasts and endothelial cells support capillary growth (which can provide oxygen and nutrients to the new/healing tissue), collagen formation and, together with angiogenesis (growth of new blood vessels), forms granulation tissue (Guo and DiPietro, 2010, Gurtner *et al.*, 2008). Granulation tissue forms a substrate for further keratinocyte migration, which will eventually restore the barrier function (Werner and Grose, 2003). Fibroblasts migrate across the wound and some are stimulated by macrophages to differentiate into myofibroblasts, which contract the wound during the healing process (Opalenik and Davidson, 2005). Fibroblasts, together with myofibroblasts, initiate the production of collagen, glycosaminoglycans and proteoglycans, all of which play a role in formation of new ECM (Guo and DiPietro, 2010, Werner *et al.*, 2007). Production of collagen is a very important function of the fibroblasts, as this increases the

strength of the wound and forms a scaffold for cells to grow and differentiate (Greenhalgh, 1998).

The final stage of wound healing, the remodeling or maturation phase, is initiated after cellular proliferation and ECM synthesis, usually within 2-3 weeks after injury and can last for years (Guo and DiPietro, 2010, Gurtner *et al.,* 2008). Healing processes halt or scale down and most endothelial cells, macrophages and myofibroblasts will either undergo apoptosis or leave the wound while type I collagen replaces type III collagen (Gurtner *et al.*, 2008). During this phase the vascular density returns to normal and the ECM regenerates to a scaffold similar to that of normal tissue (Guo and DiPietro, 2010, Gurtner *et al.*, 2008).

1.2 *S. aureus* and antibiotic-resistant strains of *S. aureus*

S. aureus is a virulent pathogen responsible for superficial and invasive skin and soft-tissue infections (SSTIs), endovascular infections, necrotizing pneumonia, septic arthritis, endocarditis, osteomyelitis, sepsis, empyema, musculoskeletal infections including pyomyositis, necrotizing fasciitis, purpura fulminans, as well as disseminated infections with septic emboli (Bagget *et al.*, 2003, Daum, 2007, Fergie and Purcell, 2001, Francis *et al.*, 2005, Fridkin *et al.*, 2005, Gonzales *et al.*, 2005a,b, Martínez-Aguilar *et al.*, 2003, Miller *et al.*, 2005, Mongkolrattanothai *et al.*, 2003). *S. aureus* is a Gram-positive coccus and a normal member of the skin microflora. It can thus rapidly colonize a wound within a few hours and can cause minor infections or more severe life-threatening infections (Chambers, 2001, Martinez *et al.*, 2009). The close association of *S. aureus* with the medical sector has resulted in rapid increase in antibiotic resistance. It is estimated that 90 to 95% of clinical *S. aureus* strains are resistant to penicillin and furthermore that 60-70% of strains are now resistant to methicillin (MRSA) (Kim, 2009).

MRSA was first reported in 1960's after methicillin, a penicillinase-resistant penicillin, was introduced to treat infections caused by *S. aureus* (Chambers, 2001). The Center for Disease Control and Prevention estimates that there are 10 000 cases of life threatening MRSA infection annually in the US and MRSA now accounts for 40-70% of all *S. aureus* infections in intensive care units (Klevens *et al.*, 2007, Ray *et al.*, 2012). Methicillin resistance is attributed to a gene (*mecA*) that encodes for a low-antibiotic affinity penicillin-binding protein, known as PBP2a (Kim, 2009). This protein confers resistance to β-lactam concentrations which would normally be inhibitory to native PBPs (Kim, 2009). The *mecA*

gene (2.1kb) is located on the *S. aureus* chromosome and is carried on a genetic element known as the staphylococcal chromosomal cassette *mec* (SCC*mec*) (Ito *et al.*, 2001). This SCC*mec* element includes the *mecA* gene, variants of a regulatory gene, *mecR1*, an insertion sequence element (IS431) and cassette chromosome recombinase (*ccr*) genes (Deurenberg and Stobberingh, 2008, Ito *et al.*, 2001). Five different SCC*mec* types have been identified to date (Type I, II, III, IV and V) (Ito *et al.*, 2003, 2004). MRSA can cause infections in patients in health care facilities (nosocomial infections), which is known as health care-associated MRSA or HA-MRSA infections, or can be acquired in the general population. The latter is known as community-acquired MRSA, or CA-MRSA (Herold *et al.*, 1998, Naimi *et al.*, 2003). The CA-MRSA strains are genetically distinct from the HA-MRSA strains, in that they predominantly contain the SCC*mec* type IV and V, whereas the HA-MRSA strains usually contain type I, II and III (Daum *et al.*, 2002, Kim, 2009). SCC*mec* type IV, like type I, confers resistance typically only to β-lactam antibiotics as it does not contain additional resistance genes, whereas the SCC*mec* II and III types confers resistance to multiple antibiotics (Daum *et al.*, 2002, Fey *et al.*, 2003, Ito *et al.*, 2004, Ma *et al.*, 2002). This is because SCC*mec* type II and III contain additional antibiotic resistant genes (Kim, 2009). Even though CA-MRSA is usually only resistant to β-lactam antibiotics, it is frequently more virulent than HA-MRSA and has serious medical implications. This could be due to the fact that CA-MRSA grows faster than HA-MRSA (CA-MRSA contains SCC*mec* type IV which is the smallest of all the SCCmec types and offers less of a metabolic burden) and as a result of containing Panton-Valentine leukocidin (PVL) (Kim, 2009). PVL is a bacterial cytolysin that is associated with skin infections, as well as necrotizing pneumonias (Gillet *et al.*, 2002, Kaneko and Kamio, 2004, Lina *et al.*, 1999). CA-MRSA isolates containing SCC*mec* type I, II, or III have also been reported (Deurenberg and Stobberingh, 2008).

Vancomycin, a glycopeptide that inhibits cell wall synthesis by binding to the D-Ala-D-Ala moiety of lipid II, served as best defence against the emerging threat of MRSA (Groves *et al.*, 1994). However, vancomycin use has led to the emergence of vancomycin-resistant pathogens, such as vancomycin-resistant enterococci (VRE). Decreased sensitivity towards vancomycin soon followed in *S. aureus*, with the emergence of heterogeneous vancomycin-intermediate *S. aureus* (hVISA), vancomycin-intermediate *S. aureus* (VISA) and vancomycin-resistant *S. aureus* (VRSA). hVISA strains show low vancomycin MIC (minimum inhibitory concentration) levels in the range of 1-4 µg/ml, whereas VISA strains have MICs in the range of 4-8 µg/ml and VRSA strains have MICs of ≥16 µg/ml (de Niederhäusern *et al.*, 2011, Liu and Chambers, 2003). Increased vancomycin resistance was

first reported in MRSA isolates from Japan in 1997, showing intermediate resistance (VISA) with an MIC of 8 µg/ml (Hiramatsu *et al.,* 1997). The first VRSA strain was isolated in Michigan in 2002 and resistance was mediated by the *vanA* gene (Chang *et al.,* 2003). Since then, numerous other VRSA strains have been isolated from all over the world (Aligholi *et al.,* 2008, CDC, 2004, Palazzo *et al.,* 2005, Saha *et al.,* 2008, Tiwari and Sen, 2006). VISA strains develop after prolonged exposure to vancomycin and result from adaptation, including thickening of the cell wall, reduced autolytic activity, as well as reduced biofilm forming ability (Howden *et al.,* 2006). VISA strains usually have lower growth rates (Cui *et al.,* 2003, Howden *et al.,* 2006, Smith *et al.,* 1999). Transfer of vancomycin resistance elements (such as *vanA*, which confers high level vancomycin resistance by converting the D-Ala-D-Ala recognition site of vancomycin to D-Ala-D-Lac) has been shown *in vitro*, as well as *in vivo*, from VRE to *S. aureus* (Cetinkaya *et al.,* 2000, de Niederhäusern *et al.,* 2011, Hughes, 2003, Noble *et al.,* 1992). High resistance levels of VRSA can be attributed to the horizontal transfer of a Inc18-like plasmid with a transposon (designated Tn1456) containing the *vanA* gene (de Niederhäusern *et al.,* 2011, Zhu *et al.,* 2008, 2010). VRSA and VRE are usually co-isolated from the same body site of an infected individual or subsequently isolated from a different body site (Zhu *et al.,* 2010). The emergence of VRSA is thus thought to occur by *in vivo* transfer of *vanA* from a *vanA*-containing *Enterococcus* isolate to an *S. aureus* isolate (Zhu *et al.,* 2010).

The first reported case of linezolid-resistant *S. aureus* (LRSA) appeared in 2001. However, recently increased reports on the occurrence of LRSA have surfaced (Endimiani *et al.,* 2011, Hill *et al.,* 2010, Sánchez García *et al.,* 2010, Tsiodras *et al.,* 2001). Linezolid, an oxazolidinone, inhibits proteins synthesis by binding to the catalytic site of the 50S ribosomal subunit and in doing so prevents initiation complex formation with the 30S ribosomal subunit, formylmethionyl-tRNA and mRNA (Ippolito *et al.,* 2008, Leach *et al.,* 2007, Swaney *et al.,* 1998, Wilson *et al.,* 2008). Resistance has been attributed to mutations in the V domain of the 23S rRNA as well as by acquisition of a chloramphenicol-florfenicol resistance gene, designated *Cfr* (Morales *et al.,* 2010, Stefani *et al.,* 2010). This gene encodes for a RNA methyltransferase which results in methylation of A2503 in the 23S rRNA gene (Kehrenberg *et al.,* 2005).

1.3 New-generation anti-staphylococcal agents

The relative ease and high frequency of pathogens to develop antibiotic resistance places an immense burden to search for new and novel antimicrobial agents active against these antibiotic-resistant pathogens. A variety of compounds are in different developmental stages for treatment of infections caused by a variety of Gram-positive bacteria, including *S. aureus*.

Ramoplanin, a lipoglycodepsipeptide produced by *Actinoplanes* ATCC 33076, has reached phase III clinical trials for the treatment of Gram-positive bacterial infections (McCafferty *et al.,* 2002). The use of ramoplanin has, however, been limited by its instability in blood and poor tolerance (van Bambeke, 2006). It is in development as treatment for systemic nosocomial infections caused by VRE as well as treatment for *Clostridium difficile*-associated diarrhoea (Gerding *et al.,* 2008, Walker *et al.,* 2005). Ramoplanin binds to the pyrophosphate moiety of lipid II, sequestering it and thereby inhibiting peptidoglycan biosynthesis, much like the lantibiotic nisin (Cudic *et al.,* 2002, Fang *et al.,* 2006).

Dalbavancin, televancin and oritavancin are all derivatives of vancomycin, termed lipoglycoproteins, and contain modifications to the disaccharide moiety of the heptapeptide core (van Bambeke, 2004). Dalbavancin and ortivancin are currently in phase III clinical trials for treatment of acute bacterial skin and skin structure infections (BSSSIs) and treatment of serious Gram-positive infections, respectively (Zhanel *et al.,* 2010). Ortivancin has been registered for two new studies (SOLO I and SOLO II) (van Hal and Paterson, 2011). Dalbavancin forms a complex with D-Ala-D-Ala in lipid II and inhibits cell wall synthesis, much like other glycopeptides, and is able to dimerise and anchor its lipophilic side chain in membranes (Chen *et al.,* 2007, Ciabatti and Malabarba, 1997, Streit *et al.,* 2004). Oritavancin has interaction with dipeptidyl residues of peptidoglycan precursors and blocks the transglycosylation step in peptidoglycan biosynthesis, hampering bacterial cell wall synthesis (Allen and Nicas, 2003). It can also dissipate the membrane potential and inhibit bacterial RNA synthesis (Zhanel *et al.,* 2010). Televancin has received FDA approval for treatment of complicated BSSSIs caused by susceptible Gram-positive bacteria, including MRSA and methicillin-susceptible *S. aureus* (MSSA) (Corey *et al.,* 2009). Televancin has a dual mode of action by binding to D-Ala-D-Ala of lipid II and hampering bacterial cell wall synthesis, as well as being able to disrupt bacterial membranes (Higgins *et al.,* 2005, Saravolatz *et al.,* 2009). Interestingly, dalbavancin, televancin and oritavancin are active against some vancomycin-resistant bacterial pathogens, even though they are derived from vancomycin. Delbavancin shows activity towards vancomycin-susceptible enterococci as well as VRE,

with an MIC of 0.03-0.12 µg/ml. However, it has poor activity against VRE strains containing the *vanA* gene (Chen *et al.,* 2007). Ortivancin is active against *vanA*- and *vanB*-containing VRE (Zhanel *et al.,* 2010). Televancin and dalbavancin are active against *vanB*-containing enterococci, as well as VISA and hVISA strains, however display poor activity towards VRSA strains (Leuthner *et al.*, 2006, Zhanel *et al.,* 2010). Ortivancin on the other hand displays good activity towards both VISA and VRSA strains (Allen and Nicas, 2003). Ortivancin shows activity towards *vanA* strains as it is able to inhibit cell wall synthesis, dissipate membrane potential, inhibit bacterial RNA synthesis and most importantly able to bind to D-Ala-D-Lac (Zhanel *et al.*, 2010).

Ceftobiprole and ceftaroline, two new cephalosporins, have been developed for treatment of MRSA. Ceftaroline has received FDA approval in 2010 for treatment of complex SSTIs, whereas the approval of ceftobiprole has been denied (van Hal and Paterson, 2011). Ceftaroline is able to bind to PBPs and inhibits their transpeptidase activity in cell wall synthesis (Villegas-Estrada *et al.*, 2008).

Iclaprim, a novel diaminopyrimidine, interacts with bacterial dihydrofolate reductases which are crucial in producing tetrahydrofolate and recycling it after reoxidation in the formation of thymidylate from deoxyuridylate, and thereby serves as a dihydrofolate inhibitor (Schneider *et al.,* 2003). Inhibition of this enzyme arrests DNA synthesis and cell division, leading to cell death (Oefner *et al.,* 2009). Iclaprim shows activity towards Gram-positive bacteria, including MRSA, VISA and F98Y trimethoprim-resistant strains (Oefner *et al.,* 2009).

Tomopenem, a novel 1β-methylcarbapenem, binds to PBPs and shows broad spectrum activity against Gram-positive and Gram-negative pathogens including MRSA, penicillin-resistant *Streptococcus pneumoniae*, *Haemophilus influenzae*, members of the family *Enterobacteriaceae,* as well as *Pseudomonas aeruginosa* (Koga *et al.,* 2005, Tanaka *et al.,* 2009, Thomson and Moland, 2004).

More recently, several new and novel antibiotics have entered human clinical trials. GSK1322322 (a hydrazinopyrimidine) is in development (phase II developmental stage) for treating complicated BSSSIs, as well as hospitalized community-acquired bacterial pneumonia (Moir *et al.*, 2012). GSK1322322 is a peptide deformylase (PDF) inhibitor and prevents the removal of N-formyl groups on the first methionine during peptide elongation, preventing protein synthesis (Johnson *et al.,* 2005). This compound shows *in vitro* activity against multidrug-resistant *S. pneumonia*, MRSA, *Moraxella catarrhalis* and *Streptococcus*

pyogenes, and promising results have been obtained in *in vivo* animal models, especially against *S. aureus* infections (Moir *et al.*, 2012). AFN-1252 (a benzofuran naphthyridinone in phase I developmental stage) is in development for treatment of susceptible and/or multidrug-resistant staphylococci, by inhibiting the enoyl-acyl carrier protein reductase (encoded by *fabI*) playing a role in bacterial fatty acid biosynthesis (Moir *et al.*, 2012). AFN-1252 shows *in vitro* activity against MSSA, MRSA, methicillin-susceptible *S. epidermidis* (MSSE), and methicillin-resistant *S. epidermidis* (MRSE) (Karlowsky *et al.*, 2007). AFN-1252 was more efficacious than linezolid when tested *in vivo* and had lower ED_{50} values when compared to that of linezolid (Moir *et al.*, 2012). AFN-1252 was safe and well tolerated in a phase I study. MUT056399 (also known as FAB001) and CG400549 are both triclosan analogs and also inhibitors of FabI, thus inhibiting fatty acid biosynthesis (Escaich *et al.*, 2011, Moir *et al.*, 2012). MUT056399 shows *in vitro* activity against MSSA, MRSA, LRSA, coagulase-negative staphylococci, multidrug-resistant strains as well as FabI-containing Gram-negative bacteria including *E. coli*, *H. influenzae*, *Neisseria gonorrhoeae*, *Neisseria meningitides*, *Helicobacter pylori* and *M. catarrhalis* (Escaich *et al.*, 2011). Strains resistant to MUT056399 could be detected at low frequency and all had mutations in the *fabI* gene (Escaich *et al.*, 2011). MUT056399 also showed promising results when tested *in vivo* against MSSA, MRSA and VISA strains (Escaich *et al.*, 2011). XF-73 is a dicationic porphyrin derivative (phase I development) in development for post-surgical staphylococcal, including MSSA and MRSA, infections (Moir *et al.*, 2012). XF-73 seems to act on the bacterial membrane and leads to inhibition of DNA, RNA and protein synthesis and a loss of K^+ and ATP, however, does not seem to lyse the bacterial cells (Ooi *et al.*, 2009). A phase I clinical study showed promising result with regards to tolerability and preliminary data indicates anti-staphylococcal activity, an additional phase I trial is being initiated for XF-73 (Moir *et al.*, 2012). Lastly, TD-1792 is a heterodimer antibiotic, generated by chemical linking of vancomycin to a cephalosporin, with reported activity against Gram-positive bacteria including MRSA and VISA strains (Blais *et al.*, 2012, Leuthner *et al.*, 2010, Long *et al.*, 2008).

1.4 *In vivo* animal models for studying skin infection and wound healing

Various animal models have been used to induce and study skin infections. These animal models include: (1) punch biopsies, resulting in excisional wounds (2) tape-stripping skin aberration models (3) burn wound models (4) skin-suture wound models, as well as (5) subcutaneous infection models.

1.4.1 Punch biopsies generating excisional wounds

Full-thickness excisional wounds were generated on the back of BALB/c mice by single punch biopsy, after which the wounds were infected with a MRSA strain (10^7 cfu/ml) and subsequently treated with nitric oxide nanoparticles (NO-np) (Martinez *et al.*, 2009). NO-np treatment resulted in reduced MRSA counts and collagen degradation was prevented by NO-np treatment. NO-np increased wound healing and caused a reduction in the size of the eschar, as compared to controls.

The antimicrobial peptide ranalexin, in combination with lysostaphin, was more effective in treating excisional wounds infected with MRSA (10^9 cfu) in New Zealand rabbits compared to the peptides on their own, as well as compared to vancomycin (Desbois *et al.*, 2010). Combined treatments reduced MRSA counts from 7.5 $\log_{10}$ cfu (control) to 3 $\log_{10}$ cfu.

1.4.2 Tape stripping skin aberration models

During tape stripping, the fur and epidermal layer on the dorsal surface of an animal (i.e. mouse) is removed by successive applications of an adhesive bandage (Kugelberg *et al.*, 2005). Kugelberg *et al.* (2005) tape stripped an area of 2 cm^2 on the back of female BALB/c mice and subsequently infected the wounds with either *S. aureus* or *S. pyogenes* (10^7 cfu). Tape stripping resulted in disruption of the skin barrier and homogeneous removal of the upper epidermal layers was achieved. The wounds were treated with 2% fusidic acid in ointment, resulting in significant reduction in bacterial counts. *S. aureus* and *S. pyogenes* infection induced an acute inflammatory response, as neutrophils, lymphocytes, histiocytes, and fibrin deposition was observed and the inflammatory response included most of the skin layers.

Tape stripping was used to study the toxicity of penicillin-conjugated nanoparticle emulsions in female CF1 mice (Greenhalgh and Turos, 2009). Mice receiving lower solid-content emulsions (5% or 2%) took longer to heal than mice with higher solid-content emulsions (7% or 9 %). Healing occurred on day 5 and 6 in the lower solid-content emulsion group, whereas mice receiving 9% took 2 days to heal. No significant inflammatory response was seen in mice receiving nanoparticle treatments, as IL-6 levels stayed the same between control and treated groups.

1.4.3 Burn wound models

1.4.3.1 Burns induced by brass blocks

The hair of BALB/c mice was removed and mice were anesthetized by ether fumes, followed by inducing a burn by applying a heated brass bar on the dorsal surface of the mice for 45 s (Kumari *et al.*, 2010). After 30 min the mice were infected with 10^2-10^{10} cfu/ml *Klebsiella pneumonia* to determine a lethal dose, which was determined at 10^8 cfu/ml *K. pneumonia*. Infected mice were subsequently treated with hydroxyl propyl methyl cellulose hydrogels (HPMC) with Kpn5 (*Klebsiella* bacteriophage Kpn 5), honey or aloe vera gel. Hydrogels showed no signs of toxicity in the mice, when using a single- and three-patch application test. Topically applied honey and aloe vera gel resulted in survival rates of 33.3% and 26.7% respectively, whereas the control had 0% survival rate. Mice receiving high phage titers (10^{10} pfu/ml), suspended in 3% HPMC, showed high survival rates of 66.7%, whereas the control had 0% survival. Phage Kpn 5 had good stability in 3% HPMC. Addition of honey and aloe vera gel to phage Kpn 5 did not result in increased survival rates.

The dorsal surface of BALB/c mice was shaved and excess hair was removed with Nair, followed by inducing a burn by placing two heated brass blocks (92-95 °C) on the mice (10-60 s) (Dai *et al.*, 2009). Mice were subsequently resuscitated with intraperitoneal injection of saline and infected with either 10^7-10^9 cfu/ml of *P. aeruginosa* (Xen 5) or with 10^8 cfu/ml *P. mirabilis* (Xen 44) on 10- and 30-s burns. Both strains were purchased from Xenogen and contain the entire bacterial *lux* operon, which allowed *in vivo* bioluminescent imaging. A 10 s burn with 10^7 cfu/ml *P. aeruginosa* Xen 5 and a 30 s burn with 10^8 cfu/ml *P. mirabilis* (Xen 44) was used in subsequent experiments. A decrease in bioluminescent signal was observed in mice receiving chitosan acetate and silver dressings as compared to the control, when mice were infected with *P. aeruginosa* Xen 5. Survival rates of the mice after 4 weeks were 73.3%, 27.3% and 13.3% for the chitosan acetate, silver dressing and control groups, respectively. No bioluminescent *P. aeruginosa* Xen 5 was observed in the blood of mice. Survival rates of mice infected with 10^8 cfu/ml *P. mirabilis* were 66.7%, 62.5% and 0% for chitosan acetate, silver dressing and the control groups, respectively. Bioluminescent *P. mirabilis* was detected in the blood of mice.

Burns were induced by applying a hot metal weight (1 g, 11 mm in diameter and 90 °C) for 2 min on the dorsal surface of female CD-1 mice (Ostorhazi *et al.*, 2010). Mice were subsequently infected with either 10^3, 10^9 or 10^{11} cfu/wound *Acinetobacter baumannii*, respectively. Treatment with synthetic peptide A3-APO resulted in <1000 cfu/mg wound and

wounds showed signs of regeneration of the epithelial layer, had no pus and showed decreased levels of inflamed granulation. The control group had large amounts of necrotic tissue with polymorphonuclear pus cells and loose granulation tissue.

1.4.3.2 Burns induced by water

Skin of Wistar rats were disinfected with ethanol and water (95 °C) was placed on the skin for 10 s to induce a burn (Ribeiro *et al.,* 2009). Damaged skin was removed with surgical scissors and forceps, 2 h after the burn was induced and treated with chitosan hydrogels. The chitosan hydrogels accelerated wound healing, which was significant for the first 9 days.

Dorsal hair of Sprague-Dawley rats was clipped and removed with depilatory cream, after which rats were placed in an insulated mold that exposed 20% of the total body surface (Hemmila *et al.*, 2010). Rats were placed in a 60 °C water bath for 25 s to induce burns, which were scrub debrided with saline. Wounds were subsequently infected with 10^6 cfu/ml *P. aeruginosa* and treated with nanoemulsion compound NB-201. Saline and 5% mafenide acetate served as controls. Nanoemulsion NB-201 was able to reduce *P. aeruginosa* cell numbers significantly, as well as decrease levels of pro-inflammatory cytokines (interleukin [IL]-1β and IL-6). The degree of hair follicle cell apoptosis in skin, compared to saline-treated controls, was also reduced.

Suzuki *et al.* (1991) developed a burn model based on skin contact with a glass chamber through which water circulates at a predetermined temperature. Kiyozumi *et al.* (2007) used this model to compare the efficacy of a chitosan hydrogel, containing DMEM/F12 medium (medium-Az-CH-LA), and a collagen sponge on deep dermal burns. Both treatments healed the wound in the same time, however re-epithelialization occurred faster (day 4) around the edges of the medium-Az-CH-LA-treated wounds, as compared to the collagen sponge-treated group (day 8). Granulation tissue thickness was also greater in this group and degradation and neovascularization occurred earlier, compared to the collagen sponge-treated group.

1.4.3.3 Burns induced by ethanol

Stieritz and Holder (1975) developed an ethanol bath burn model in mice, where an asbestos board was placed against the dorsal surface of female CF1 mice, exposing 30% of the total body surface area. Ethanol was spread over the exposed surface and set alight for 10 s. *P. aeruginosa* infected burn wounds were induced by subcutaneous injection (100 cfu).

1.4.4 Skin-suture wound model

Sutures were soaked in a 1:100 dilution of *S. aureus* and/or *S. pyogenes*, before being placed between the skin layers and the *panniculus carnosus* of male MF1 mice (Boon and Beale, 1987). Subsequently, a 1 cm incision was made through the skin, along the length of the suture and mice were treated orally with either amoxicillin or amoxicillin-clavulanic acid. Amoxicillin and amoxicillin-clavulanic acid were both effective in reducing *S. pyogenes* numbers in wounds. However, amoxicillin failed to eliminate *S. pyogenes* from a mixed infection, containing *S. aureus* and *S. pyogenes*. *S. aureus* numbers were reduced by amoxicillin-clavulanic acid treatment and controlled the *S. aureus* infection, whereas amoxicillin was ineffective.

Sutures, which had been soaked in bacterial suspensions (10^8 cfu/ml *S. aureus* or *S. pyogenes*), were inserted under the skin of female MF1 mice and male golden Syrian hamsters and an incision made along the length of the suture (Gisby and Bryant, 2000). The wound was closed with an adhesive skin closure and treated with mupiricin cream, mupirocin ointment, their respective vehicle placebos, or fusidic acid cream. Oral administration of erythromycin, cephalexin and flucloxacillin was also investigated as treatment. In female MF1 mice, mupirocin cream was not significantly better than mupirocin ointment in reducing bacterial numbers. It had similar efficacy to oral flucloxacillin, however was significantly more effective than oral erythromycin. Similar efficacy to cephalexin against *S. pyogenes*, but superior efficacy against *S. aureus* was observed for mupirocin cream. It had similar efficacy to fusidic acid cream against *S. aureus* but was significantly superior against *S. pyogenes*. In a hamster model, infected with *S. aureus,* mupirocin cream had similar efficacy to mupirocin ointment and was not significantly different from fusidic acid cream or neomycin-bacitracin cream. It had, however, better efficacy than oral erythromycin and cephalexin. The authors concluded that mupirocin cream was as effective as, or superior to, oral and other topical agents commonly used for skin infections.

1.4.5 Subcutaneous infection

Fernandez *et al.* (2010) studied the effect of ceftobiprole, cefazolin, vancomycin, or linezolid on *S. aureus* infection, as well as ceftobiprole, medocaril, cefepime, or meropenem-cilastatin on *P. aeruginosa* infection, in female SKH1 mice. Mice received a subcutaneous injection of Cytodex (dextran microbeads) inoculum containing *S. aureus* Smith OC 4172 on the left flank and Cytodex suspension containing MRSA OC 8525 on the right flank. Ceftobiprole was shown to be more effective in reducing bacterial number in the skin than cefazolin,

vancomycin, or linezolid. Skin lesions were 19 to 29% lower than those for the other groups (treated with cefazolin, vancomycin, or linezolid). Lesion size, in mice treated with ceftobiprole, was 34% less compared to cefazolin or linezolid treated groups, when MRSA infections were studied. Later, immunocompromised female SKH1 mice received a subcutaneous injection of Cytodex *P. aeruginosa* OC 4351 (with an inducible AmpC β-lactamase) and *P. aeruginosa* OC 4354 (overproducing AmpC β-lactamase) in the left and right flank, respectively. Ceftobiprole, showed similar results when compared to meropenem-cilastatin. Both of these treatments were more effective than cefepime against *P. aeruginosa* OC 4351. Ceftobiprole showed similar results to meropenem-cilastatin and was more effective than cefepime in reducing the size of the lesions caused by either of *P. aeruginosa* strains.

Immune response in different mouse strain backgrounds (C57BL6, BALBc and DBA2 mice) as a result of subcutaneous *S. aureus* infection was studied by Nippe *et al.* (2011). Mice were inoculated subcutaneously with 2×10^7 cfu of *S. aureus.* C57BL/6 mice were more susceptible than BALB/c and DBA/2 mice. They had significantly higher footpad swelling and bacterial load, as well as increased dissemination of bacteria into inguinal lymph nodes and kidneys. Resistance correlated with a *S. aureus*-specific Th2-cell response in BALB/c and DBA/2 mice, whereas susceptible C57BL/6 mice generated a Th1-cell response.

1.5 Bioluminescent imaging (BLI) for monitoring infections in real time

The use of bioluminescent pathogens has become a powerful tool to monitor infection in real time in animal model systems and can have significant power to increase the success rate of preclinical studies of antimicrobial agents. Classical methods have drawbacks where large quantities of animals are needed for statistically significant data and animals need to be euthanized to determine pathogen numbers, thus making comparisons more difficult with inter sample differences (Andreu *et al.*, 2011). Bioluminescent imaging (BLI) overcomes this problem as it is possible to use the same animal during the course of the experiment. This reduces the number of animals needed for each experiment as well as eliminate inter sample differences. The bioluminescent system also has lower background noise, as compared to fluorescence based reporter systems, especially *in vivo* (Andreu *et al.*, 2011). *In vivo* BLI is made possible by various imaging systems such as the *In Vivo* Imaging System (IVIS), manufactured by Caliper Life Sciences (Hopkinton, MA), which detects the bioluminescent signal emitted from *lux*-tagged pathogens. A specialized charged coupled device (CCD)

camera captures the photons emitted, converts the photons to electrons and generates an image by encoding it into electrical patterns. Background noise is substantially reduced by using a cooled CCD camera (-90°C). Bacterial bioluminescence has been used to study promoters in a variety of Gram-negative organisms (Waidmann *et al.,* 2011). The *lux* operon from *Photorhabdus luminescens* is ideal for *in vivo* BLI and has been extensively studied and used in this regard, as the luciferase is thermally stable (Meighen, 1991). Bacterial bioluminescence is encoded by several *lux* genes and involves the production of detectable light (emitted at 490 nm) by the oxidation of luciferin and reduced flavin mononucleotide ($FMNH_2$), catalysed by a luciferase (Meighen, 1991). *LuxAB* encodes for the α and β subunit of the luciferase and *luxCDE* encodes for a transferase, synthetase and reductase, respectively. These proteins play a role in the conversion of fatty acids into long chain fatty aldehydes needed for the bioluminescent reaction (Meighen, 1991). The *luxCDABE* operon has been successfully cloned into Gram-negative bacteria including, *Salmonella enterica* serovar Typhimurium, *E. coli*, *Citrobacter rodentium* as well as *Campylobacter jejuni* (Allen and Griffiths, 2001, Beeston and Surrette, 2002, Belkin *et al.,* 1996, Contag *et al.,* 1995, Wiles *et al.,* 2004). After the introduction of Gram-positive ribosome binding sites and reshuffling of the gene order (from *luxCDABE* to *luxABCDE*) it was also functionally expressed in Gram-positive bacteria (Francis *et al.,* 2000). Another approach for expression in Gram-positive bacteria was to introduce optimized Gram-positive translational signals in front of *luxA, C* and *E* (Qazi *et al.,* 2001). Bioluminescent Gram-positive bacteria have also been constructed using a novel Gram-positive *lux* transposon cassette, which is integrated into the host chromosome. This overcame problems with plasmid based approaches (Francis *et al.,* 2001). Bioluminescent pathogens have been used to study infection, including skin infections, implant related infections (including biofilm formation), lung infections, muscle infections, endocarditis, peritonitis infections, as well as bacterial meningitis (Bernthal *et al.,* 2010, Dai *et al.,* 2009, Engelsman *et al.,* 2008, Francis *et al.,* 2000, 2001, Kadurugamuwa *et al.,* 2003, 2005a,b, Kuklin *et al.,* 2003, Li *et al.,* 2008, Mortin *et al.,* 2007, Xiong *et al.,* 2005). The first *in vivo* BLI study was conducted by Contag *et al.* (1995) with mice infected with a *lux*-tagged *S. enterica* serovar Typhimurium. Since then various other bioluminescent pathogens have been used for BLI, including *S. aureus, P. aeruginosa, A. baumannii, S. pneumonia, C. rodentium* as well as eukaryotic pathogens including *Candida albicans* (Dai *et al.,* 2009, 2011, Francis *et al.,* 2000, 2001, Kadurugamuwa *et al.,* 2003, Wiles *et al.,* 2006).

In vivo BLI, however, has some drawbacks and certain factors need to be considered when conclusions are drawn from *in vivo* experiments. Photon intensity is reduced 10 fold

with an increase of 1 cm in tissue depth (Contag *et al.*, 1995). Oxygen and adenosine-5'-triphosphate (ATP) is also very important as they are needed by the luciferase to oxidize luciferin (Andreu *et al.*, 2011, Meighen, 1991). Bioluminescent signal emitted from these pathogens is thus an indicator of the metabolic state of the cells, as the luciferase is an energy requiring oxygenase needing ATP, as previously mentioned (Andreu *et al.*, 2011). *In vivo* BLI of pathogens in oxygen scarce environments could be problematic, however, some successful studies have been reported. *C. rodentium* was able to colonize and emit a bioluminescent signal from the murine colon and bioluminescence from *S. enterica* serovar Typhimurium was detected from the cecum of animals (Contag *et al.*, 1995, Wiles *et al.*, 2006). Some studies showed that bioluminescence correlated well with viable cell numbers, however, discrepancies have been reported in others *in vitro* as well as *in vivo* (Francis *et al.*, 2000, 2001, Kadurugamuwa *et al.*, 2003, Marincs, 2000, Marques *et al.*, 2005). Detection limit is another factor to consider. It has been shown that the detection limit of bioluminescence from *Bacillus anthracis* ranged from 3 x 10^3 to 2 x 10^5 in the ear, superficial parotid lymph node, spleen and right lung, respectively (Glomski *et al.*, 2007). Wiles *et al.* (2004) reported that 10^3 cfu was the detection limit for bioluminescence from *C. rodentium* in the foci of mice. Thus, for accurate conclusions to be drawn there is still a need to verify the *in vivo* bioluminescent measurements with classical methods if infection studies are conducted.

2. Bacteriocins of Gram-positive Bacteria: Next-Generation Antimicrobials?

2.1 Introduction and classification

Bacteriocins are bacterial ribosomally synthesized proteins which usually show antibacterial activity against closely related species. A few bacteriocins, however, have a broader spectrum of activity, i.e. bactericidal or bacteriostatic against species genetically not closely related to the producer strain (De Vuyst and Vandamme, 1994, Jack *et al.*, 1995, Kleanhammer, 1993). Bacteriocins have been classified and re-classified as the wealth of knowledge increased and various classification systems have been proposed. The latest classification system has been proposed by Rea *et al.* (2011) and is built on that proposed by Cotter *et al.* (2005). The classes include class I (post-translationally modified bacteriocins), class II (unmodified bacteriocins) and the bacteriolysins (formerly class III bacteriocins). Class Ia (lantibiotics) are small peptides (<5 kDa and contain 19-28 amino acids) which undergo post-translational modification. They contain unusual amino acids such as dehydroalanine, dehydrobutyrine and D-alanine, as well as thioether bridges which form *meso*-lanthionine and β-methyllanthionine

as a result of post-translational modifications. Lantibiotics can further be divided into 12 subclasses based on the sequence of the propeptides or the composition of the associated modification/transport systems (Cotter *et al.,* 2005b, Piper *et al.,* 2009, Willey and van der Donk, 2007). Class Ib (labyrinthopeptins) undergoes post-translational modification and have labionin, a carbacylic amino acid, in the peptide sequence. Class Ic (sactibiotics) are cyclic peptides that have cross-linkages between cysteine residues and α-carbons of other amino acids. Examples include subtilosin A (which has linkages between three cysteine residues and α-carbons of two phenylalanines and one threonine) and thuricin CD, a two peptide bacteriocin. Thuricin CD has linkages between three cysteine residues and α-carbons of serine and two threonines in the one peptide (Trn-α) and linkages between three cysteine residues and α-carbons of threonine, alanine, tyrosine in the other peptide (Trn-β) (Rea *et al.,* 2011). Class Ic can further be sub-divided into two groups, including single- and two-peptide sactibiotics (Rea *et al.,* 2011).

Class IIa are the pediocin-like bacteriocins. These peptides are all active against *Listeria monocytogenes,* have a conserved N-terminal amino acid sequence, YGNGVXCXK/NXXC (where X is any amino acid), termed the "pediocin-box" and a double glycine processing site (Cotter *et al.,* 2005b, Rea *et al.,* 2011). Variants with an alternate YGNGL sequence have also been identified as class IIa bacteriocins (Atrih *et al.,* 2001, Yamazaki *et al.,* 2005). It has thus been proposed that the N-terminal "pediocin-box" of class IIa bacteriocins be amended to include this sequence with YGNG[V/L] (Beluguesmia *et al.,* 2011b). Class IIb are two-peptide unmodified bacteriocins that require both peptides for optimal activity. The individual peptides possess little or no activity (Oppegard *et al.,* 2007). Examples include plantaricin S, lactococcin G and plantaricin JK (Jiménez-Díaz *et al.,* 1993, Rogne *et al.,* 2008, 2009). Class IIc are circular bacteriocins that undergo post-translational modifications, which leads to their N- and C-terminus being covalently linked. They are heat stable and show resistance to proteolytic degradation (Rea *et al.,* 2011). Examples of class IIc bacteriocins include enterocin AS-48 and carnocyclin A (Jimenez *et al.,* 2005, Martin-Visscher *et al.,* 2009). Class IId contains the unmodified, linear, non-pediocin-like bacteriocins. These are all of the bacteriocins that do not fall in the other groups, based on the current classification systems, for example lactococcin A (Nissen-Meyer *et al.,* 2009).

Bacteriolysins, formerly known as the class III bacteriocins, are non-bacteriocin lytic proteins (Rea *et al.,* 2011). They are large antimicrobial proteins that are heat-labile. Examples include millericin B, helveticin J and enterolycin A (Beukes *et al.,* 2000, Hickey *et al.,* 2003, Joerger and Kleanhammer, 1986).

2.2 Biosynthesis of bacteriocins

2.2.1 Class I

The operon encoding class I bacteriocins (lantibiotics), such as nisin, include a structural gene, genes encoding accessory proteins responsible for modifications (i.e. proteolytic processing of the leader peptide, dehydration, cyclization), transport genes (encoding an ABC-superfamily transport protein for peptide translocation), regulatory genes and immunity genes conferring resistance to the producer strain (de Vos *et al.*, 1995, Jack *et al.*, 1995, Sahl *et al.*, 1995). The structural gene encodes an inactive precursor peptide (LanA) with a N-terminal extension or leader peptide connected to the C-terminal of the propeptide (McAuliffe *et al.*, 2001, Sahl and Bierbaum, 1998). The propeptide is modified and the leader peptide is cleaved to produce a mature, active peptide (McAuliffe *et al.*, 2001). LanB dehydrates serines and threonines in the propeptide to form 2,3-didehydroalanine (Dha) and 2,3 didehydrobutyrine (Dhb), respectively. LanC couples dehydrated residues to cysteines, via the reaction between the double bond of Dha and/or Dhb with the thiol (-SH) group of the cysteine to form *meso*-lanthionines (Ala-S-Ala) and β-methyllanthionines (Abu-S-Ala), respectively (Héchard and Sahl, 2002, McAuliffe *et al.*, 2001, Sahl *et al.*, 1995). The peptide will then be transported via the ABC transporter (LanT) and in most cases the leader peptide will be cleaved by a protease (LanP) to release the active peptide (Kleanhammer, 1993, McAuliffe *et al.*, 2001, Sahl and Bierbaum, 1998, Willey and van der Donk, 2007). In some lantibiotics, such as mersacidin and lacticin 481, there is only one bifunctional enzyme responsible for dehydration and cyclization of the propeptide, designated LanM (Kuipers *et al.*, 2011). Their LanT is also bifunctional in that it intracellularly processes the modified peptide, before it gets translocated (Willey and van der Donk, 2007). Two-component lantibiotics, such as lacticin 3147, contain two modifying enzymes (LtnM1 and LtnM2). One for each pre-peptide (LtnAα and LtnAβ) and after modification they are translocated by one LtnT enzyme (Kuipers *et al.*, 2011). Lacticin 3147 contains an additional post-translational modifying enzyme (LtnJ), which converts dihydroalanines to D-alanines in the two peptides (Ryan *et al.*, 1999b).

2.2.2 Class II

The genes involved in class II bacteriocin production include a structural gene (or 2 structural genes in the case of two-peptide bacteriocins), an immunity gene, genes encoding a membrane-associated ATP-dependent binding casette (ABC) transporter and an accessory protein (Ennahar *et al.*, 2000, Kleanhammer, 1993). The structural genes encode a precursor

peptide with a leader peptide, which becomes active once the leader peptide is cleaved by a protease (Eijsink *et al.*, 2002, Nes and Holo, 2000 Nissen-Meyer and Nes, 1997). Class IIa bacteriocins have a leader peptide with double-glycine residues, which acts as the processing site, and will be secreted once the leader peptide has been cleaved (Kleanhammer, 1993, Michiels *et al.*, 2001). Class IIa peptides have a 3 component regulatory system which includes an inducer peptide (or pheromone peptide), a transmembrane histidine kinase (pheromone receptor) and a cytosolic response regulator (Nes and Eijsink, 1999). Once the inducer peptide exceeds a basal concentration, it will activate the transmembrane histidine kinase which will in turn interact with a response regulator. The phosphate group of the activated histidine kinase will be transferred to a conserved Asp residue in the response regulator and will then serve as transcriptional activator for bacteriocin biosynthesis (Cho *et al.*, 2001, Nes and Eijsink, 1999). Some two-peptide bacteriocins (class IIb) also use a 3 component regulatory systems (as described above), however others seem to be expressed constitutively (Nissen-Meyer *et al.*, 2011). Circular bacteriocins (class IIc) are also produced as precursor peptides with an N-terminal leader peptide extension and undergo head to tail cyclization by the formation of an amide bond between the N- and C-terminal residues (Gabrielsen *et al.*, 2012). The class IId bacteriocins are diverse and can contain a *sec*-type signal peptide, which will be secreted by the general secretion pathway, while others are produced without a leader peptide. Non-pediocin-like linear bacteriocins (also class IId) are synthesized with a *sec*-independent double-glycine-type leader sequence and are transported by dedicated ABC-transporters (Iwatani *et al.*, 2011).

2.3 Bacteriocin mode of action

2.3.1 Class I

Lantibiotics, especially nisin, have been extensively studied for their mode of action. Nisin has a dual mode of action, which includes inhibition of bacterial cell wall synthesis and pore formation (McAuliffe *et al.*, 2001). Nisin inhibits bacterial cell wall synthesis by binding to lipid II, which then serves as a receptor site for targeted pore-formation. The A and B N-terminal rings of nisin form a pyrophosphate cage that binds the pyrophosphate moiety of lipid II and the C-terminal tail inserts into the phospholipid bilayer, forming pores (Hsu *et al.*, 2004). Eight nisin molecules and four lipid II molecules are needed for pore formation (Hasper *et al.*, 2004). Nisin sequesters lipid II from the septum, where cell wall biosynthesis is most active, in patches on the membrane (Hasper *et al.*, 2006). The N-terminal AB ring

structure of nisin is conserved amongst other lantibiotics and can be seen as a lipid II binding and sequestering motif (Hasper *et al.,* 2006). Other lantibiotics including epidermin and galledermin also have this lipid II binding motif in the N-terminal part of the peptide, however, the C-terminal parts are much shorter. They can thus inhibit cell wall synthesis, but cannot form pores in bacterial membranes (Bonelli *et al.,* 2006, Willey and van der Donk, 2007). Some class II lantibiotics are also pore formers, such as lacticin 481, where others can only inhibit cell wall biosynthesis, such as mersacidin (Brötz *et al.,* 1998, Wiedemann *et al.,* 2006). Mersacidin inhibits the transglycosylation step in peptidoglycan synthesis, much like vancomycin (Brötz *et al.,* 1997). Mersacidin, however, seems to target the disaccharide moiety of lipid II, whereas vancomycin uses the D-Ala-D-Ala pentapeptide of lipid II (Brötz *et al.,* 1998, Hasper *et al.,* 2006). Some lantibiotics, such as nisin and subtilin, are also able to inhibit the germination/outgrowth of bacterial spores (Liu and Hansen, 1993, Montville *et al.,* 2006, Scott and Taylor, 1981). Nisin was shown to target lipid II and that inhibition of *Bacillus anthracis* spore outgrowth was only achieved with lipid II-mediated pore formation and not only as a result of lipid II binding (Gut *et al.,* 2011). Nisin has also been shown to induce autolysis (Bierbaum and Sahl, 1985).

2.3.2 Class II

Class IIa mainly permeabilize bacterial membranes by forming ion-selective pores in sensitive cells by recognizing specific protein receptors in the membrane (Drider *et al.,* 2006). This leads to dissipation of the proton motif force (PMF), efflux of intracellular material and depletion of intracellular ATP (Chikindas *et al.,* 1993). Class IIa bacteriocins target a mannose permease (EIIt) belonging to the phosphotransferase system (PTS) (Diep *et al.,* 2007, Héchard *et al.,* 2001, Nes and Diep, 2009). Mannose permeases of the PTS consists of four domains (including IIA, IIB, IIC, and IID) arranged in two to four subunits and the extracellular loop of the membrane-located protein MptC has now been shown to be the recognition site of class IIa bacteriocins (Kjos *et al.,* 2010). The N-terminal part of class IIa bacteriocins will form a three-stranded antiparallel β-sheet, whereas the C-terminal part will form amphiphilic α-helices in a hairpin-like structure (Drider *et al.,* 2006). Initial binding is mediated by the electrostatic interaction of the positively charged amino acids in the hydrophilic β-sheet of the peptide and the bacterial membranes, after which the C-terminal region of the peptide will insert into the hydrophobic regions of the cell membrane and induce pore formation (Belguesmia *et al.,* 2011b, Chen *et al.,* 1997). The C-terminal of class IIa bacteriocins is less conserved than the N-terminal and determines the antimicrobial spectrum of the specific peptide. This was shown by generating hybrid peptides, by interchanging

corresponding modules from various pediocin-like bacteriocins. The hybrid peptides displayed activity corresponding to the peptide from which the original C-terminal region was derived and not the N-terminal region (Fimland *et al.,* 1996). Mesentericin Y105 is an exception to the general mode of action of class IIa bacteriocins and has also been shown to uncouple mitochondrial respiration (Maftah *et al.,* 1993).

Two-peptide bacteriocins (class IIb) require both peptides for optimal activity and induce membrane permeability by forming pores in sensitive cells, thereby once again causing an efflux of small molecules (Garneau *et al.,* 2002). The peptides do not interact with each other without the presence of membrane-like structures and will only interact and structure each other in the presence thereof (Hauge *et al.,* 1998, 1999). The peptides could thus bind to membrane proteins before they interact with each other and penetrate the hydrophobic regions of the cell membrane, as all characterized two-peptide bacteriocins contain a GxxxG motifs which is often involved in helix–helix interactions between transmembrane helices in membrane proteins (Nissen-Meyer *et al.,* 2010, Senes *et al.,* 2000, 2004). However, to date no receptor has been identified for two-peptide bacteriocins and studies indicate that some two-peptide bacteriocins don't need a receptor for activity (Marciset *et al.,* 1997, Nissen-Meyer *et al.,* 2010).

Circular bacteriocins (class IIc), like most other bacteriocins, cause membrane permeability, dissipation of the proton motif force and an efflux of intracellular material (Martin-Visscher *et al.,* 2011). Structural analysis of circular bacteriocins revealed that they share a compact globular structure, comprised of repeated α-helical motifs surrounding a hydrophobic core (Martin-Visscher *et al.,* 2009). Basic amino acid residues result in a positive charge on the surface of circular bacteriocins, thought to play a role in initial binding to the cell membrane (Borrero *et al.,* 2011). The first recognition site for circular bacteriocins has recently been reported and a maltose ABC transporter was shown to play a role in antimicrobial activity of garvicin ML (Gabrielsen *et al.,* 2012)

The Class IId bacteriocins are very diverse and contain a broad group of peptides. Some of these peptides (including lactococcin A and lactococcin B) have been shown to function similarly to the class IIa bacteriocins in that they also use the mannose permease of the PTS system as receptor (Diep *et al.,* 2007). Lactococcin 972 inhibits the growth of susceptible cells by interfering with septum formation and does not target the membrane (Martínez *et al.,* 2000). It was later shown that this bacteriocin specifically interacts with lipid II and is the first non-lanthionine-containing bacteriocin identified to do so (Martínez *et al.,*

2008). Lacticin Q was shown to induce membrane permeabilization without the presence of a receptor and is the first bacteriocin from Gram-positive bacteria which uses a toroidal-pore mechanism (Yoneyama *et al.,* 2009a,b).

2.4 Biomedical applications of bacteriocins from Gram-positive bacteria

The frequent use of antibiotics has led to a crisis in antibiotic resistance in human and animal pathogens (Neu, 1992). This has led to high selection pressures for pathogens to develop antibiotic resistance, including the well-known example of MRSA. Antibiotic resistance and the emergence of multidrug-resistant bacterial pathogens have led to the investigation of alternative antimicrobial agents, including bacteriocins from Gram-positive bacteria, to treat and prevent infections in both humans and animals.

2.4.1 Treatment of infections

2.4.1.1 Topical infections

S. aureus is commonly isolated from skin infections and is becoming a serious problem, with the emergence of MRSA and VRSA (Chang *et al.,* 2003, Daum, 2007). Lacticin 3147 shows activity towards antibiotic-resistant human pathogens including MRSA and VRE strains (Galvin *et al.,* 1999). Nisin has been under investigation for topical use as antimicrobial and has been incorporated in topical formulations (Valenta *et al.,* 1996). Patents have been filed for the use of nisin and lantibiotic combinations for the treatment of skin infection, as well as for mersacidin as treatment for skin- and systemic-infections (Dawson *et al.,* 2009, Walsh *et al.,* 2003). De Kwaadsteniet *et al.* (2010), however, reported on the inability of nisin F to treat subcutaneous *S. aureus* Xen 36 infections in mice when C57BL/6 mice were infected with 4.9 x 10^6 cfu *S. aureus* Xen 36 and treated subcutaneously with 256 AU nisin F. Nisin treated groups also showed infiltration of polymorphonuclear neutrophils.

Propionibacterium acnes is a member of the normal skin flora of humans and is implicated in acne vulgaris, causing inflamed lesions (Leyden, 2001). Gallidermin, epidermin, mersacidin and lacticin 3147, all lantibiotics, are active against *P. acnes* and have the potential to be used in the treatment of acne (Galvin *et al.,* 1999, Jung, 1991a,b, Kellner *et al.,* 1988, Niu and Neu, 1991). A patent has been filed for the use of bacteriocin-like inhibitory substances (BLIS) as treatment for acne (Margolis and Bowe, 2006).

2.4.1.2 Systemic infections

Systemic infections affect the whole body, or multiple organs, and are caused by a variety of pathogens (Czuprynski *et al.,* 2002, Harbarth *et al.,* 1998, Klug *et al.,* 1997). *L. monocytogenes* is a foodborne pathogen responsible for sepsis, meningitis, fetal loss, as well as gastroenteritis (Voetsch *et al.,* 2007). Pediocin PA-1 reduced *L. monocytogenes* fecal numbers, as well as *L. monocytogenes* in the liver and spleen, when administered to ICR mice (Dabour *et al.,* 2009). Pediocin PA-1 showed no deleterious effect on the intestinal flora of the mice, which is a desired characteristic for an antimicrobial. Piscolin 126 reduced the outgrowth of *L. monocytogenes* in the liver and spleen of BALB/c mice when injected intravenously into the tail vain (Ingham *et al.,* 2003). None of the mice showed any signs of localized inflammation around the site of injection, vomiting, depression or other signs of toxicity. Rihakova *et al.* (2010) reported on the *in vivo* activity of divercin V41 and its structural variants against *L. monocytogenes.* Recombinant divercin RV4 was the most potent and significantly reduced *L. monocytogenes* numbers in the spleen of BALB/c mice. No side effects were observed when the mice were monitored for 72 h after intravenous administration of the peptides. Bacteriocin Abp118, produced *in vivo* by *Lactobacillus salivarius* UCC118, showed good activity against *L. monocytogenes* in infected mice, while a mutant lacking the ability to produce this bacteriocin was ineffective in protecting the mice (Corr *et al.,* 2007).

C. difficile is a major causative agent of hospital-acquired diarrhoea (Pépin *et al.,* 2005). Nisin and lacticin 3147 has *in vitro* activity against clinical *C. difficile* strains (Bartoloni *et al.,* 2004, Rea *et al.,* 2007) and has the potential to be used as treatment. Thuricin CD is a two-peptide bacteriocin (Trn-α and Trn-β), produced by *Bacillus thuringiensis* 6431, showing activity towards *C. difficile*, while showing low activity against members of the normal intestinal microflora (Rea *et al.,* 2010).

Nisin showed good activity against *S. pneumoniae in vitro*, as well as *in vivo* when mice were infected intraperitoneally with 10^2-10^3 *S. pneumonia* Felton and treated with nisin (Goldstein *et al.,* 1998). Mice receiving 0.16 mg/kg nisin intravenously twice had survival rates of 100%, whereas a single intravenous dose of 0.63 mg/kg protected 80% of the mice.

Mutacin B-Ny266 protected 100% of mice infected intraperitoneally with *S. aureus* Smith (Mota-Meira *et al.,* 2005). Treatment (also intraperitoneally) followed directly after infection with 1, 3 and 10 mg/kg mutacin B-Ny266. Nisin F was, however, ineffective in controlling *S. aureus* Xen 36 infection in the peritoneal cavity in mice (Brand *et al.,* 2010). A slight reduction in bioluminescent signal was observed in mice, infected with 10^8 *S. aureus*

Xen 36 and treated with 640 AU nisin F, within the first 15 min, however no difference in bioluminescent signal was observed thereafter. Microbisporicin, a lantibiotic, was effective in protecting ICR (CD-1) mice infected intraperitoneally with 10^6 cells of *S. aureus* Smith 819 (Castiglione *et al.*, 2008). Protection was observed when microbisporicin was administered both intravenously and subcutaneously. Mice survived high doses of microbisporicin (≥200 mg/kg), indicating a low toxicity profile. Recently, lacticin 3147 was shown to prevent the systemic spread of *S. aureus* Xen 29 in a murine model, when infected intraperitoneally with 10^6 cfu/ml (Piper *et al.*, 2012). *S. aureus* numbers in the spleen, liver and kidney were in the range of 10^5 cfu/ml organ, whereas control mice had numbers in the range of 10^6 cfu/ml organ.

H. pylori is a pathogenic organism that causes chronic bacterial infections in humans and is an important organisms causing ulcers. This organism causes gastric ulcers, as well as duodenal ulcers, and upper GIT disorders such as chronic gastritis, peptic ulcer disease, tissue lymphoma and gastric cancer (Isreal and Peek, 2001, Korman and Tytgat, 1995, Kusters *et al.*, 2006). Nisin shows activity towards *H. pylori* and can serve as treatment for peptic ulcers (Delves-Broughton *et al.*, 1996). Nisin (produced by AMBI, Purchase, New York), has successfully undergone phase I clinical trials and is being considered as treatment for stomach ulcers caused by *H. pylori* (Hancock and Chapple, 1999). However, nisin has not been further developed in this regard. Lacticins A164 and BH5 (produced by *Lactococcus lactis* subsp. *lactis* A164 and *L. lactis* BH5, respectively) showed strong *in vitro* antibacterial activity against *H. pylori* strains when using a broth micro-dilution assay (Kim *et al.*, 2003). Spent culture supernatants of *Lactobacillus acidophilus* LB showed strong antimicrobial activity against *H. pylori in vitro* (Conconnier *et al.*, 1998). However, was unable to completely diminish numbers of *H. felis* in the stomachs of BALB/c mice infected intragastrically. No inflammatory reaction was observed in the infected mice treated with the spent culture supernatants (Conconnier *et al.*, 1998). *Lactobacillus johnsonni* La1 supernatants also inhibited *H. pylori* growth *in vitro*, but were unable to cure patients of *H. pylori* during a randomized, double-blind, controlled clinical trial. *H. pylori* was still detected in gastric biopsies and the supernatants only have a suppressive effect *in vivo* (Michetti *et al.*, 1999).

2.4.1.3 Device-related infections

Biomedical implant-related infections can cause complications with prosthetic devices and can force patients back for repair or replacement surgery (Campoccia *et al.*, 2005). It would be advantageous to the patient to minimize unwanted microbial adhesion to such devices.

Nisin adsorbed to salinized surfaces resulted in reduced growth and cell division of *L. monocytogenes* on nisin covered surfaces (Bower *et al.,* 1995). Nisin-coated polyvinyl chloride (PVC) suction catheter tubing prevented colonization of *S. aureus*, *S. epidermidis*, and *Streptococcus faecalis*, whereas microbial colonization occurred in untreated catheter tubing (Bower *et al.,* 2002). *In vivo* studies confirmed these results and nisin-coated Teflon® FEP intravenous catheters in sheep showed bactericidal properties up to 5 h, however no activity was reported after 7 days. The authors suggested that the antimicrobial activity of nisin may only have short-term benefits in environments such as blood. PVC® tracheotomy tubes pretreated with nisin, showed promise in providing an antimicrobial barrier to prevent bacterial infection (Bower *et al.,* 2002). Nisin, in combination with RNAIII-inhibiting peptide, showed promising results *in vivo* to inhibit *S. epidermidis*, as well as methicillin-resistant *S. epidermidis,* colonization on implanted Dacron® grafts in Wistar rats, with less than 10 cfu remaining on implants (Ghiselli *et al.,* 2004). Incorporation of bacteriocins into bone cement has been reported to prevent bacterial colonization of implants. Gallidermin and a novel peptide (NI01) incorporated into polymethylmehacrylate (PMMA) bone cement was able to significantly reduce biofilm formation of clinical *S. epidermidis* 156 on the bone cement (Sandiford *et al.,* 2012). Nisin F incorporated into brushite bone cement prevented the growth of *S. aureus* Xen 36 *in vivo* in a murine model (van Staden *et al.,* 2012). No bacteria were detected in mice which received bone cement containing 5% (w/w) nisin and no adverse effects on blood parameters could be identified.

2.4.1.4 Upper respiratory tract infections

Tuberculosis is regarded as the second greatest killer worldwide, after HIV/AIDS, caused by a single infectious agent (WHO, 2012). *Mycobacterium tuberculosis* infected, and caused tuberculosis, in 8.8 million people in 2010, of which 1.4 million succumbed to this disease (WHO, 2012). Multidrug-resistant cases of tuberculosis have been on the increase over the last two years, with an increase from an estimated 150,000 cases in 2008 to 650,000 cases in 2010 (WHO, 2011/2012). Antimycobacterial activity of bacteriocins has been reported. Bacteriocin (Bcn2-5) showed better *in vitro* activity against *M. tuberculosis* H37Rv when compared to rifampicin (Sosunov *et al.,* 2007). Cytotoxicity was observed in a dose dependant manner, with no cytotoxicity observed at 0.1 mg/L. Bcn5 was unable to increase survival of C57BL/6JCit (B6) mice infected intravenously with *M. tuberculosis* H37Rv 2 x 10^7 cfu/mouse. A Bcn5-phosphatidylcholine-cardiolipin liposome complex was, however, able to increase survival of animals as compared to untreated controls (Sosunov *et al.,* 2007). These bacteriocins thus have the potential to be used as alternative tuberculosis-drugs or can

be used in combination with other treatments to treat tuberculosis. This could help to impede the increase in antibiotic resistance in *M. tuberculosis* strains, especially with the development of multidrug-resistant strains.

When tested *in vivo*, nisin F showed promise in the treatment of upper respiratory tract infections in immunocompromised Wistar rats, caused by *S. aureus* K. Rats were infected intranasally with 4 x 10^5 cells of *S. aureus* K and treated with 8192 arbitrary units (AU) of nisin F. Symptoms of pneumonia were detected in the trachea and lungs of control animals, whereas nisin F-treated animals showed no signs of pneumonia (de Kwaadsteniet *et al.*, 2009). Mersacidin was able to protect mice and prevented MRSA colonization, when mice were infected intranasally with MRSA and treated with this peptide (Kruzewska *et al.*, 2004). No MRSA could be detected in the blood, lungs, liver, kidney, spleen or nasal scrapings of mice treated with mersacidin. No adverse effects were observed in cytokine profiles of mersacidin treated mice (Kruszewska *et al.*, 2004). Nisin was, however, ineffective in preventing *S. aureus* colonization when applied to the nasal tract of cotton rats (Kokai-Kun *et al.*, 2003).

Otitis media is a common disease diagnosed in children, with long term side effects including impaired hearing and delayed speech (Ryding *et al.*, 1997, Segal *et al.*, 2005, Teele *et al.*, 1990). Peptide ST4SA showed promising results when tested against Gram-positive middle ear pathogens (Knoetze *et al.*, 2008).

2.4.2 Bioactive ingredients in personal care products

The use of bacteriocins in health care products has also been proposed. Epidermin and gallidemin have activity against staphylococci and streptococci isolated from skin and have been proposed for use in deodorants (van Kraaij *et al.*, 1999). The use of nisin in mouth wash, deodorants, as well as cosmetics has also been proposed (Molitor and Sahl, 1991, van Kraaij *et al.*, 1999). Lantibiotics were shown to be effective in the prevention of tooth decay and gingivitis (Galvin *et al.*, 1999, Howell *et al.*, 1993). Nisin reduced plaque build-up and gingivitis in beagle dogs and treated groups had a lower gingival index score, as compared to untreated controls (Howell *et al.*, 1993). Patents have been filed on the use of nisin in oral cavity applications (Blackburn *et al.*, 1993). Lacticin 3147 shows activity towards *Streptococcus mutans*, which plays a role in dental decay in humans and could be used for oral hygiene (Galvin *et al.*, 1999). BLIS K12 is a commercial product which contains *Streptococcus salivarius* (which produces salivaricin A2 and B) that inhibits bacteria causing bad breath (Tagg, 2004). A strain of *S. mutans*, producing mutacin 1140 which is active

against tooth decay bacteria, has been modified (termed SMaRT) to produce less lactic acid and evaluated as a replacement therapy to eliminate decay causing strains of *S. mutans* (Hillman *et al.,* 2000). Preclinical tests showed that the SMaRT strain reduced tooth decay and displaced decay-causing *S. mutans*. No adverse reactions were reported in first Phase 1 clinical trials. Lozenges containing *S. salivarius,* producing salivaricin A, are marketed as oral care products (Boakes and Wadman, 2008).

2.4.3 Treatment of Mastitis in dairy cows

Mastitis, inflammation of the mammary glands, is caused by microbial infection of the mammary glands of cows and causes huge economic losses in the dairy industry (Ryan *et al.,* 1998). Lacticin 3147 has been investigated as treatment for bovine mastitis and showed activity against mastitic staphylococci and streptococci (Ryan *et al.,* 1998, Twomey *et al.,* 2000). A teat seal containing lacticin 3147 was effective in reducing infection, when *Streptococcus dysgalactiae* M (1.5 x 10^4 cfu) was administered into the teat sinus of dairy cows. Lacticin 3147-containing teat seals did not elicit significant irritancy and only a temporary increase in somatic cell count (Ryan *et al.,* 1999a). A bacteriocin produced by *Bacillus subtilis* LFB112 was active against *S. aureus, Streptococcus bovis* and *S. equi* subsp. *zooepidemicus* causing disease in animals (Xie *et al.,* 2009). Nisin has also shown promise in treating and/or prevention of mastitis and is the active compound in two commercial products (Consept® and WipeOut®) which are used as preventative measure for mastitis (Cotter *et al.,* 2005b).

2.4.4 Anti-inflammatory agents

Phospholipase A2 is an enzyme that releases arachidonic acids from cellular phospholipids and catalyses a rate-limiting step leading to the formation of potent mediators of inflammation and allergy, including the prostaglandins, leukotrienes and hydroxyeicosatetraenoic acids (Irvine, 1982, Johnson *et al.,* 1983, Zipser and Laffi, 1985). Phospholipase A2 also plays a role in the formation of lyso-PAF, a precursor of platelet-activating factor (PAF) which is a pro-inflammatory mediator (Braquet *et al.,* 1987). Cinnamycin-like lantibiotics show inhibitory activities towards human phospholipase A2 (Fredenhagen *et al.,* 1990). The lantibiotics duramycin, duramycin B and C and cinnamycin were shown to inhibit phospholipase A2 indirectly, by sequestering the substrate phosphatidylethanolamine (Marki *et al.,* 1991). These lantibiotics thus have potential as anti-inflammatory drugs (van Kraaij *et al.,* 1999).

2.4.5 Regulation of blood pressure

Angiotensin-converting enzyme plays an important role in the regulation of blood pressure and fluid balance (Zhang *et al.*, 2000). It achieves this function by catalyzing the conversion of angiotensin I to angiotensin II and histidylleucine (Kido *et al.*, 1983). Ancovenin, a lantibiotic produced by *Streptomyces* spp. was shown to be an inhibitor of angiotensin converting enzyme (Kido *et al.*, 1983). Cinnamycin-like lantibiotics, cinnamycin and duramycin, can also inhibit angiotensin-converting enzyme (Kaletta *et al.*, 1991, Kessler *et al.*, 1991, Shiba *et al.*, 1991). These lantibiotics can have potential in the regulation of blood pressure.

2.4.6 Carrier molecules for increased absorption of bioactive molecules

Insulin and many other therapeutic proteins are poorly absorbed through mucosal membranes and for that reason must be injected, rather than taken orally (Bower *et al.*, 2001). Properties of certain compounds can enhance absorption of these therapeutic proteins and include molecules that are cationic, that are active at a low pH and molecules that have a relatively small molecular size (Bower *et al.*, 2001, Natsume *et al.*, 1999). Lantibiotics, such as nisin, have some of these properties and could potentially be used to enhance the absorption of therapeutic molecules through mucosal membranes (Bower *et al.*, 2011).

2.4.7 Contraceptives and potential applications in the urogenital tract

Sexually transmitted diseases and HIV/AIDS hold a great health risk for sexually active individuals. It is estimated that a large majority of pregnancies occurring globally are unwanted, with 133 million unintended pregnancies occurring every year (Yedery and Reddy, 2005). A safe and effective alternative to commercially available contraceptives would we useful to reduce the risk of sexually transmitted diseases and unplanned pregnancies. Nisin has shown good contraceptive activity *in vitro* and *in vivo* (Aranha *et al.*, 2004, Reddy *et al.*, 2004). Nisin caused no abnormalities when applied intravaginally in rats and caused inhibition of sperm motility and prevented conception (Aranha *et al.*, 2004). Intravaginal application of nisin also prevented conception in rabbits and showed no inflammation or damage to the vaginal epithelium when applied for 2 weeks (Reddy *et al.*, 2004). In another study by Reddy *et al.* (2011) it was shown that nisin had no negative influence on rats, as well as their reproductive organs when administered orally. It had no effect on the reproductive performance of rats, as well as their offspring (Reddy *et al.*, 2011). Subtilosin A, produced by *B. amyloliquefaciens*, was shown to have potent spermicidal activity in a dose dependant

manner, while showing low toxicity when using an EpiVaginal (VEC-100) human ectocervical tissue model (Sutyak *et al.*, 2008a). Subtilosin also showed spermicidal activity when tested in various other animal systems (Silkin *et al.*, 2008) Lacticin 3147 has also been proposed as potential spermicidal agent (Silkin *et al.*, 2008).

Subtilosin A was shown to be active against *Gardneralla vaginalis*, a major causative agent of bacterial vaginosis, whilst lacking activity against vaginal isolates of *Lactobacillus* (Sutyak *et al.*, 2008b).

2.4.8 Biosurfactants

Surfactants are used to stabilize emulsions, such as oil in water, and are amphiphilic molecules (Singh and Cameotra, 2004). They contain hydrophilic and hydrophobic moieties that partition at the interface of fluids which have different polarities and hydrogen bonding. Nisin has been shown to be a good emulsifier and was able to stabilize oil droplets in water (Bani-Jaber *et al.*, 2000). Emulsifying properties of nisin were dependent on nisin concentration and pH of the solution. Nisin has the potential to be used as alternative surfactant in pharmaceutical emulsions. These biosurfactants will have advantages because they are biodegradable and less toxic than some of the chemical surfactants and will have antimicrobial activity (Bower *et al.*, 2001).

2.4.9 Other

Antiviral activity against Herpes simplex virus has been reported for lanthiopeptin and enterocin CR35 (Naruse *et al.*, 1989, Wachsman *et al.*, 1999). Duramycin is being developed for the treatment of reduced mucociliary clearance in cystic fibrosis (Kuipers *et al.*, 2011). Duramycin caused improvements in pulmonary function parameters in cystic fibrosis patients when inhaled over 5 days, during a phase II clinical study (Grasemann *et al.*, 2007) and showed no adverse effect on blood parameters of humans, when treated intranasally with aerosolized duramycin (Zeitlin *et al.*, 2004). Duramycin has also entered phase II clinical trial for treating dry eyes (Grasemann *et al.*, 2007). By opening calcium-activated chloride channels, duramycin is able to encourage hydration of the epithelial tissue (Kuipers *et al.*, 2011).

2.5 Cytotoxicity of bacteriocins

2.5.1 In vitro *on human cell lines*

Bacteriocins have numerous potential biomedical applications. However, cytotoxicity of bacteriocins needs to be determined before these peptides can be implemented in the pharmaceutical industry. Some bacteriocins have shown cytotoxic effects on human cell lines, however, this seems to be selective and dose-dependent. Subtle changes have also been shown to influence cytotoxicity. Cytotoxicity testing will thus have to be conducted on each peptide as safety assessment, before these peptides can reach end stages of clinical development. Gallidermin was less toxic than nisin A and the eukaryotic AMPs, magainnin I and II and melittin, towards two epithelial cell lines, HT29 and Caco-2 cells (Maher and McClean, 2006). A concentration dependent increase in membrane permeability was seen, with gallidermin showing significantly lower membrane permeability than the other peptides. The effect of a bacteriocin produced by *Lactococcus* sp. HY 449 has been tested on human fibroblasts (HNF, ATCC CCL-28) (Oh *et al.*, 2006). Cell proliferation decreased in a dose-dependent manner and a cytotoxicity test revealed that the LC_{50} of the bacteriocin was 50 mg/ml after 24 h of treatment (Oh *et al.*, 2006). Villarante *et al.* (2011) studied the effect of dialyzed and undialyzed bacteriocin (pediocin K2a2-3) fractions on the growth of two cancer lines, HT29 and HeLa cells. Inhibition of HT29 cells did not differ significantly, however undialysed bacteriocin inhibited significantly more of the HeLa cells (52.3 ± 6.0%), whereas only 15.6 ± 4.0% were inhibited by the dialysed bacteriocin. Toxicity was attributed to the low pH of the undialyzed fraction, pH 2, and that the HeLa cells were more sensitive towards low pH than the HT29 cells. The authors, however, concluded that the inhibitory effect on the HT29 cells were as a result of the bacteriocin and not that of the pH of the media. Carnobacteriocins, Cbn BM1 and Cbn B2, showed no cytotoxicity towards Caco-2 cells, as no significant effect was observed on growth rate and viability, when tested in combination at a final concentration of 100 mg/L (100x higher than needed for antimicrobial activity) for 48 h. Cytotoxicity was only observed when Cbn B2 (100 mg/L) was used independently. However, the growth rate and the viability was still approximately 80% of that of the control (Jasniewski *et al.*, 2009). Nisin, pediocin PA-1 and colicin E6 showed toxicity in a dose dependent manner when tested against Simian virus 40-transfected human colon (SV40-HC) and Vero monkey kidney (Vero) cells (Murinda *et al.*, 2003). Highest toxicity was recorded at 700 AU/ml. Colicins E1, E3 and E7 on the other hand showed lower toxicity towards these cells. Enterocin S37, a bacteriocin produced by *Enterococcus faecalis* S37, showed dose dependent toxicity when tested against undifferentiated Caco-2/TC7 cells (Belguesmia *et al.*,

2011a). This bacteriocin was more cytotoxic towards undifferentiated Caco2-TC7 cells than nisin. However, no significant effects were seen on differentiated Caco-2/TC7 cells. Nisin also showed lower hemolytic activity (1.1% lysis) than enterocin S37 (74.2% lysis) at a concentration of 10 µg/ml, when tested against sheep erythrocytes. Microcin E492 has shown cytotoxicity towards HeLa in a dose dependent manner. Apoptosis was induced at lower levels of microcin E492, whereas at higher concentrations necrosis was induced (Hertz *et al.*, 2002). Plantaricin A, a bacteriocin produced by *Lactobacillus plantarum*, has been shown to increase cell proliferation, enhance migration and influence the expression of TGF-β1, FGF7, VEGF-A and IL-8 *in vitro* in human keratinocytes (Pinto *et al.*, 2011). Subtle changes in amino acid composition of a peptide can also influence its hemolytic activity. Subtilosin A variants, with an isoleucine at position 6 instead of a threonine, showed higher levels of hemolytic activity (Huang *et al.*, 2009).

Because of the selective toxicity of some bacteriocins towards human cells it can be hypothesized that they use cell surface receptors to exert activity. Detailed information on the initial interaction of bacteriocins and subsequent cytotoxicity towards human cell lines is not available and only a few papers have addressed this topic. Hydrophobicity has been proposed to promote interaction with mammalian cell membranes thereby causing toxicity (Maher and McClean, 2006). However, more complex systems have been described. Pyocin S2, produced by *P. aeruginosa*, was shown to be active against some normal and malignant human cells. Pyocin S2 used certain carbohydrate moieties for attachment to the human cells, whereas it recognizes an iron-regulated outer membrane protein in sensitive bacteria (Watanabe and Saito, 1980). Tumor cells have also been shown to be selectively inhibited by colicins A and E1, which bind to the Vitamin B12 receptor BtuB (Cramer *et al.*, 1995, Lazdunski *et al.*, 1998).

2.5.2 In vivo cytotoxicity, pharmacokinetics and pharmacodynamics

Bacteriocins, both class I and class II, show relatively low levels of toxicity when tested *in vivo* in animal models, as mentioned in the section on biomedical applications of bacteriocins. A bacteriocin derived from *Lactococcus* sp. HY449 showed no irritancy on human skin, with a patch test (Oh *et al.*, 2006). Pediocin PA-1 also showed no negative side effects and did not alter the normal intestinal flora when administered to mice (Bernbom *et al.*, 2009). Pediocin AcH was further shown to be non-immununogenic and did not elicit a response when administered to mice and rabbits (Bhunia *et al.*, 1990). There are, however, a few exceptions, such as a natural subtilosin A variant with higher hemolytic activity and cytolycin, produced

by *E. faecalis*, with high toxicity levels towards both prokaryotic and eukaryotic cells (Huang *et al.*, 2009).

Mutacin 1140 has been shown to bind strongly to human serum with up to 92.7 ± 2.0% bound to serum components (Ghobrial *et al.*, 2010a). Human serum also negatively affected the antimicrobial activity of mutacin 1140 against *S. pneumonia*, however, strangely increased the activity against *S. aureus*. Further studies revealed that mutacin 1140 could be detected in blood plasma of Sprague-Dawley rats for up to 6 h after a single intravenous administration of 25 mg/kg (Ghobrial *et al.*, 2010b). The elimination terminal half-life of mutacin 1140 was determined to be 1.6 h. A hypersensitive reaction was observed with rapid injection of mutacin 1140 which could be blocked by pretreatment with diphenhydramine. Intravenous administered duramycin (Moli1901) has been shown to be excreted in the feces and that it accumulated mainly in the liver, when administered to rats and mice (McNulty *et al.*, 2003). Aerosolized duramycin showed high accumulation in the respiratory tract of mice and rats and also got excreted in the feces 2 days after treatment. Finally, duramycin was eliminated from the pulmonary system and once again excreted in the feces of mice and rats, with an elimination half-life of 52 days, following intratracheal administration (McNulty *et al.*, 2003).

2.6 Resistance towards bacteriocins

2.6.1 Class I

Nisin has been applied to food products for biopreservation all over the world and remarkably, very little resistance has been reported. As is the case with antibiotics, however, it is expected that resistance will develop once used in a clinical setting and mechanisms of resistance therefore needs to be elucidated. In the laboratory, spontaneous resistance to nisin can easily be induced upon incubation below the MIC, with subsequent increase in concentration (Kramer *et al.*, 2004, Severina *et al.*, 1998). Nisin resistance in *L. monocytogenes* can be ascribed to changes in fatty acid composition as well as the phospholipid composition. Decreased ratios of C15/C17 fatty acids, more zwitterionic phosphatidylethanolamine, less anionic phosphatidylglycerol and cardiolipin and a requirement for divalent cations (Mg^{2+}, Ca^{2+}, Mn^{2+} and Ba^{2+}) have been reported to be involved in resistance (Crandall and Montville, 1998, Mazzotta and Montville, 1997, Ming en Daeschel, 1993, 1995). Decreased phosphatidylglycerol results in a less negative cell surface

charge and can thus reduce the binding affinity of cationic antimicrobial peptides (Chatterjee *et al.,* 2005). Increased straight chained, monounsaturated and saturated fatty acids, as well as less branched polyunsaturated fatty acids have been reported in resistant strains of *L. monocytogenes* and *C. botulinum* (Mazzotta and Montville, 1997, Ming en Daeschel, 1993). Increased C16:1 and C18:1 monounsaturated fatty acids were also identified in resistant *P. acidilactici* (Goulhen *et al.,* 1998). Spontaneous nisin resistance in *L. monocytogenes* varied from 10^{-2} to 10^{-7} in a strain dependent manner (Gravesen *et al.,* 2002a) and *L. monocytogenes* mutants were detected at a frequency of 10^{-6} to 10^{-8} which were resistant to 50 µg/ml nisin (Harris *et al.,* 1991). It has also been proposed that nisin resistance in *L. monocytogenes* was due to shielding of lipid II (Gravesen *et al.,* 2001, 2004). Increased expression was observed in a protein homologous to the glycosyltransferase domains of high molecular weight PBPs, as well as a protein histidine kinase which was directly involved in nisin resistance. Nisin resistance in *M. flavus* appeared to be independent of lipid II levels (Kramer *et al.,* 2004).

Nisin resistance in *L. lactis* was ascribed to various complex changes by preventing nisin from reaching the cytoplasmic membrane, reducing the acidity of the medium (resulting in the binding of nisin to the cell wall), preventing the insertion of nisin into the membrane, as well as possibly by transportation of nisin across the membrane or export of nisin out of the membrane (Kramer *et al.,* 2006). Resistant strains showed increased thickness of the cell wall at the septum, indicating a potential shielding of lipid II to prevent nisin binding and translocation of lipid II. The resistant strains also showed increased D-alanyl esters and galactose in the cell walls, resulting in a less negatively charged cell wall. Shielding of lipid II appeared to be the main mechanism of resistance towards nisin (Kramer *et al.,* 2006).

S. aureus with a disrupted *dltA* (gene products which are involved in the synthesis of D-alanyl esters for substitution in lipoteichoic acids, resulting in the incorporation of positive charges) was more sensitive towards various cationic antimicrobial peptides including gallidermin and nisin (Peschel *et al.,* 1999). Wild type *S. aureus* strains with additional copies of the *dlt* operon were less sensitive to cationic antimicrobial peptides as they had higher amounts of D-alanylation of their lipoteichoic acids and thus a more positive charge to hinder initial binding of the cationic AMPs (Peschel *et al.,* 1999).

Bacillus subtilis mutants (s*igX*, and *dltA* mutants) were also shown to be more sensitive towards nisin than wild type strains (Cao and Helmann, 2004). The extracytoplasmic-function (ECF) σ^X factor plays a role (in part) in regulation of expression of genes playing a role in phosphatidylethanolamine biosynthesis (*pssA*, *psd* and *ybfm*) as well as

D-alanylation of lipoteichoic and wall teichoic acids (*dlt*) (Chatterjee *et al.*, 2005). Activation of (ECF) σ^X factor thus results in nisin resistance by inducing genes which causes alterations of cell surface properties (Cao and Helmann, 2004).

A mutation in a putative histidine kinase (designated *nsaS*, nisin susceptibility associated) in *S. aureus* was recently shown to result in high level resistance to nisin (Blake *et al.*, 2011). The *nsaSR* operon and a downstream operon (encoding an ABC transporter) forms part of a locus which encodes proteins that is paralogous to the *graRS*/*vraFG* cluster conferring resistance to vancomycin and polymixin B (Blake *et al.*, 2011). NsaRS was further shown to regulate a downstream transporter NsaAB during nisin-induced stress and that NsaRS plays a role in sensing cell damage, after which it will modify gene expression of genes playing a role in cell envelope modification, conferring resistance (Kolar *et al.*, 2011). Another group independently identified a two-component regulatory system (which they termed BraSR and BraDE, bacitracin resistance associated) as the main regulatory element controlling bacitracin and nisin resistance in *S. aureus* (Hiron *et al.*, 2011). They showed that the two ABC transporters are involved in signal transduction and resistance, BraDE is involved in sensing and signalling through BraSR, whereas VraDE will finally aid in detoxification and conferring resistance. Two-component systems in *L. monocytogenes* have also been identified as playing a role in resistance towards nisin A (Cotter *et al.*, 2002, Collins *et al.*, 2012). Two-component regulatory systems (where genes encoding a sensor kinase/response regulator pair lie adjacent to genes encoding an ABC transporter) seem to play a major role in antimicrobial peptide resistance in not only *S. aureus*, but also the *Firmicutes* (Jordan *et al.*, 2008).

An enzyme, nisinase, can also potentially confer resistance to nisin. Nonproteolytic nisin inactivating enzymes has been reported for several *Bacillus* spp., with putative dehydroalanine reductase activity (Jarvis, 1967, Jarvis and Farr, 1971).

2.6.2 Class II

Resistance to class IIa bacteriocins commonly occur at frequencies of 10^{-9} to 10^{-3} in *L. monocytogenes* and can be very stable in some cases (Gravesen *et al.*, 2002b, Tessema *et al.*, 2009). Inactivation of the *rpoN* gene (encoding the σ^{54} subunit of the bacterial RNA polymerase) in *L. monocytogenes* was shown to confer resistance to mesentericin Y105 and sensitivity was restored upon complementation with the wild type *rpoN* gene (Robichon *et al.*, 1997). The σ^{54} subunit is an alternative sigma factor for the transcription of a set of genes, including the *mpt* operon which encodes for a mannose permease of the PTS (Man-PTS)

(Belguesmia *et al.,* 2011b, Gravesen *et al.,* 2002b). *L. monocytogenes* resistance towards class II bacteriocins, as well lactococcin A, correlated with a reduction in the expression of the Man-PTS (Dalet *et al.,* 2001, Héchard *et al.,* 2001, Kjos *et al.,* 2011). Down-regulation of Man-PTS thus appears to be one way to confer class II bacteriocin resistance. This was further supported by the fact that lower levels of Man-PTS expression were found in natural bacteriocin-resistant *L. monocytogenes* isolates (Kjos *et al.,* 2011). The role of Man-PTS was further investigated by heterologous expression of the *mptACD* operon of *L. monocytogenes* in *L. lactis*. It was shown that *mptC* alone could confer resistance and as a result was identified as the target site of class IIa bacteriocins (Kjos *et al.,* 2010). Transcriptional analysis of pediocin PA-1-resistant *E. faecalis* revealed that there was reduced expression of the *mpt* operon, as well as genes encoding glycolytic enzymes. The resistant strains were relieved from catabolite repression, more specifically glucose repression, and metabolism was shifted to mixed acid fermentation (Opsata *et al.,* 2010). Some reports revealed that resistance can be more complex, as normal or even higher expression of genes in the Man-PTS system was found in the resistant strains. The presence of recombinant divercin RV41 caused a down-regulation of *rpoN* and *glpQ* gene expression and an up-regulation of *mptR*, *mptC*, and *pde*. The expression of *mptB* and *mptD* genes remained unchanged in the presence of recombinant divercin RV41 (Calvez *et al.,* 2008). Three genes including, *rpoN*, *glpQ* (encoding a glycerolphosphoryl diester phosphodiesterase) and *pde* (putative phosphodiesterase) were identified to play a role in divercin V41 resistance in *E. faecalis* (Calvez *et al.,* 2007). Mutants, deficient in synthesis of σ^{54} subunit of the bacterial RNA polymerase and glycerophosphoryl diester phosphodiesterase, differed from both the *pde* mutant and the wild-type strain in fatty acid and polysaccharide composition (Calvez *et al.,* 2010). Sakacin P-resistant *L. monocytogenes* strains showed differences in growth, stress tolerance, as well as biofilm-forming ability. Differences in polysaccharide, fatty acid, and protein regions in cell membranes were observed in resistant strains as compared to the wild type (Tessema *et al.,* 2009). Resistance in *L. monocytogenes* has also been attributed to cell envelope changes including membrane fluidity and composition, especially alanine content, as well as cell surface charge (Vadyvaloo *et al.,* 2002, 2004). Class II bacteriocin resistance is a complex phenotype and several resistance mechanisms can together impart resistance in bacteria at either a low or a high level. Low-level resistance (2 to 4-fold) to class IIa bacteriocins can be attributed to alterations in membrane lipid composition, whereas high-level resistance (1000-fold) can be attributed to inactivation of the *mptACD* operon (Belguesmia *et al.,* 2011b). Immunity genes have been identified in non bacteriocin-producing bacterial strains and this immunity was termed “resistance through immune

mimicry" (Draper *et al.,* 2009, Fimland *et al.,* 2002, Møretrø *et al.,* 2005). Efflux pumps which expel the bacteriocins from the cell and non-specific extracellular proteases have also been proposed to be involved in clas II bacteriocin resistance (Kjos *et al.,* 2011).

2.6.3 Cross-resistance

Cross-resistance has been reported between different bacteriocins. Nisin resistance conferred cross-resistance to pediocin PA-1 and leuconocin S in *L. monocytogenes* (Crandall and Montville, 1998). Curvaticin 13 resistance in *L. monocytogenes* conferred resistance to camocin CP5 and pediocin AcH, two class IIa bacteriocins. No cross-resistance was, however, conferred to the class I bacteriocin nisin (Bouttefroy and Millière, 2000). Leucocin A-resistant strains of *L. monocytogenes* showed no significant cross-resistance towards nisaplin and the synthetic peptide ESF1-7GR, however were resistant to pediocin PA-2 (Ramnath *et al.,* 2000). Cross-resistance has been detected between pediocin PA-1 and bavaricin A in *L. monocytogenes*, however, once again no cross-resistance to nisin was detected (Rasch and Knøchel, 1998). Cross-resistance between pediocin PA-1, leucocin A and carnobacteriocin B2 have been reported in *L. monocytogenes*. Some cross-resistance between nisin and leucocin A has also been reported (Gravesen *et al.,* 2004). Acquired nisin (A or Z) resistance in *L. monocytogenes* reduced susceptibility towards pediocin and divergicin. A divergicin resistant strain showed increased resistance to nisin Z, but strangely decreased resistance to nisin A, and pediocin resistant strains exhibited increased resistance to nisin Z, pediocin and divergicin but also decreased resistance to nisin A (Naghmouchi *et al.,* 2007). Recently, *L. lactis* mutans have been isolated that are simultaneously resistant towards lactococcin 972 and nisin, as well as lysozyme and bacteriophage c2 (Roces *et al.,* 2012).

Almost no or very low levels of cross-resistance between bacteriocins and antibiotics have been found (Liu *et al.,* 2011). However, some cross-resistance has been reported. Bacteriocin resistance was reported to decrease sensitivity towards various antibiotics, including ampicillin, chloramphenicol, erythromycin and tetracycline (Naghmouchi *et al.,* 2007). Bacteriocins and antibiotics can potentially be used together to prevent or hamper the emergence of resistant pathogens, because they have different modes of action and acquiring resistance towards two different antimicrobials is very unlikely (Diep *et al.,* 2007).

2.7 Delivery systems for bacteriocins aimed at biomedical applications

The stability or mode of *in vivo* application of bacteriocins can be problematic and needs to be overcome. Drug delivery systems will play an integral role in the successful application of bacteriocins in the biomedical industry. These delivery systems would potentially be able to protect the peptides from proteolytic enzymes, which will result in the peptide staying active for prolonged periods of time, and will be able to release the bacteriocins in a controlled manner to exert its antimicrobial activity.

Most studies aimed at the delivery of bacteriocins have dealt with antimicrobial packaging films or materials aimed at food preservation and very few studies have been conducted to generate delivery systems for biomedical applications of bacteriocins (Bastarrachea *et al.,* 2011). Some of these systems, aimed specifically at medical applications, have previously been described in this literature overview and include bacteriocin-absorbed catheters, bacteriocin-impregnated bone cements, bacteriocin-liposome complexes, as well as nisin-coated vascular implants. Nisin has also been encapsulated into poly(L-lactide) (PLA) nanoparticles by semicontinuous compressed CO_2 anti-solvent precipitation (Salmaso *et al.,* 2004). Nisin was released in the active form for up to 1000 h and release was dependent on the salt concentration and the pH of the release medium. Nisin released from the PLA nanoparticles was able to inhibit the growth of *Lactobacillus delbrueckeii* spp. *bulgaricus*, when nisin-loaded PLA nanoparticles were incubacted in MRS containing the sensitive strain. These nanoparticles have been investigated for food preservation, however, could also potentially be used in antimicrobial pharmaceutical products. Pectin/HPMC was shown to protect nisin tablets during tests simulating colonic delivery. The authors reported that pectin on its own was not suitable in protecting nisin core tablets and that a 80:20 ratio of pectin:HPMC was the optimum composition to protect nisin, with the tablets maintaining integrity during a 6 h dissolution test (Urgurlu *et al.,* 2007).

3. Electrospun Nanofiber Scaffolds for Enhanced Wound Healing and Tissue Regeneration

3.1 Electrospun nanofibers

Nanofibers are polymeric fibers in the nanometer range with a large surface area to volume ratio and advanced mechanical properties (Agarwal *et al.,* 2008, Chew *et al.,* 2005, Maretschek *et al.,* 2008). These properties, as well as ease of fabrication, have led to various

applications in diverse fields, including filtration, protective clothing, biosensors and biomedical engineering (Agarwal *et al.,* 2008, Gibson *et al.,* 2001, Huang *et al.,* 2003, Theron *et al.,* 2008, Yoon *et al.,* 2006). In biomedical engineering the nanofibers are attractive substrates for controlled release of various drugs and growth factors, for tissue engineering, medical prostheses, for the immobilization of enzymes and as wound dressings (Chew *et al.,* 2005, Jiang *et al.,* 2005, Kim *et al.,* 2007, Liang *et al.,* 2007, Maretschek *et al.,* 2008, Porter *et al.,* 2009, Quaglia, 2008).

During electrospinning an electric field will be applied to a polymer solution or melt, causing the electrically charged polymer to form a Taylor cone (Huang *et al.,* 2003, Maretschek *et al.,* 2008). Once the electric forces overcome the surface tension a charged polymer jet will eject out of the Taylor cone and accelerate towards the collector. The polymer solution becomes very thin as it moves towards the collector, the solvent evaporates and fibers are collected (Agarwal *et al.,* 2008, Liang *et al.,* 2007, Zhou *et al.,* 2008). These fibers are in the non-woven form and contain a high surface area to volume ratio. Three components needed for electrospinning include a high voltage supply, a long capillary tube and a collector (Liang *et al.,* 2007). Various parameters influence successful electrospinning of polymer solutions into nanofibers, including solution properties (viscosity, molecular weight of polymer(s) used, solvent(s) used), electric field applied, distance between needle tip and collector, temperature of the solution and humidity (for more information see Deitzel *et al.,* 2001, Huang *et al.,* 2003, Jacobs *et al.,* 2010).

Modifications of conventional electrospinning have appeared with co-axial electrospinning, emulsion electrospining and electrospinning using dual spinnerets. Co-axial electrospinning utilizes a specialized needle setup to produce core shell structured nanofibers (Sun *et al.,* 2003). During co-axial electrospinning a polymer solution in the outer parts of the needle will form the shell and the solution on the inner part of the needle will form the core, thus encapsulating the core material in the outer shell. The core material can be a polymer solution or a bioactive molecule to be encapsulated (antibiotics, growth factors, living organisms) or combinations thereof. Emulsion electrospinning is another approach to produce core-shell structured nanofibers without the need of the specialized needle setup (Xu *et al.,* 2006). During emulsion electrospinning an aqueous solution (bioactive molecule, hydrophilic polymer or a combination thereof) will be placed in an organic polymer solution and emulsified, with subsequent electrospinning using the conventional electrospinning setup (Maretschek *et al.,* 2008). Molecules in the aqueous phase will thus also be encapsulated.

3.2 Nanofiber wound dressings and scaffolds for tissue regeneration

It is beyond the scope of this review to cover all of the biomedical applications of electrospun nanofibers (for detailed reviews see Agarwal *et al.*, 2008, Jayakumar *et al.*, 2012, Meinel *et al.*, 2012) and will mainly focus on electrospun nanofibers as wound dressing materials and for the delivery of compounds influencing wound healing. Wound dressing materials have evolved over the last few decades from merely substrates that cover wounds, to bioactive materials which promote wound healing (Zahedi *et al.*, 2010). Optimal wound dressings should facilitate wound healing by forming a barrier to prevent microbial colonization, be able to treat infection in such instance, absorb wound educate, allow gas exchange, be non-immunogenic and non-toxic and must be easily removable without causing damage (Zahedi *et al.*, 2010). Electrospun nanofibers have various properties that make them suitable as wound dressing materials such as high oxygen permeability, variable pore size, a high surface area to volume ratio and morphological similarity to the extracellular matrix (EM) (Smith and Ma, 2004, Zahedi *et al.*, 2010, Zhou *et al.*, 2008). Various natural and synthetic polymer/polymer blends have been electrospun into nanofibers to generate potential wound dressing materials. The ability to incorporate a variety of bioactive molecules (such as antimicrobials, anti-inflammatories) into the nanofibers can increase the desirable wound healing (and wound dressing) properties.

3.2.1 Nanofibers electrospun without the addition of bioactive molecules

Hydrophobicity seems to be a very important characteristic influencing cell attachment and proliferation as more promising results are usually obtained with a higher degree of hydrophilicity in the nanofiber scaffolds. Blending of hydrophobic and hydrophilic polymers by either co-electrospinning, coating of fibers post-electrospinning or co-axial electrospinning is thus attractive ways to modify scaffold properties to enhance cell attachment and proliferation.

Chitosan has received enormous attention as potential wound dressing biomaterial as it has beneficial properties such as biodegradability, biocompatibility, antibacterial- and wound healing-activity (Jayakumar *et al.*, 2010). Chitin, a structural polysaccharide from arthropods, has been electrospun into nanofibers by Min *et al.* (2004) using 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) as solvent. Nanofibers had diameters ranging from 40 to 600 nm with an average diameter of 110 nm. Chitosan nanofibers were formed by deacetylation of the chitin nanofibers by treatement with 40% NaOH at 100 °C for 120 min or for 1 day at 60 °C (Min *et al.*, 2004b). Human oral- and epidermal-keratinocytes (HOK and HEK,

respectively), as well as human gingival fibroblasts (HGF), seeded onto electrospun chitin nanofibers (163 nm) showed better cell attachment and spreading as compared to microfibers (8.77µm), however both showed inadequate cell attachment and spreading (Noh *et al.*, 2006). Higher rates of attachment and proliferation were obtained on chitin nanofibers when coated with type I collagen and this significantly promoted the cellular response (Noh *et al.*, 2006). Chitin nanofibers were stable for 7 days after implantation in Sprague-Dawley rats, however lymphocytes and macrophages (inflammatory cells) were observed on the fiber matrix. Signs of degradation were observed in the nanofibers 14 days after implantation and inflammatory cells reduced during this period. Chitosan/polyethylene glycol (PEG) nanofibers were stabilized in 1M NaOH, resulting in the dissolution of PEG, leaving chitosan nanofibers with diameters ranging from 50 to 200 nm, which promoted cell attachment and proliferation of human fibroblasts, endothelial cells and keratinocytes (Tchemtchoua *et al.*, 2011). RT-PCR analysis revealed reduced keratin 14 expression, as well increased keratin 10 and involucrin expression in keratinocytes cultured on chitosan nanofibers, indicating differentiation of keratinocytes and low level cytotoxicity of the nanofibers. Chitosan nanofibers were fully biocompatible and promoted recovery of full-thickness wounds in BALB/c mice.

Blending chitin or chitosan with other polymers have resulted in scaffolds with enhanced properties. Human epidermal fibroblasts (HEF) showed good attachment and proliferation on chitin/polyglycolic acid (PGA) blend nanofiber scaffolds, with promising results obtained as the PGA content increased (Park *et al.*, 2006a). A chitin/PGA (75:25) blend showed an increase in adherent cells when coated with BSA. HEF and HEK also showed increased attachment and spreading on chitin/silk fibroin (SF) blend fibers (75:25) (Park *et al.*, 2006b). Water-soluble carboxyethyl chitosan (CECS)/PVA (50:50) nanofibers, cross-linked with glutaraldehyde vapour, showed no toxicity towards mouse fibroblasts (L929) and promoted cell attachment and proliferation (Zhou *et al.*, 2008). Nanofibers consisting of type I collagen, chitosan, and poly(ethylene oxide) (PEO) have been electrospun with subsequent cross-linking, using glutaraldehyde vapor (Chen *et al.*, 2008b). Nanofibers showed good biocompatibility and promoted proliferation of NIH3T3 fibroblasts. These nanofibers were also comparable with a commercial collagen sponge, when wound healing was evaluated in SD rats (Chen *et al.*, 2008b). Fibroblasts showed good viability on chitosan (CS)-poly(L-lactic acid-co-ε-caprolactone) P(LLA-CL) scaffolds, with best viability observed on the CS: P(LLA-CL) 1:2 nanofiber scaffold (Chen *et al.*, 2008a). Electrospun chitin/poly(ε-caprolactone) (PCL) nanofibers were washed in ethanol and neutralised with 0.1M sodium carbonate dissolved in 70% ethanol (Kim *et al.*, 2012). Cellular attachment and proliferation

of human dermal fibroblasts (HDF) increased on scaffolds, as the chitin content in the nanofibers increased and showed increased density, formation of actin filaments and good overall morphology on the chitin/PCL nanofibers. Blending other polymers has also shown promising results. Enhanced NIH3T3 fibroblast attachment and proliferation was observed on nanofibers prepared by co-electrospinning keratin or gelatin with poly(lactic acid) (PLA), as compared to plain PLA nanofibers (Yuan *et al.*, 2008). Xylan, natural hemicellulose (polysaccharide) present in hardwood, and PVA was successfully co-electrospun into nanofibers and subsequently cross-linked with glutaraldehyde vapour (Krishnan *et al.*, 2012). Nanofiber diameters increased with cross-linking time and xylan/PVA fibers cross-linked for 48 h improved human foreskin fibroblast attachment, cell morphology and proliferation and also showed best stability upon incubation. Normal human fibroblasts were able to adhere and proliferate on polyethyleneimine (PEI) nanofibers, electrospun with succinic anhydride and subsequently cross-linked with 1,4-butanediol diglycidyl ether, and exhibited a normal phenotype when evaluated with immunofluorescence (Khanam *et al.*, 2007). PEI nanofibers was shown to be non-toxic and promoted cell-nanofiber scaffold interactions.

Post-electrospinning modifications can also alter adherence and proliferation of mammalian cells. Increased attachment and proliferation of HOK, HEK and HGF were obtained on collagen-coated chitin nanofibers, as previously mentioned (Noh *et al.*, 2006). HOK, HEK and NHGF were able to attach and proliferate on silk fibroin nanofibers (Min *et al.*, 2004a). Nanofibers coated with type I collagen however showed increased adhesion and proliferation by cells. PCL nanofibers were coated with collagen using either a co-axial electrospinning setup (where PCL is the core and collagen the shell) or by immersion of PCL nanofibers in a collagen solution (Zhang *et al.*, 2005). HDF showed better attachment and viability on co-axial electrospun nanofibers, than on post-electrospinning collagen coated nanofibers. No improvement over the controls was, however, observed. Poly(propyl carbonate) (PPC) and poly(cyclohexyl carbonate) (PCHC) was synthesized and electrospun into nanofibers with subsequent surface modification using low-power deep UV irradiation (Welle *et al.*, 2007). L929 fibroblasts cultured on PPC nanofibers showed promising attachment and viability on the scaffolds. PGA, poly(L-lactic acid) (PLLA) and poly(lactic-co-glycolic acid) (PLGA) has been electrospun into nanofibers and their surfaces chemically modified using oxygen plasma treatment and in situ grafting of hydrophilic acrylic acid (Park *et al.*, 2007). Fibroblast adherence and proliferation was increased on modified nanofiber scaffolds, as compared to unmodified. Electrospun quaternized chitosan (QCh)/PVA nanofibers were cross-linked with the addition of a photo-cross-linking agent and UV light-

irradiation, to render the nanofibers insoluble in aqueous environments (Ignatova *et al.*, 2006). QCh/PVA nanofibers showed strong antimicrobial activity and were able to reduce viable cells of *S. aureus* and *E. coli*. Adherence, proliferation and/or viability of mammalian cells on these scaffolds were however not evaluated by the authors. A comprehensive *in vivo* wound healing evaluation on cross-linked PVA, wool protein-coated PVA (PVA-p-coated), wool protein/PVA co-electrospun (PVA-p-cospin), PVA containing silver nanoparticles (PVA-nAg), PCL, wool protein-coated PCL (PCL-p-coated), polyacrylonitrile (PAN), cross-linked polyacrylonitrile-polyurethane blend nanofibers (PAN-PEU) and poly(vinylidene fluoride-*co*-hexafluoropropene) (PVdF-HFP) nanofibers was conducted by Liu *et al.* (2010). Wounds covered with all nanofibers showed good fluid retention and wool protein seemed to enhance wound healing on day 10, as PVA-p-coated and PVA-p-cospin showed improved results over the control. PVA-p-coated also showed better healing on day 16, however PVA-p-cospin did not show improvements on the control. Cross-linked PVA showed enhanced wound healing over the control on day 16 and thus hydrophilic nanofibers were shown to increase wound healing ability, over the hydrophobic nanofibers. Very low levels of increased wound healing were however observed as compared to controls. Only PVA-nAg nanofibers showed antimicrobial activity towards *E. coli.*

Electrospun collagen nanofibers have shown promise as wound dressing and tissue regeneration scaffolds, however (partial) denaturation of collagen has been reported by some authors when electrospinning in HFIP or 2,2,2-trifluoroethanol (Matthews *et al.*, 2002, Yang *et al.*, 2008, Zhong *et al.*, 2005). Polyurethane (PU) nanofibers improved wound healing in full-thickness excisional wounds generated on male guinea pigs, when compared to Tegaderm™ (Khil *et al.*, 2003). Histological analysis revealed that the PU nanofiber-treated group showed lower inflammatory cells, whereas inflammatory cells were still present in the Tegaderm™-treated groups. PU nanofiber-treated groups also showed increased epithelialization and a well-organized dermis could be observed (Khil *et al.*, 2003). PCL and gelatin nanofibers, electrospun using needleless Nanospider™ technology, were evaluated as wound dressings (Dubský *et al.*, 2012). Gelatin nanofibers needed cross-linking with glutaraldehyde vapour. HDF and human dermal keratinocytes grew well on both gelatin and PCL nanofibers, however, gelatin nanofibers showed most promising results, with increased wound closure rate of full-thickness wounds in Wistar rats. Increased epithelialisation, increased depth of granulation tissue and increased myofibroblasts were observed in gelatin nanofiber-treated wounds.

Hemoglobin and myoglobin was successfully electrospun into fibers for potential delivery of oxygen to healing tissues (Barnes *et al.,* 2006). Hemoglobin and myoglobin fibers were subsequently cross-linked with glutaraldehyde vapor and increased stiffness was observed with an increase in cross-linking time. Porosity of hemoglobin fibers was concentration-dependent and increased as concentration increased. Platelet rich plasma was successfully electrospun into nano- and micro-fibers by Wolfe *et al.* (2011). Human platelet rich plasma was subjected to a freeze-thaw-freeze process, lyophilized to form a powdered preparation rich in growth factors (PRGF) and electrospun in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP). Protein release was observed from the fibers for at least 35 days, with the highest release observed on day 1. Human adipose derived stem cells and human umbilical artery smooth muscle cells were able to penetrate the PRGF fibers and increased penetration was observed as concentration (for electrospining) increased. Penetration was hypothesized to be a result of growth and chemotactic factors inherent to platelet rich plasma.

3.2.2 Nanofibers co-electrospun with bioactive molecules

Various drugs and proteins influencing wound healing (including antibiotics, silver nanoparticles, analgesics, growth factors, vitamins, antifungal agents, as well as herbal extracts) have been co-electrospun into nanofibers for controlled release. Polymer-drug interaction and solubility of the drug in the polymer solution will influence distribution of the drug in the nanofiber scaffold and will thus influence drug release profile. Drug stability in the solution is also an important factor to consider and addition of drugs and proteins to the solution can alter solution properties, thus influencing the electrospinning process.

3.2.2.1 Antibiotics

A low concentration of rifampin, co-electrospun with PLLA, was released from PLLA nanofibers when incubated in 0.05 M Tris-HCl buffer (Zeng *et al.,* 2003). Rifampin was only released from the nanofibers when proteinase K was added to the buffer solution and degradation was thus the main factor influencing rifampin release (Zeng *et al.*, 2003).

Cefazolin was successfully co-electrospun into PLGA nanofibers without significantly altering scaffold properties, however cefazolin-containing nanofibers had slightly larger diameters as compared to PLGA nanofibers without antibiotic (Katti *et al.,* 2004).

Tetracycline hydrochloride (TH) was released from poly(ethylene-co-vinyl acetate) (PEVA) nanofibers with a high initial burst, releasing 65% of the antibiotic in 120 h, whereas a 50:50 blend of PLA and PEVA released 50% in the same time period (Kenawy *et al.,* 2002). PLA showed a very high initial burst release, where after release was negligible. All nanofiber scaffolds and Actisite®, a commercial drug delivery system, showed an initial burst release upon incubation. TH increased fiber diameter and resulted in formation of less uniform fibers, when co-electrospun with PVA (Kanawung *et al.,* 2007). Most of TH was released within a 6-h period and increasing TH concentration reduced total release. A decrease in total release was also observed when the PVA fibers were coated by electrospinning PVA-sodium alginate nanofibers on top of the PVA-TH nanofibers.

Mefoxin was released from poly(D,L-lactide) (PDLLA) nanofibers with a very high burst release within 48 h (Zong *et al.,* 2002). Mefoxin also showed a high burst release from PLGA and poly(lactide-co-glycolide)/poly(lactic acid)/poly(ethylene glycol)-b-poly(lactic acid) PLGA/PEG-b-PLA diblock copolymer/PLA nanofibers (Kim *et al.,* 2004). The mefoxin-loaded nanofibers showed an inhibitory effect on the growth of *S. aureus* when tested in liquid culture, as well as on solid agar plates. Electrospun PLGA and PLGA/PEG-PLA blend fibers decreased abdominal adhesions in Sprague-Dawley rats, which underwent midline celiotomy (Zong *et al.,* 2004). PLGA and PLGA/PEG-PLA fibers reduced abdominal adhesion with 40% and 22% incidence of adhesions, respectively, whilst the control showed an incidence of 78%. A further significant decrease in abdominal adhesion was observed upon co-electrospinning with mefoxin and mefoxin-loaded PLGA/PEG-PLA fibers completely inhibited formation of abdominal adhesions.

Biteral was released with a high initial burst release from PCL nanofibers, releasing 80% of the antibiotic within 3 h, and total release was seen after 18 h (Bölgen *et al.,* 2007). The nanofibers prevented abdominal adhesions in Wistar-Albino rats and improved healing.

Gentamycin sulfate (GS) was encapsulated into PCL fibers by co-axial electrospinning, where PCL forms the shell and GS the core of the fibers (Huang *et al.,* 2006). GS release was dependent on fiber degradation, as *Pseudomonas* lipase added in the PBS release buffer resulted in GS release. The characteristic burst release was thus not observed in these fibers.

In a study aimed at the release of multiple compounds Takur *et al.* (2008) used a dual spinneret electrospinning setup to co-electrospin lidocaine hydrochloride (LH), an anesthetic, and mupirocin, an antibiotic, into PLLA fibers. LH showed an initial burst release and

mupirocin a low burst release, followed by a more sustained release (biphasic). Mupirocin showed a much higher release profile, without the presence of LH. Addition of LH thus altered the release profile of mupirocin in the dual setup. A dual spinneret setup was also used to generate scaffolds containing both PLGA and poly(ester urethane) urea (PEUU) (Hong *et al.*, 2008). TH was co-electrospun with the PLGA for drug release. Plain spun TH-PGLA nanofibers showed a high initial burst release with negligible release after 3 h. TH release was increased in the PEUU/TH-PLGA mixed nanofibers, with release being observed up to 96 h. PEUU/TH-PLGA nanofibers were able to inhibit the growth of *E. coli* for up to 7 days of incubation and prevented abscess formation and wound dehiscence in an abdominal model with fecal contamination in Lewis rats.

3.2.2.2 Silver nanoparticles

Silver (Ag) has been recognized as a strong antimicrobial compound for centuries and forms part of various commercial antimicrobial wound dressings (Zahedi *et al.*, 2008). Electrospinning of Ag into nanofibers to generate antimicrobial nanomaterials is thus well established and actively researched by various groups. Son *et al.* (2006) incorporated silver nanoparticles (nAg) in cellulose acetate nanofibers by co-electrospinning with silver nitrate ($AgNO_3$). Subsequent irradiation at 240 nm resulted in generation of nAg, with an average diameter of 21 nm, on the surface of the nanofibers. nAg-nanofibers showed strong antimicrobial activity towards *S. aureus, E. coli, K. pneumoniae* and *P. aeruginosa* after 18 h of incubation. Co-electrospun PCL and silver-loaded zirconium phosphate nanoparticles produced nanofibers that showed strong antimicrobial activity and was able to inhibit the growth of *S. aureus* and *E. coli* by 99.27% and 98.44%, respectively (Duan *et al.*, 2007). Importantly, HDF were able to attach and proliferate when cultured on these scaffolds. Gelatin, co-electrospun with $AgNO_3$, was subsequently cross-linked with glutaraldehyde to stabilize the nanofibers (Rujitanaroj *et al.*, 2008). Ag^+ from the gelatin nanofibers inhibited the growth of *P. aeroginosa*, *S. aureus*, *E. coli* and MRSA (Rujitanaroj *et al.*, 2008). Antimicrobial nanofibers were also generated by co-electrospinning Nylon 6 with a nAg suspension (8 nm in diameter) (Park *et al.*, 2009). Strong antimicrobial activity towards *S. aureus* and *K. pneumonia* was observed in a dose dependant manner, as the nAg content was increased from 100-1000 ppm in the nanofibers. PVA-nAg nanofibers were shown to inhibit *E. coli* growth (Liu *et al.*, 2010). In another study PVA, co-electrospun with $AgNO_3$, was cross-linked at 150 °C and needed UV irradiation to form nAg in the nanofibers (Chun *et al.*, 2010). PVA-nAg nanofibers however showed reduced adherence and proliferation of human epidermal keratinocytes and fibroblasts, indicating cytotoxicity of nAg. Ag^+ ions and nAg

were both evenly cytotoxic and nitrate ions were also shown to effect cell adherence and proliferation.

Cytotoxic effects of nAg have also been reported by other authors. nAg was shown to induce mitochondria-dependent apoptosis in NIH3T3 mouse fibroblasts (Hsin *et al.*, 2008). Reactive oxygen species (ROS) formation and activation of c-Jun NH_2-terminal kinases (JNK) was associated with nAg-induced apoptosis and subsequent inhibition of ROS and JNK inhibited apoptosis. nAg caused lung function changes and resulted in an increased inflammatory response when inhaled by Sprague-Dawley Rats (Sung *et al.*, 2008). Thickened alveolar walls and small granulomatous lesions could be observed in exposed animals. nAg and gold nanoparticles (nAu) were shown to be cytotoxic towards J774 A1 murine macrophages and cytotoxicity increased as size of the particles decreased (Yen *et al.*, 2009). It was however observed that nAg was not as toxic as nAu. nAg and nAu were observed inside the macrophages and it was hypothesized that the nanoparticles entered cells via endocytosis. nAg resulted in the formation of reactive oxygen species (ROS), reduced ATP, caused damage to mitochondria as well as DNA and resulted in cell cycle arrest (in G_2/M phase) in human lung fibroblast cells (IMR-90) and human glioblastoma cells (U251) (AshaRani *et al.*, 2009). nAg were observed in endosomes near the cell membrane, in the cytoplasm, lysosomes, as well as in the nucleus of treated cells. nAg also seemed to enter cells via endocytosis. nAg also caused apoptosis in mouse peritoneal macrophages (RAW264.7) and nAg could be observed within the treated cells (Park *et al.*, 2010). Later nAg was shown to exert cytotoxicity towards L929 fibroblasts and mouse peritoneal macrophage cells (RAW264.7), with the macrophages being more tolerant to nAg (Park *et al.*, 2011). A recent study showed that polymer stabilizers could alter the cytotoxicity of nAg. Poly(oxyethylene)-segmented imide (POEM), poly(styrene-co-maleic anhydride)-grafting poly(oxyalkylene) (SMA) and PVA was used as polymer stabilizers by Lin *et al.* (2012). SMA-nAg and PVA-nAg showed lower cytotoxicity as compared to POEM-nAg, however cytotoxicity was still observed in a dose dependent manner in mouse skin fibroblasts (L929), human hepatocarcinoma cells (HepG2), and mouse monocyte macrophages (J774A1). Once again nAg entered L929 fibroblasts and HepG2 cells in a dose dependent manner. PVA-nAg showed no hemolysis up to 10 ppm, whereas POEM-nAg and SMA-nAg showed hemolysis at 4 ppm and 6 ppm, respectively. Metallic silver in commercial wound dressings has also resulted in discoloration (blue-gray colour) of treated wounds (Jayakumar *et al.*, 2012). Safety concerns thus exist with regards to long term safe usage of silver.

3.2.2.3 Analgesics and anti-inflammatory drugs

Paracetamol has been co-electrospun into acid-labile fibers, prepared by reacting 3,9-dimethylene-2,4,8,10-tetraoxaspiro [5.5] undecane with 1,10-decanediol or PEG with subsequent co-polymerization with D,L-lactide (Qi *et al.*, 2008). Resulting fibers were designated $PLAOE_D$, $PLAOE_P$ and PDLA. Fibers were produced with mean diameters of 940, 1490, and 1260 nm for $PLAOE_D$, $PLAOE_P$ and PDLA, respectively. Paracetamol was released from the fibers in a biphasic manner with an initial burst release, followed by a sustained release and release of paracetamol increased as pH decreased. $PLAOE_D$ and PLAOE fibers released a higher percentage of paracetamol, compared to PDLA fibers. Diclofenac sodium (DS) was released from poly(ε-caprolactone/D,L-lactide) nanofibers with an initial burst, releasing up to 45% in 24 h (Nikkola *et al.*, 2006). DS showed low total drug release with a high initial burst release in the first 1-1.5 h from PCL nanofibers (Kanawung *et al.*, 2007). Ibuprofen also showed a burst release from PLGA nanofibers, releasing up to 85% over a 4 day period (Jiang *et al.*, 2004). Ibuprofen release was improved by co-electrospinning ibuprofen into PLGA-PEG-g-chitosan nanofibers and further improvements were obtained by covalent binding of ibuprofen to PLGA-PEG-g-chitosan nanofibers. Ketoprofen was co-electrospun into PVA nanofibers with subsequent incubation in methanol to cross-link the PVA nanofibers (Kenawy *et al.*, 2007). PVA nanofibers cross-linked for 24 h showed a lower release profile, especially a lower initial burst release, as compared to PVA nanofibers cross-linked for 1 h and PVA nanofibers without cross-linking. Ketoprofen release was dependent on temperature and a higher total release was observed at 37 °C, compared to 20 °C. Meloxicam was co-electrospun into PVA for transdermal delivery (Ngawhirunpat *et al.*, 2009). Higher skin permeation was observed for meloxicam in meloxicam-loaded nanofibers, as compared to films, when skin permeation tests were conducted using shed snake skin of *Elaphae obsolete*. Skin permeation flux was shown to be time-dependent.

3.2.2.4 Growth factors

Epidermal growth factor (EGF) was slowly released from silk/PEO fibers in a time-dependent manner, with an initial burst release (Schneider *et al.*, 2009). EGF-silk nanofibers promoted wound healing in a three-dimensional wound healing model and was able to increased re-epithelialization and wound closure of model wounds.

Nanofibers were electrospun from amine-terminated PCL-PEG/PCL with functional amine groups on the surface, to which recombinant EGF (rhEGF) was subsequently immobilized by chemical conjugation (Choi *et al.*, 2008). Keratinocyte-specific gene (keratin

1 and loricrin) expression was upregulated when human primary keratinocytes (HPK) were cultured on rhEGF-PCL-PEG/PCL nanofibers and rhEGF-PCL-PEG/PCL nanofibers improved early wound healing in diabetic C57BL/6 mice. When rhEGF was added as solution on PCL-PEG/PCL nanofibers (i.e. not conjugated), it showed less promising results. In another study by Choi *et al.* (2011) amine-functionalized PCL/PEG block copolymers were electrospun using a co-axial electrospinning setup, forming the shell. The core consisted of a PVA solution containing basic fibroblast growth factor (bFGF). After electrospinning EGF was immobilized on the surface of the electrospun nanofibers, to generate scaffolds containing multiple growth factors (bFGF/EGF). Release of bFGF was observed from the fibers with a small initial burst release, followed by a sustained release whereas very low release, 2% in 7 days, was observed for the EGF. The bFGF/EGF nanofibers increased cell proliferation of HDF and HPK after 7 days of incubation. Increased wound healing in diabetic C57BL/7 mice treated with bFGF/EGF nanofibers was also observed at day 7. Nanofibers containing either bFGF or EGF also showed improved wound healing over plain nanofibers and controls. Wounds treated with bFGF/EGF nanofibers showed expression of keratin 1, 5 and 14. The bFGF-nanofibers induced similar levels of expression of keratin 14 and a 75% lower expression of keratin 5, whereas EGF-nanofibers induced lower levels of keratin 5 and 14 expression. This indicates that bFGF/EGF nanofibers resulted in remodeling of the wound into normal epidermis-like tissue (Choi *et al.*, 2011).

Two model proteins, BSA and epidermal growth factor (EGF), was encapsulated in electrospun fibers by first encapsulating the proteins into nanoparticles, mixing the particles and then subsequently electrospinning the solution into fibers (Dong, 2009) The proteins were found in different domains of the fibers. This method was also used to encapsulate dextran and BSA into electrospun fibers. The release of these compounds was not studied by the authors.

3.2.2.5 Vitamins

Vitamins play an important role in wound healing, where vitamin A plays a role in collagen synthesis and influencing epithelial differentiation, whereas vitamin C is essential in intracellular matrix of tissues (Zahedi *et al.*, 2010). Vitamin A acid and vitamin E was successfully co-electrospun into cellulose acetate nanofibers for dermal delivery (Taepaiboon *et al.*, 2007). Addition of vitamins did not alter fiber properties and fibers had diameters of 253 and 247 nm, for vitamin A and vitamin E, respectively. An initial burst release of both vitamins was observed, followed by a sustained release with close to 100% being released in

acetate buffer containing 0.5 vol % Tween 80 and 10 vol % methanol over 6 h and 24 h, for vitamin A and vitamin E, respectively. Lower release profiles were obtained in acetate buffer containing 0.5 vol % Tween 80.

3.2.2.6 Antifungals and herbal extracts

Itraconazole, and antifungal agent, was co-electrospun into non-biodegradable PU fibers (Verreck *et al.*, 2003). PU nanofibers with 10% itraconazole showed a linear drug release profile, whereas nanofibers containing 40% showed a biphasic release profile.

Centella asiatica-herbal extract was co-electrospun into gelatin nanofibers, with subsequent cross-linking with glutaraldehyde vapour (Sikareepaisan *et al.*, 2008). *Centella asiatica* is a medicinal plant widely known for its traditional medical applications including its wound healing ability (Sikareepaisan *et al.*, 2008). Release of asiaticoside was measured, as this compound was identified as the most active in wound healing (Shukla *et al.*, 1999). Asiaticoside was released from the fibers with a high initial burst release within the first 2 days.

4. Conclusion

Wound healing is a complex process and infection can cause a delay in normal wound healing and recovery. Increased antibiotic resistance is becoming problematic and alternative antimicrobials can play an integral role in the future of successful wound treatment. Cytotoxicity of certain antimicrobials is of concern and alternatives need to be explored. Antimicrobial peptides, especially bacteriocins produced by Gram-positive bacteria, have enormous potential as next generation antimicrobials for treatment of various bacterial infections, including topical skin infections.

A novel approach to generate antimicrobial wound dressings would be to co-electrospin bacteriocins of LAB into nanofibers. Because of the large surface area to volume ratio, high amounts of bacteriocins could potentially be incorporated and can then directly be delivered to a site of infection. A controlled localized released of the bacteriocins can be obtained to inhibit and/or prevent the growth of pathogenic bacteria. These antimicrobial materials can have various applications, not only as wound dressing materials but also as coating for biomedical devices to combat device related infections.

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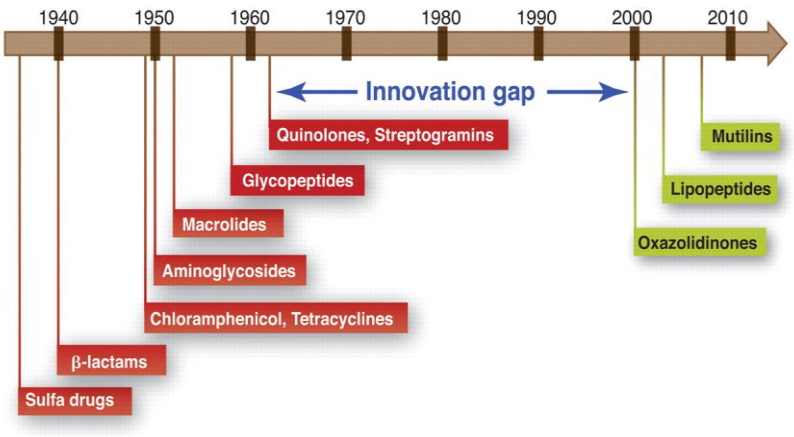

Fig. 1 Classes of antibiotics introduced in the past (Fischbach and Walsh, 2009).

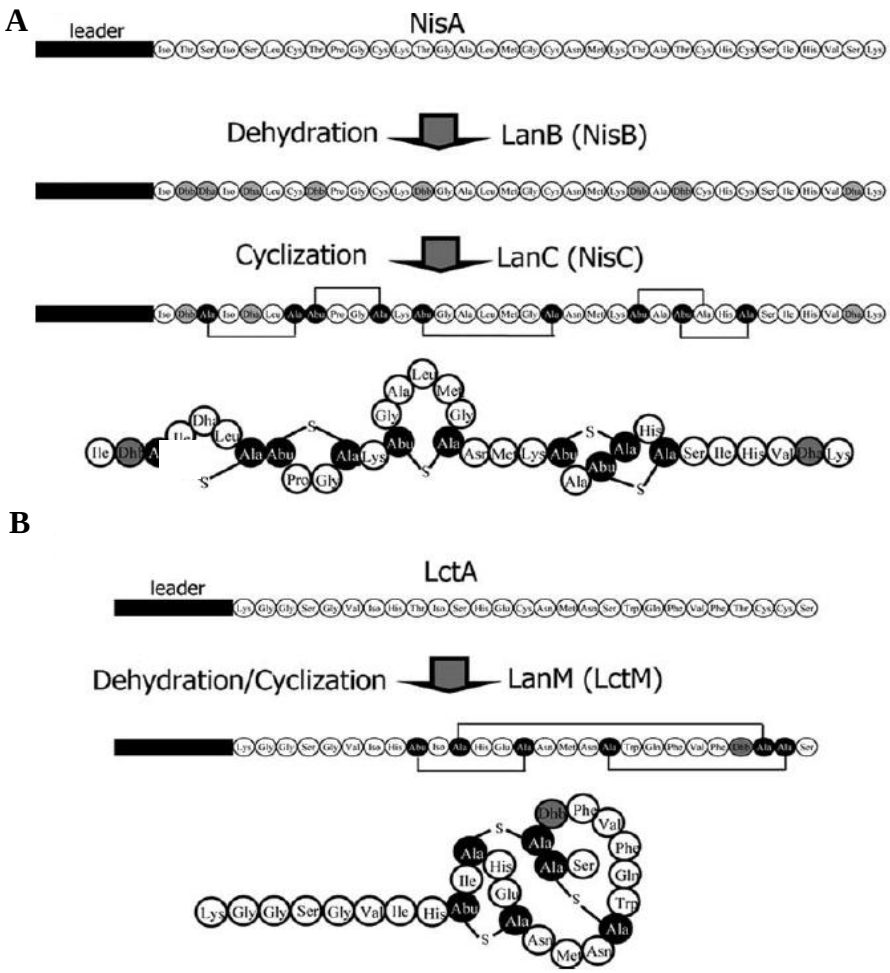

Fig. 2 Post-translational modification of nisin (**A**) and lacticin 481 (**B**). Nisin modification enzymes include two distinct enzymes responsible for dehydration and cyclization, whereas lacticin 481 is modified by one bifunctional enzyme (Cotter *et al.*, 2005a).

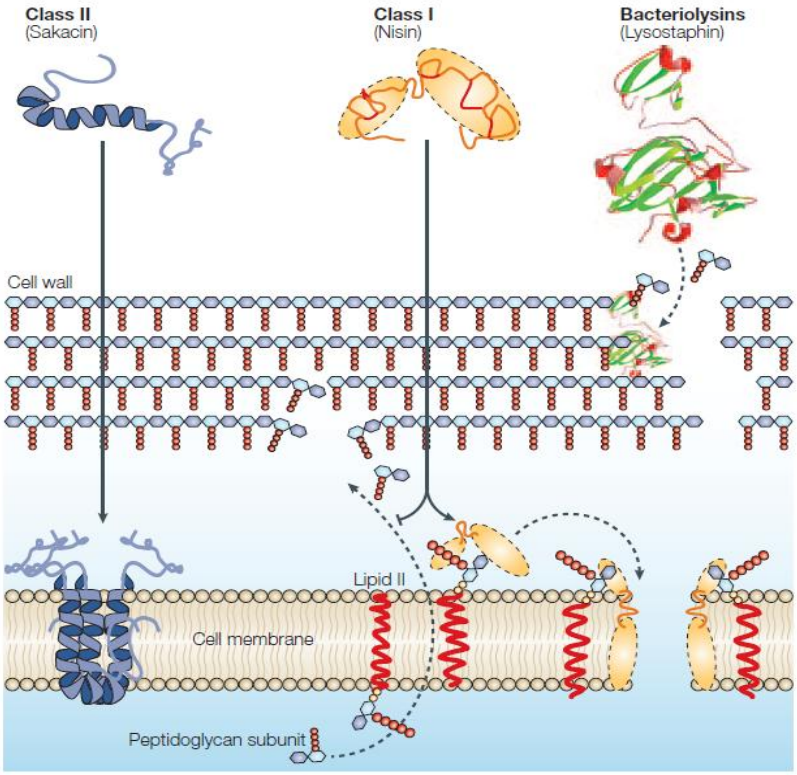

Fig. 3 Mode of action of different bacteriocin classes (Cotter *et al.*, 2005b).

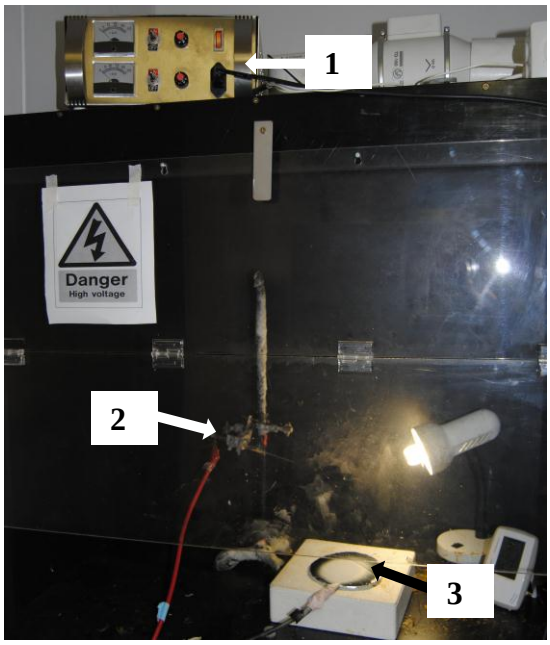

Fig. 4 Electrospinning apparatus used in this study to generate nanofibers. **1**-High voltage power supply, **2**-capillary tube containing polymer solution and **3**-collector with nanofibers visible.

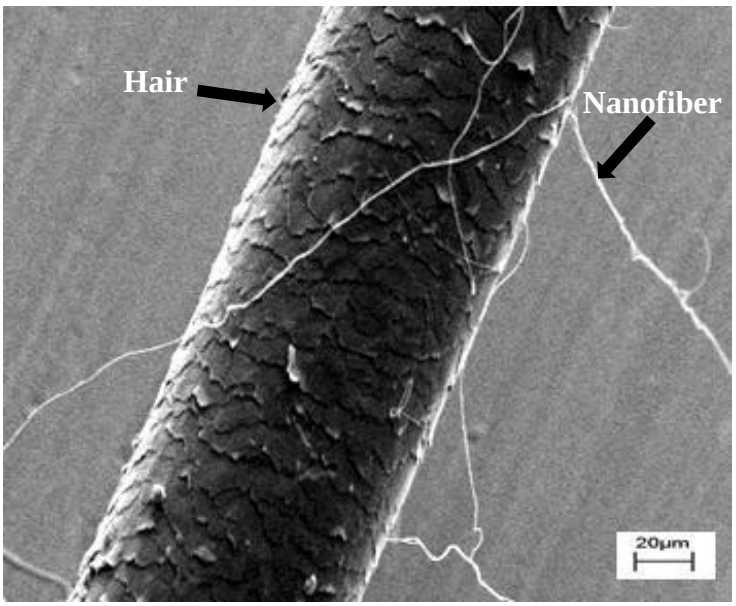

Fig. 5 Electrospun PEO:PDLLA nanofibers (generated in this study) versus human hair.

AIMS OF THIS STUDY

1. Evaluate the feasibility of co-electrospinning bacteriocins of lactic acid bacteria into nanofibers.

2. Evaluate the feasibility of co-electrospinning viable *L. plantarum* 423 cells into nanofibers.

3. Develop and characterize a suitable nanofiber drug delivery scaffold for bacteriocins of lactic acid bacteria.

4. Evaluate the nanofiber drug delivery system as wound dressing material to treat *S. aureus*-induced skin infections in mice.

CHAPTER 3

Encapsulation of *Lactobacillus plantarum* 423 and its Bacteriocin in Nanofibers

Abstract

Plantaricin 423, produced by *Lactobacillus plantarum* 423, was encapsulated in nanofibers that were produced by the electrospinning of 18% (w/v) poly(ethylene oxide) (200 000 Da). The average diameter of the nanofibers was 288 nm. Plantaricin 423 activity decreased from 51 200 AU/ml to 25 600 AU/ml and from 204 800 AU/ml to 51 200 AU/ml after electrospinning, as determined against *Lactobacillus sakei* DSM 20017^T and *Enterococcus faecium* HKLHS, respectively. Viable cells of *L. plantarum* 423 encapsulated in nanofibers decreased from 2.3 x 10^{10} cfu/ml before electrospinning to 4.7 x 10^8 cfu/ml thereafter. Cells entrapped in the nanofibers continued to produce plantaricin 423. As far as we could determine this is the first report on the encapsulation of a bacteriocin and cells of *L. plantarum* in nanofibers. The method may be used to design a drug delivery system for bacteriocins and the encapsulation of probiotic lactic acid bacteria.

Introduction

Lactic acid bacteria (LAB) are generally regarded as safe and their antimicrobial peptides (bacteriocins) have been used in the preservation of many food products (De Vuyst and Vandamme, 1994). Various claims have been attributed to LAB with probiotic properties, e.g. reduction or prevention of gastrointestinal disorders, including inflammatory bowel disease, alleviation of lactose intolerance, lowering of serum cholesterol levels, stimulation of the immune system and control of tumor growth (Franz *et al.*, 1999, Saarela *et al.*, 2000) Some probiotic claims may be associated with the production of antimicrobial peptides (bacteriocins).

Bacteriocins are produced by many LAB, including species that are associated with humans, viz. the *Lactobacillus acidophilus* group (*L. acidophilus*, *Lactobacillus amylovorus*, *Lactobacillus crispatus*, *Lactobacillus gallinarum*, *Lactobacillus gasseri* and *Lactobacillus johnsonii*), *Lactobacillus reuteri*, *Lactobacillus casei*, *Lactobacillus fermentum* and *Lactobacillus plantarum* (De Vuyst and Vandamme, 1994, Tahara and Kanatani, 1997). Many of the bacteriocins produced by these species are active against foodborne pathogens such as *Listeria*, *Clostridium*, *Bacillus* and *Staphylococcus* spp. (Schillinger *et al.*, 1996).

The antimicrobial activity of bacteriocins in the gastrointestinal tract (GIT) is difficult to prove. Maré *et al.* (2006) showed that plantaricin 423 was produced by cells of *L. plantarum* 423 colonizing the ileum of piglets. Studies with real-time PCR have shown that

the gene encoding plantaricin 423 is constitutively expressed at conditions simulating the GIT (Ramiah *et al.*, 2007). Different levels of plantaricin 423 production have also been recorded in each of the sections of a GIT model (Botes *et al.*, 2008), supporting the hypothesis that plantaricin 423 may be produced *in vivo*. Plantaricin 423 inhibited the growth of *Bacillus cereus*, *Clostridium sporogenes*, *Enterococcus faecalis*, several *Lactobacillus* spp., *Oenococcus oeni*, *Listeria innocua*, *Listeria monocytogenes*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Propionibacterium acidipropionici*, *Propionibacterium* sp., *Staphylococcus carnosum* and *Streptococcus thermophilus* (van Reenen *et al.*, 1998), and excluded *Enterobacteriaceae*, but not lactic acid bacteria, from the cecum and colon in Wistar rats (Ramiah *et al.*, 2009).

Promising results were reported with lantibiotics regarding the control of pathogens in airways. Kruszewska *et al.* (2004) reported on growth repression of methicillin-resistant *Staphylococcus aureus* (MRSA) strains in the respiratory tract of mice with mersacidin, a lantibiotic produced by *Bacillus* sp. HIL Y-85,54728. Similar studies with nisin, a lantibiotic produced by *Lactococcus lactis* subsp. *lactis*, did not eradicate *S. aureus* from the nasal tract of rats (Kokai-Kun *et al.*, 2003). Nisin F, a lantibiotic produced by *L. lactis* subsp. *lactis*, proved effective in the treatment of sinusitis in rats (de Kwaadsteniet *et al.*, 2009), but less effective in treatment of skin infections in mice (de Kwaadsteniet *et al.*, 2010). Reports on bacteriocins of LAB active against Gram-negative bacteria (Caridi, 2002, Ivanova *et al.*, 1998, Messi *et al.*, 2001, Todorov and Dicks, 2005a,b) sparked a renewed interest in these peptides and their interaction with intestinal pathogens. Data generated from genome sequences of probiotic bacteria such as *L. plantarum* WCFS1, *L. acidophilus* NCFM and *L. johnsonii* NCC 533 (Alterman *et al.*, 2005, Kleerebezem *et al.*, 2003, Pridmore *et al.*, 2004) and the application of DNA micro-array technology (de Vries *et al.*, 2006) may provide valuable answers to *in vivo* gene expression.

Studies on the medical applications of bacteriocins need to be done under strictly controlled conditions, demanding the design of special drug delivery systems. In this chapter, a brief description of a novel drug delivery system for LAB and their bacteriocins are given.

Materials and Methods

Production and Isolation of Bacteriocins

L. plantarum 423 was cultured in MRS broth (Biolab Diagnostics, Midrand, South Africa) at 30 °C for 24 h. Cells were harvested (8000g, 10 min, 4 °C), the pH of the cell-free supernatant

adjusted to pH 6.5-7.0 with 1N NaOH and then heated at 80 °C for 10 min. Plantaricin 423 was precipitated from the cell-free supernatant with 70% saturated ammonium sulfate (Sambrook *et al.*, 1989) and desalted against distilled water by using a 1000 Da cutoff dialysis membrane (Spectrum Inc., CA, USA). The sample was freeze-dried and stored at -20 °C.

Bacteriocin activity tests were performed by using the agar-spot method described by van Reenen *et al.* (1998). *Lactobacillus sakei* DSM 20017^T and *Enterococcus faecium* HKLHS, cultured in MRS broth at 30 °C, were used as target strains. Antimicrobial activity was expressed as arbitrary units (AU) per ml. One AU was defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition (van Reenen *et al.*, 1998).

Preparation of Nanofibers

Poly(ethylene oxide) (PEO, 200 000 Da), obtained from Sigma-Aldrich (St. Louis, MO, USA), was dissolved (18%, w/v) in a solution of plantaricin 423 (204 800 AU/ml, as determined against *E. faecium* HKLHS) and carefully mixed. In another experiment, *L. plantarum* 423 was cultured at 37 °C for 24 h, harvested (8000g, 2 min) and the cells resuspended in sterile distilled water to ±2 x 10^{11} cfu/ml. From this suspension, 100 ul (2.3 x 10^{10} cfu/ml) was mixed with 500 µl (18%, w/v) PEO dissolved in sterile distilled water.

The polymer solutions were each placed in a tapered sterile glass tube and fixed at a 15° angle (Fig. 1). The positive electrode (cathode) was placed in the polymer solution, and the anode on a collector plate which was covered with sterile foil and positioned 15 cm from the cathode. A constant electric field of +10 kV was applied to the polymer solution and -5 kV to the collector. The relative humidity was kept constant at 50% and the temperature at 25 °C. Nanofibers, ejected from the tip of the tapered glass tube, were collected at the anode (Fig. 1). Nanofibers were coated with gold particles and visualized using a Leo® 1430VP Scanning Electron Microscope. The diameter of the nanofibers was determined by using the SEM Image Studio software (version 10.1).

Evaluation of the Bacteriocin and Cell-Encapsulation in Nanofibers

Nanofibers with plantaricin 423 were collected from the metal surface, and 25 mg suspended in 500 µl sterile distilled water by vigorous mixing on a vortex. The antimicrobial activity of the solution was determined by using the agar-spot method as described before. *L. sakei* DSM 20017^T and *E. faecium* HKLHS served as target strains. The number of viable cells encapsulated in the nanofibers was determined by vortexing 25 mg fibers in 500 µl sterile saline (0.75%, w/v, NaCl). Serial dilutions were made in sterile saline and plated onto MRS

agar. Colonies were counted after 48 h of incubation at 37 °C. Plantaricin 423 production by encapsulated cells was evaluated by overlaying the colonies with *E. faecium* HKLHS embedded in MRS agar. The plates were incubated at 37 °C for 48 h and then inspected for formation of inhibition zones.

Scanning Electron Microscopy of Lactobacillus plantarum 423 cells

L. plantarum 423 was cultured, as described before, placed on a glass slide and air dried. Images of cells were recorded with a Leo® 1430VP Scanning Electron Microscope (SEM). Samples were coated with a thin layer of gold to increase conductivity for imaging.

Results

The activity of plantaricin 423 in the cell-free supernatant was recorded as 51 200 AU/ml against *L. sakei* DSM 20017^T and 204 800 AU/ml against *E. faecium* HKLHS. After electrospinning, the activity of plantaricin 423 in the nanofibers was 25 600 AU/ml against *L. sakei* and 51 200 AU/ml against *E. faecium* HKLHS (Fig. 2). The diameter of the fibers ranged from 169 nm to 465 nm, with a mean diameter of 288 nm (Fig. 3).

The cell numbers of *L. plantarum* 423 decreased from 2.3 x 10^{10} cfu/ml before electrospinning to 4.7 x 10^8 cfu/ml thereafter, as determined by dissolving the fibers in sterile saline. Viable cells of *L. plantarum* 423 encapsulated in nanofibers and inhibition zones produced by encapsulated cells are presented in Fig. 5a and b, respectively.

Discussion

Several methods are used to produce ultrafine fibers, e.g. self assembly of polymers (Hartgerink *et al.*, 2001, Nagai *et al.*, 2006) template synthesis (Pender and Sneddon, 2000), phase separation (Tan and Lim, 2004) and electrospinning (Chew *et al.*, 2005, Zhou *et al.*, 2008). Electrospinning is a versatile and relatively easy technique to produce large amounts of ultrathin fibers. During the electrospinning process, an electric field is applied to a polymer solution to form a Taylor cone (Taylor, 1969, Yarin *et al.*, 2001). When the electric forces overcome the surface tension of the solution, a charged jet is ejected from the Taylor cone and accelerates toward the collector. The solvent evaporates and nanofibers are formed (Agarwal *et al.*, 2008, Liang *et al.*, 2007). The quality and characteristics of the final product are determined by the elasticity, viscosity, temperature, conductivity and surface tension of the polymer solution, strength of the electric field, distance between the Taylor cone and collector, and humidity (Huang *et al.*, 2003, Liang *et al.*, 2007).

Electrospinning has been used to incorporate antibiotics (Kenawy *et al.*, 2002, Zeng *et al.*, 2003), growth factors, proteins (Chew *et al.*, 2005, Li *et al.*, 2008, Maretschek *et al.*, 2008) and silver nanoparticles (Son *et al.*, 2006). As far as we could determine, we are the first to report on the encapsulation of viable cells of *L. plantarum* and a bacteriocin. The slight decrease in antimicrobial activity of plantaricin 423 recorded after electrospinning could be due to the high voltage applied. However, Kim *et al.* (2007) have reported only a slight decrease (10%) in the activity of lysozyme after electrospinning. Human β-nerve growth factor released from electrospun fibers was still able to differentiate PC12 cells into neurons (Chew *et al.*, 2005).

Judged from the decrease in cell numbers (from 2.3 x 10^{10} cfu/ml before electrospinning to 4.7 x 10^{8} cfu/ml when encapsulated), only 2% of the *L. plantarum* survived after encapsulation into nanofibers (Fig. 5). A higher percentage of cells might have been encapsulated, but could have been damaged by the high voltage, sudden change in osmotic pressure and whipping or shearing action of electrospinning. It is also possible that cells could have lost their viability after encapsulation. Only 0.1% of *Escherichia coli* cells encapsulated in PEO nanofibers survived, whereas 74% of *Micrococcus luteus* cells survived electrospinning (Gensheimer *et al.*, 2007). It would seem as if smaller cells and Gram-positive bacteria are more resistant to electrospinning. Further research is needed to determine the exact fate of the cells. The small size of the fibers and the large surface to volume ratio provide for encapsulation of high cell numbers, thus compensating for the loss in viability.

In this chapter the feasibility of encapsulating lactic acid bacteria and bacteriocins into electrospun nanofibers was demonstrated. Encapsulation of bacteriocin-producing cells and cells with probiotic properties may have specific food and medical applications. Through careful selection of polymers, higher concentrations of bacteriocins and higher cell numbers may be spun into nanofibers.

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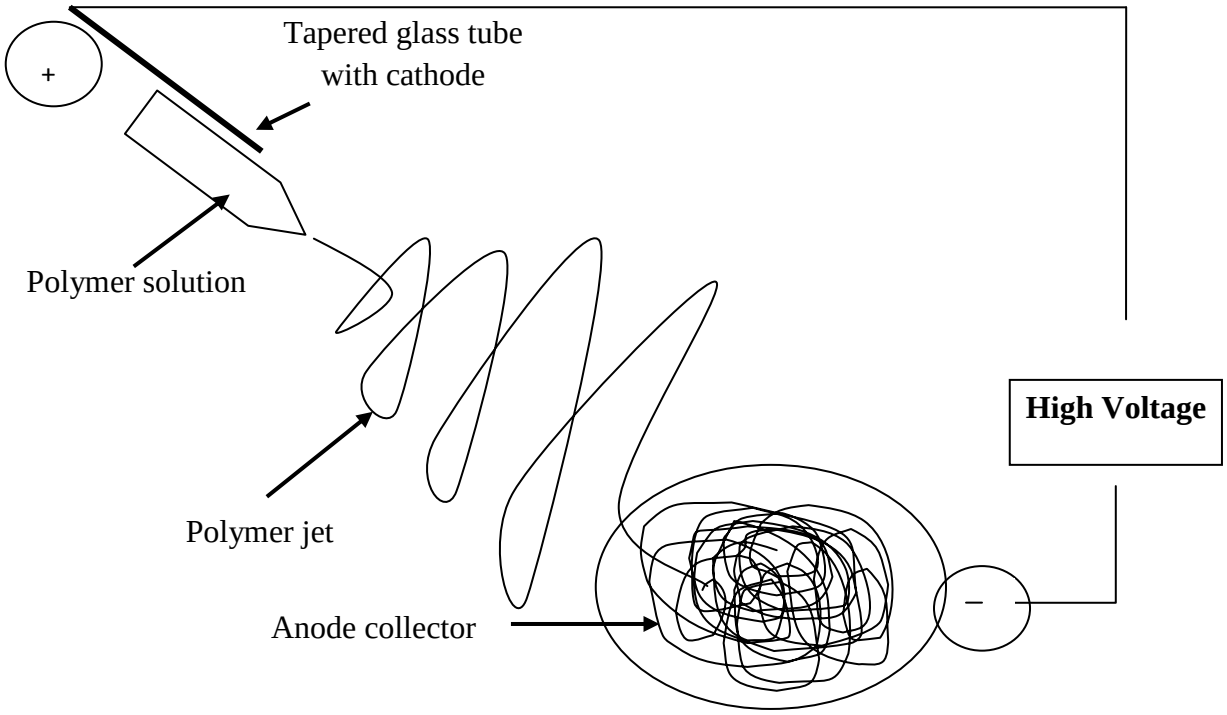

Fig. 1 Schematic representation of the electrospinning process with a high voltage supply, tapered glass tube and collector.

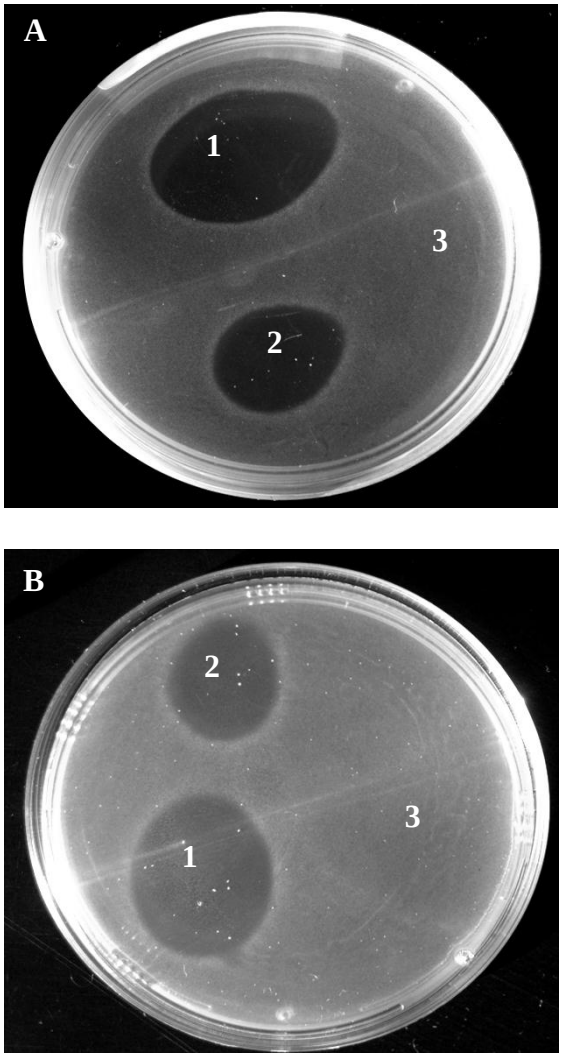

Fig. 2 Activity of plantaricin 423 encapsulated in PEO nanofibers. The sensitive strains were (**A**) *E. faecium* HKLHS and (**B**) *L. sakei* DSM 20017^{T}. **1**-Plantaricin 423 before electrospinning (positive control), **2**-Plantaricin 423 after electrospinning and **3**-PEO polymer suspension (negative control).

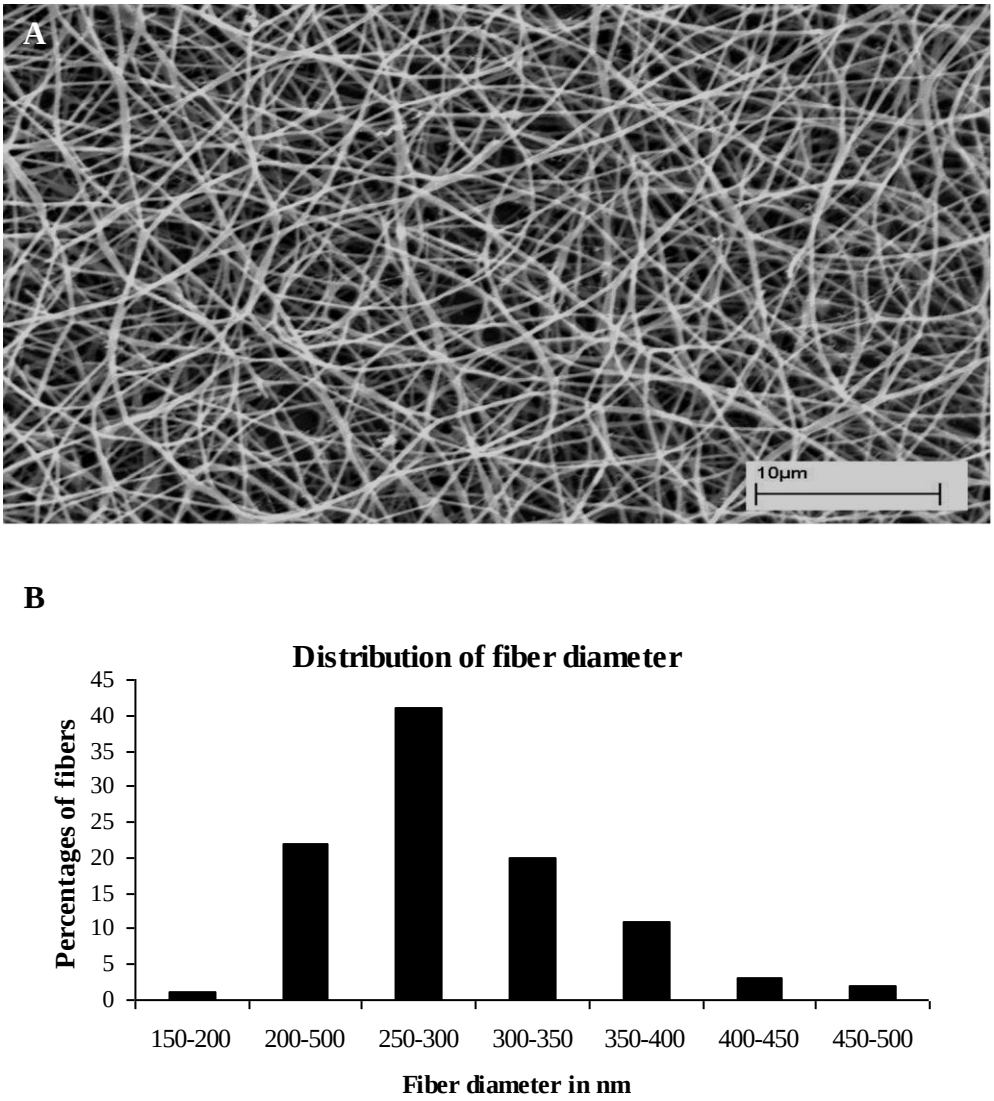

Fig. 3 (**A**) SEM image of 18% (w/v) PEO nanofibers co-electrospun with plantaricin 423. (**B**) Variation of nanofiber sizes, ranging from 169 nm to 465 nm in diameter. Most nanofibers were in the 250 to 300 nm range (mean diameter = 288 nm).

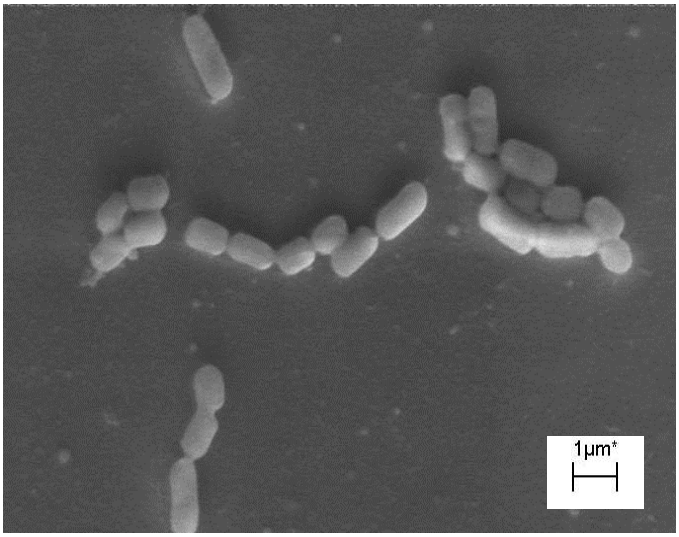

Fig. 4 SEM image of *L. plantarum* 423 cells.

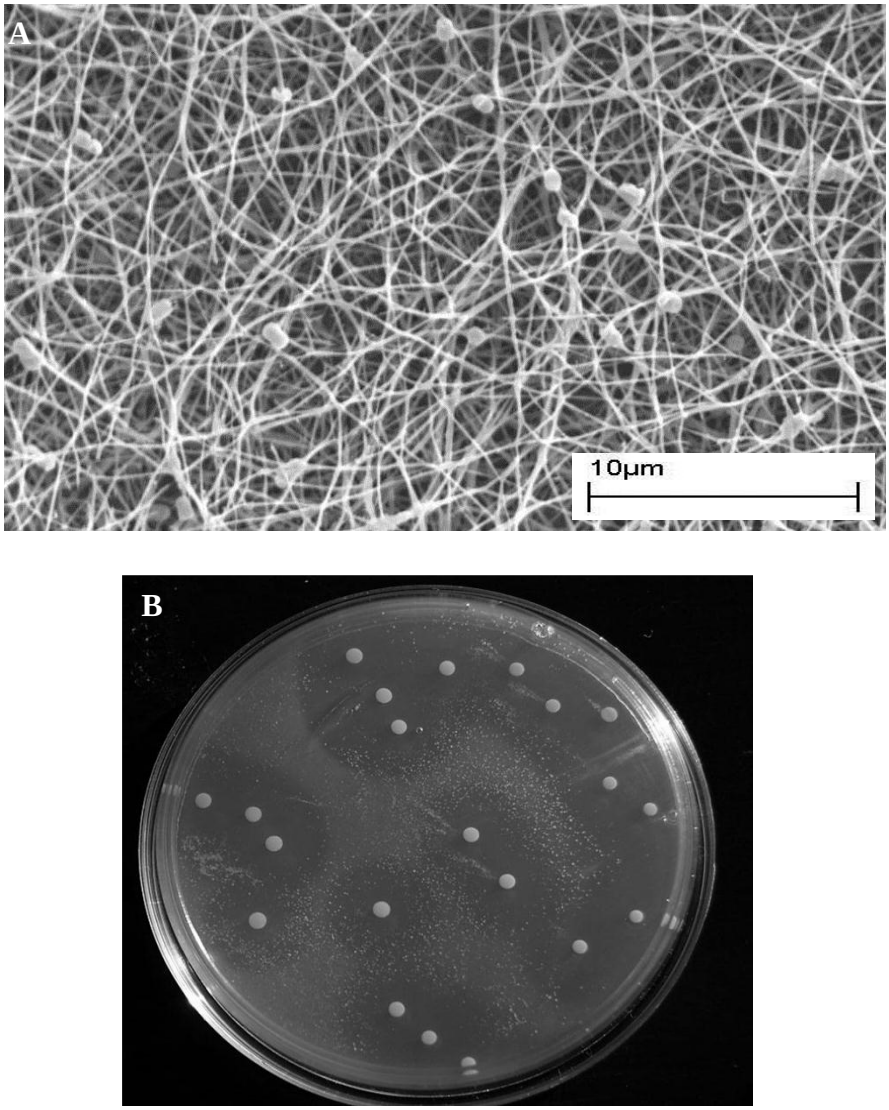

Fig. 5 (**A**) Viable cells of *L. plantarum* 423 encapsulated in 18% (w/v) PEO nanofibers, clearly showing a "bead" structure where cells were entrapped. (**B**) Growth inhibition of *E. faecium* HKLHS by plantaricin 423 produced from encapsulated cells.

CHAPTER 4

Release of Bacteriocins from Nanofibers Prepared with Combinations of Poly(D,L-lactide) (PDLLA) and Poly(Ethylene Oxide) (PEO)

Abstract

Plantaricin 423, produced by *Lactobacillus plantarum* 423, and bacteriocin ST4SA, produced by *Enterococcus mundtii* ST4SA, were electrospun into nanofibers prepared from different combinations of poly(D,L-lactide) (PDLLA) and poly(ethylene oxide) (PEO) dissolved in *N,N*-dimethylformamide (DMF). Both peptides were released from the nanofibers with a high initial burst and retained 88% of their original antimicrobial activity at 37 °C. Nanofibers have the potential to serve as carrier matrix for bacteriocins and open a new field in the development of controlled antimicrobial delivery systems for various applications.

Introduction

Bacteriocins (ribosomally synthesized antimicrobial peptides) and bacteriocin-like inhibitory substances (BLIS) are produced by several species of bacteria, including lactic acid bacteria that are generally recognized as safe (Cotter *et al.*, 2005, Dicks *et al.*, 2009, Todorov and Dicks, 2005). Bacteriocins are diverse peptides and can be divided into the Class I (lanthionine-containing post-translationally modified bacteriocins), Class II (non-lanthionine-containing bacteriocins that do not undergo extensive post-translational modification) and the bacteriolysins (Cotter *et al.*, 2005). Each of these groups can be further sub-divided. Bacteriocins are membrane active peptides and most form pores in the cell membranes of sensitive bacteria due to the cationic and amphiphilic nature of these peptides, however there are exceptions (Bauer and Dicks, 2005). Pore formation leads to the disruption of the proton motive force (PMF) and the efflux of intracellular material (Knoetze *et al.*, 2008). Bacteriocins can have a narrow (active against closely related species) or broad spectrum of activity (Jack *et al.*, 1995, Kleanhammer, 1993). Class Ia bacteriocins, or lantibiotics, use lipid II as target for their mode of action and kill sensitive bacteria by pore formation, or by hampering cell wall formation (McAuliffe *et al.*, 2001). Lantibiotics can also inhibit the germination of spores (McAuliffe *et al.*, 2001) Class IIa, or pediocin-like bacteriocins, mainly form pores in the membranes of sensitive cells (Cotter *et al.*, 2005).

More than 95% of *Staphylococcus aureus* isolates are resistant to penicillin. Of these, 60 to 70% are resistant to methicillin (Kim, 2009). A few bacteriocins are active against methicillin-resistant strains of *S. aureus* (MRSA), and versus other bacteria of interest in human health, such as *Pseudomonas aeruginosa*, *Streptococcus pneumonia* and vancomycin-

resistant enterococci (Chatterjee *et al.*, 1992, Galvin *et al.*, 1999). Bacteriocin ST4SA, produced by *Enterococcus mundtii* ST4SA, is a class IIa bacteriocin and is active against several pathogens, including *Acinetobacter baumannii*, *P. aeruginosa*, *S. pneumonia*, *S. aureus* and *Enterococcus faecium* (Knoetze *et al.*, 2008). Bacteriocin ST4SA survived conditions in the animal gastro-intestinal tract (Dicks and ten Doeschate, 2010, Ramiah *et al.*, 2009) and was constitutively expressed (Granger *et al.*, 2008). Nisin F, produced by *Lactococcus lactis* subsp. *lactis* F10, was effective in the treatment of *S. aureus* in respiratory tract infections (de Kwaadsteniet *et al.*, 2009). Plantaricin 423, produced by *Lactobacillus plantarum* 423, displaced *Clostridium sporogenes* and *Enterococcus faecalis* from Caco-2 cells (Ramiah *et al.*, 2008). Plantaricin 423 is also a class IIa bacteriocin and shows activity against *Bacillus cereus*, *C. sporogenes*, *E. faecalis*, *Lactobacillus brevis* (*Lactobacillus* spp.), *Listeria innocua*, *Listeria monocytogenes*, *Propionibacterium* sp., *Propionibacterium acidipropionic* and *Staphylococcus carnosum* (van Reenen *et al.*, 1998). Bacteriocins may thus offer an alternative to control bacterial infections, administered alone or in combination with antibiotics (Knoetze *et al.*, 2008). The challenge would be to protect bacteriocins from proteolytic enzymes, keep the peptides in contact with the infected surface and extend the contact time for as long as possible.

Electrospun nanofibers, prepared from selected polymers, may be used as carrier matrix for bacteriocins and other antimicrobial peptides (Heunis and Dicks, 2010). Nanofibers have been used to release antibiotics, proteins, growth factors, silver nanoparticles, plasmid DNA, as well as viable cells (Chew *et al.*, 2005, Heunis and Dicks, 2010, Heunis *et al.*, 2010, Kenawy *et al.*, 2002, Kim *et al.*, 2004, 2007, López-Rubio *et al.*, 2009, Luu *et al.*, 2003, Maretschek *et al.*, 2008, Rujitanaroj *et al.*, 2008). Specific structures can be designed, such as oxygen permeable membranes, matrices with a high surface to volume ratio and scaffolds that are morphologically similar to the extracellular matrix (Li *et al.*, 2002, Smith and Ma, 2004, Yang *et al.*, 2005, Zeng *et al.*, 2003, Zhou *et al.*, 2008).

In this chapter the feasibility of electrospinning bacteriocins into nanofibers consisting of polymer blends was investigated, as well as the potential of the nanofibers to serve as a delivery system for the bacteriocins. Plantaricin 423 and bacteriocin ST4SA were used as model bacteriocins (class IIa) in this study. These bacteriocins were dissolved in *N*,*N*-dimethylformamide (DMF) and electrospun into biodegradable nanofiber blends of poly(D,L-lactide) (PDLLA) and poly(ethylene oxide) (PEO). The nanofiber scaffolds were evaluated (*in vitro*) for the release of the peptides and antimicrobial activity.

Materials and Methods

Isolation of Antimicrobial Peptides

L. plantarum 423 and *E. mundtii* ST4SA were cultured, separately, in MRS broth (Biolab, Biolab Diagnostics, Midrand, South Africa) for 24 h at 37 °C. Cells were harvested (8000g, 10 min, 4 °C), the pH of the cell-free supernatant adjusted to between pH 6.5-7.0 with 10M NaOH and then heated at 80 °C for 10 min to inactivate proteolytic enzymes. Peptides were precipitated from the cell-free supernatant with 70% saturated ammonium sulfate (Sambrook *et al.*, 1989) and desalted against distilled water by using a 1000 Da cut-off dialysis membrane (Spectrum Inc., CA, USA). Protein concentrations were determined by using the Micro-bicinchoninic Acid (BCA) kit (Pierce, Rockford, IL). Readings were recorded at 562 nm. Samples were freeze-dried and stored at -20 °C.

Electrospinning of Nanofibers

Freeze-dried peptides were dissolved in *N*,*N*-dimethylformamide (DMF) and centrifuged (6000g, 1 min) to obtain the supernatants. From each of these supernatants, 2 ml was used as solvent for poly(D,L-lactide) (PDLLA, 75 000-120 000 Da, Sigma-Aldrich) blended with poly(ethylene oxide) (PEO, 200 000 Da, Sigma-Aldrich) (20-24%, w/v,) at a ratio of 10:90, 30:70 and 50:50, respectively. Solutions were heated to 40 °C on a hot plate and then electrospun using a gravity system described by (Heunis *et al.*, 2010). A constant electric field of +10 kV was applied to the polymer solution and -5 kV to the collector. The collector was placed 15 cm from the polymer solution. The relative humidity was kept constant between 50-55%.

Scanning Electron Microscopy of Electrospun Fibers

Images of the nanofibers were recorded with a Leo® 1430VP Scanning Electron Microscope (SEM). Samples were coated with a thin layer of gold to increase conductivity. Fiber diameters were determined by using the SEM Image Studio Software (version 10.1).

Fluorescent Labeling of Antimicrobial Peptides and Polymers

Freeze-dried plantaricin 423 (20 mg) was suspended in 1 ml DMF and the supernatant was reacted with 20 mg rhodamine B isothiocyanate (RBITC) or fluorescein isothiocyanate (FITC) from Sigma-Aldrich, dissolved in the same volume. The solution was placed on an orbital shaker and incubated in the dark at 4 °C for 48 h. Unbound fluorescent marker was removed by dialyzing against sterile distilled water for 3 weeks at 4 °C. Dialyses bags with

1000 Da cut-off were used. Labeled peptides were freeze-dried, dissolved in DMF and electrospun into nanofibers as described before.

PEO (0.2048 mg) was dissolved in 2 ml sterile distilled water containing RBITC (20 mg/ml). The polymer solution was placed on an orbital shaker and incubated in the dark at 25 °C for 48 h. Unbound fluorescent marker was removed through dialysis as described before and labeled PEO was freeze-dried. PDLLA (0.2048 mg) was dissolved in 2 ml DMF and was labeled with FITC (20 mg/ml) for 48 h on an orbital shaker and incubated in the dark at 25 °C.

The labeled peptides and polymer solutions were electrospun into nanofibers as described before. Nanofibers were excited at 472 nm and 572 nm, respectively, by using a Xenon-Arc burner light source (Olympus Biosystems GMBH). Fluorescent images were captured with an Olympus Cell^R system attached to an IX-81 inverted fluorescent microscope. An Olympus Plan Apo N 60×/1.4 Oil objective was used. Images were taken with an F-view-II cooled CCD camera (Soft Imaging Systems) and the background subtracted using the Cell^R imaging software.

Differential Scanning Calorimetry (DSC)

Thermal analysis was carried out with a TA Q100 Differential Scanning Calorimeter (DSC) (TA Instruments) which was calibrated against an indium standard. An empty aluminium pan was the reference. Fiber samples (15-18 mg) were run at a 10 °C/min in three cycles under nitrogen atmosphere (flow rate 50 ml/min). In the first cycle, samples were heated from -80 °C to 180 °C and isothermed for 1 min, cooled back to -80 °C and isothermed for 5 min. Finally, the samples were heated for a second time at 10 °C/min to 180 °C. The crystalline melting temperature (T_m) and the glass transition temperature (T_g) were recorded in the second heating cycle.

Release of Antimicrobial Peptides and Degradation of Nanofibers

Nanofibers (25 mg) were placed in 2 ml sterile phosphate buffered saline (PBS, pH 7.4) and incubated at 37 °C on an orbital shaker (30 rpm). At pre-determined time intervals the PBS was replaced with 2 ml sterile PBS (pH 7.4). The protein concentration in each sample was determined by using the BCA protein assay kit as described before. The initial concentration of bacteriocin in the nanofibers was determined by dissolving 25 mg fibers in DMF. This suspension was diluted in PBS 7.4 before the protein concentration was determined by using

the BCA protein assay kit as described before. Nanofibers were freeze-dried and then visualized by SEM. Changes in the weight of nanofibers were recorded.

Antimicrobial Activity Tests

Enterococcus faecium HKLHS (lab culture collection) was cultured in MRS broth at 37 °C for 24 h. *Listeria monocytogenes* EGD-e (obtained from Dr. Colin Hill, Cork, Ireland) was cultured in Listeria enrichment broth (Merck, Germany), supplemented with chloramphenicol (7.5 µg/ml), at 37 °C for 24 h. Both strains were used as sensitive organisms in antimicrobial determinations. Antimicrobial activity of the PEO 50:PDLLA 50 blend was determined using a modified Kirby Bauer method. Sections of approximately 1 cm^2 nanofibrous membrane were placed on agar plates seeded either with *E. faecium* HKLHS or *L. monocytogenes* EGD-e. The plates were incubated at 37 °C for 24 h and then inspected for the presence of inhibition zones. In a separate experiment, 25 mg of PEO 50:PDLLA 50 nanofibers, containing plantaricin 423, was placed in 50 ml MRS broth, inoculated with *E. faecium* HKLHS (10^5 cfu/ml) and incubated at 37 °C for 12 h. Changes in optical density readings were recorded at 600 nm. Activity of bacteriocins after electrospinning was determined by placing 25 mg of PEO 50:PDLLA 50 nanofibers containing either plantaricin 423 or bacteriocin ST4SA, separately, in PBS pH 7.4 and incubated for 18 h at 37 °C. Released bacteriocin concentration was determined by BCA as previously described, diluted and 10 µl spot on plates seeded with *E. faecium* HKLHS. Plates were incubated at 37 °C for 24 h and then inspected for the presence of inhibition zones. Plantaricin 423 and bacteriocin ST4SA not electrospun served as controls.

Results

Nanofibers produced from blends of PEO and PDLLA, containing plantaricin 423, have a regular texture and smooth surface, as revealed by scanning electron microscopy (Fig. 1). The PEO 90:PDLLA 10 blend produced fibers ranging from 200 to 450 nm, with most of the fibers in the 300-350 nm range (Fig. 1F). Fibers with similar dimensions were produced from the PEO 50:PDLLA 50 blend (Fig. 1D). Fibers with slightly less variation (200-400 nm) and mostly in a smaller size range (250-300 nm) were produced from the PEO 70:PDLLA 30 blend (Fig. 1E). Mean fiber diameters ranged from 361 ± 83, 286 ± 34 and 336 ± 32 nm for the PEO 90:PDLLA 10, PEO 70:PDLLA 30 and PEO 50:PDLLA 50 blend, respectively.

Fluorescent microscopy images have shown that PEO and PDLLA are homogenously distributed in the fiber structure and indicate a large degree of miscibility when used in a

50:50 combination (Fig. 2). This blend was selected based on characteristics best suited for a drug delivery system, as discussed in this chapter.

It can be seen that the DSC curve of the PEO 50:PDLLA 50 blend yielded a single glass transition temperature (T_g) of 9 °C, which falls between the T_g values recorded for PEO (-54 °C) and PDLLA (49 °C) (Fig. 3). DSC curves provide a good indication of the macromolecular nature of the polymer, i.e., crystallinity, T_g and crystalline melting point. In our study, PDLLA had no melt endothermic response as expected from an amorphous polymer, contrary to PEO where a melting endotherm was recorded at 64 °C. From the insert in Fig. 3, it is evident that a single T_g was detectible at 9 °C, which falls between both T_gs of PEO (-54 °C) and PDLLA (49 °C). It is therefore suggested that the blend is miscible in the amorphous phase. DSC analysis was also performed on the PEO 50:PDLLA 50 blend, containing plantaricin 423, and the T_g value decreased from 9 °C (in the blend without peptide) to -12 °C. This T_g was still between that of the component polymers and therefore also suggested to be miscible in the amorphous phase.

Fluorescently labeled plantaricin 423 was electrospun into nanofibers prepared from a PEO 50:PDLLA 50 blend. The bacteriocin was unevenly distributed in these fibers, indicating that the peptide has better interaction with one of the polymers (Fig. 4). Due to the amphiphilic nature of bacteriocins, we expected the peptide to show better association with one of the two polymers. Fluorescent images of nanofibers prepared from a PEO 50:PDLLA 50 blend are shown in Fig. 5. Signals of the plantaricin 423 and PEO are overlapping (Fig. 5C), suggesting that the peptide is associated with the polymer. This was verified with the co-localization of the signals (Fig. 5E). These results were confirmed with fluorescent images of labeled plantaricin 423 and PDLLA. Green and red fluorescent signals could still be differentiated in the overlay, indicating that the peptide is closer associated with PEO. These results were supported with much less co-localization seen between the signals from the fluorescently labeled PDLLA and plantaricin 423 (Fig. 5J). From cumulative release studies, it is evident that the PEO 90:PDLLA 10 blend released the highest concentration (78%) of plantaricin 423 (Fig. 6). An initial release of 46% was recorded within the first 2 h, followed by 32% over the following 8 days (Fig. 6). This could be ideal in the control of infections. A rapid release of antimicrobial peptides during the first 2 h would kill most of the pathogens, whereas a slow and constant release over the following few days would keep infection under control. The PEO 70:PDLLA 30 and PEO 50:PDLLA 50 blends yielded less promising results with 69% and 60% of the peptides being released over 8 days (Fig. 6).

SEM images taken from PEO 50:PDLLA 50 nanofibers, containing plantaricin 423, after 8 days of incubation in PBS buffer (pH 7.4) did not reveal any significant structural changes or signs of deterioration of the fibrous structure (Fig. 7I-L), suggesting that these nanofiber scaffolds would be stable for at least 8 days. Slight textural changes have been recorded for the PEO 70:PDLLA 30 blend, containing plantaricin 423, after 8 days (Fig. 7E-H). However, significant morphological changes and signs of deterioration were observed for nanofibers prepared from the PEO 90:PDLLA 10 blend, containing plantaricin 423 (Fig. 7A-D). Fibrous structures were visible, but most of the nanofibers formed a film-like structure after 2 h. Nanofibers prepared from the PEO 90:PDLLA 10 blend showed high weight loss within the first few hours with only 24% of the original weight remaining. Eighty percent weight loss was seen over 8 days at 37 °C, whereas nanofibers prepared from the PEO 70:PDLLA 30 and PEO 50:PDLLA 50 blends lost approximately 60% and 40% of their original weight, respectively (Fig. 8).

A clear zone of growth inhibition was recorded surrounding PEO 50:PDLLA 50 nanofibers for at least 6 days (Fig. 9). This suggested that the nanofibers retained their antimicrobial activity for at least 6 days on solid medium. The peptides retained 88% of their original antimicrobial activity after 18 h of incubation at 37 °C in PBS pH 7.4. As little as 25 mg nanofibers inhibited the growth of *E. faecium* HKLHS in 50 ml MRS broth for up to 10 h, whilst cells in the control (without nanofibers) reached stationary phase after 10 h (Fig. 10).

Discussion

Electrospinning is a versatile technique to produce large amounts of fibers, in the micrometer to nanometer range, in a short amount of time. A variety of natural and synthetic polymers have been electrospun into fibers and some have been used to control the release of various compounds (Chew *et al.*, 2005, Kim *et al.*, 2007, Maretschek *et al.*, 2008, Min *et al.*, 2004, Zhou *et al.*, 2008). Blending hydrophilic and hydrophobic polymers directly in a suitable solvent before electrospinning is an easy way to change the hydrophobicity, thermal stability and mechanical strength of the fibers (Nijenhuis *et al.*, 1996). Since blending is an attractive way to modify polymer properties, this method was chosen to modify the hydrophobicity/hydrophilicity of the electrospun fibers by the addition of PEO, to help facilitate the release of the bacteriocin from the nanofibers (See addendum 1).

Fibers were all in the nanometer range and addition of bacteriocin did not influence the fiber structure as can be seen in Fig. 1.

Polymers were labeled to elucidate fiber morphologies after electrospinning. Fluorescent microscopy images showed that there is a large degree of miscibility between the PEO and PDLLA when these polymers are used in a 50:50 combination (Fig. 2). However, to confirm these results and to determine the miscibility of the polymers, DSC analysis was performed on the PEO 50:PDLLA 50 fiber blend, see Fig. 3. This fiber blend was chosen as it retained its structure the best, had the least amount of weight loss and still released enough bacteriocin to inhibit the growth of a sensitive strain. Nijenhuis *et al.* (1996) have studied thermal properties using DSC of polymer blends containing various ratios of PEO and the poly(L-lactide) (PLLA) isomer. The results showed that blends containing up to 50 wt% PEO were miscible in the amorphous phase. In this work, PDLLA which is an amorphous grade unlike the semi crystalline poly(D-lactide) PDLA was used. A good indication of a miscible polymer blend is the shift of the *T*g to a value between the *T*gs of the pure polymers. The *T*g value of a mixed phase is determined by the weight fraction of each polymer in that phase, and by the *T*gs of the pure polymers. DSC curves of the second heating cycle of the PEO 50:PDLLA 50 blend and the corresponding homopolymers are shown in Fig. 3. A single T_g (9 °C) can be seen that is between the T_g values for PEO (-54 °C) and PDLLA (49 °C) which suggests that the PEO 50:PDLLA 50 blend is miscible in the amorphous phase. DSC analysis performed on the PEO 50:PDLLA 50 blend, containing plantaricin 423, revealed that the T_g value still fell between the T_g of the component polymers and is therefore still miscible to an extent. It could be due to the peptide that is more associated with the PEO domains or simply restricting chain mobility. Investigation of the distribution of bacteriocins in the final fiber structure after electrospinning could yield important information in designing polymeric drug delivery systems for these peptides.

Fluorescent microscopy images revealed that the bacteriocin was better associated with PEO than with PDLLA, even though the peptide was associated with both these polymers (Fig. 5). Bacteriocins are amphiphilic peptides with both hydrophilic and hydrophobic residues (Cotter *et al.*, 2005). Because of this amphiphilic nature it is possible for the peptide to interact better with one polymer than with the other. These results indicate that the bacteriocin is better associated with the hydrophilic polymer, in this case PEO, but were also associated with the PDLLA to a lesser extent. This would explain the high initial burst release within the first few hours.

Cumulative release studies revealed that nanofibers with a higher PEO content showed a higher release of the bacteriocin than those with a lower PEO content (Fig. 6). An initial burst release was seen within 2 h, after which a more sustained release was seen. This could

be an ideal release profile in the control of infections. A rapid release of antimicrobial peptides during the first 2 h would kill most of the pathogens, whereas a slow and constant release over the following few days would keep infection under control. The PEO 70:PDLLA 30 and PEO 50:PDLLA 50 blends released lower amounts of the peptide, with the PEO 70:PDLLA 30 and PEO 50:PDLLA 50 blends releasing 69% and 60% of plantaricin 423, respectively (Fig. 6). Blends of different polymers have earlier shown to facilitate the release of molecules from electrospun fibers. Lysozyme release from nanofibers was facilitated by blending PEO and poly(ε-caprolactone) (PCL), with a 90:10 blend releasing most of the lysozyme with a high initial burst release (Kim *et al.*, 2007). Higher concentrations of cytochrome C were also released from fibers consisting of poly(L-lactide) PLLA and high concentrations of both poly(L-lysine) (PLL) and poly(ethylene imine) (PEI), respectively (Maretschek *et al.*, 2008). Fibers containing 50% PLL released most of the protein (75%) with a high initial burst release.

SEM images revealed that as the PEO content increased, significant morphological changes can be seen as the incubation time increased (Fig. 7) The PEO 90:PDLLA 10 blend showed the most significant morphological changes over the 8 day period. This could be due to the high content of PEO in the nanofibers as PEO swells readily in an aqueous environment and dissolves easily. The resulting destruction of the nanofiber scaffold explains the higher release of plantaricin 423 as observed over 8 days (Fig. 6). Nanofibers retained their structure better in the PBS buffer (pH 7.4), as the PEO content decreased. This can be observed in the PEO 50:PDLLA 50 blend, with this blend showing no significant signs of structural changes or deterioration in the buffer medium (Fig. 7I-L). Slight structural changes were seen for the PEO 70:PDLLA 30 blend after 8 days (Fig. 7E-H). These results suggest that the PEO 50:PDLLA 50 blend nanofibers would be stable for at least 8 days and show no significant signs of degradation. The ideal nanofiber drug delivery systems has to retain its structure, have high oxygen permeability, variable pore size and a high surface to volume ratio. Nanofibers prepared from the PEO 90:PDLLA 10 blend showed very high weight loss over the 8 day period, which is not very desirable (Fig. 8). The PEO 70:PDLLA 30 showed lower weight loss, however the PEO 50:PDLLA 50 showed the best results and lost the least amount of its original weight (Fig. 8). It is very important that the nanofiber scaffolds retain their integrity and with this in mind, the PEO 50:PDLLA 50 would be the preferred choice as drug delivery system in this work.

Bacteriocins retained their activity after electrospinning and were able to inhibit the growth of a sensitive strain on solid media as well as in liquid broth (Fig. 9 and 10). The

bacteriocins showed activity on the solid media for at least 6 days of consecutive transfer. Growth inhibition in liquid broth was observed and may be obtained over longer periods of time by electrospinning higher concentrations of bacteriocin in the nanofibers, by using more nanofibers (weight), or by replacing the nanofibers in the broth with new nanofibers at certain time intervals. Only 25 mg of nanofibers, containing plantaricin 423, was able to inhibit actively growing cells of *E. faecium* HKLHS, indicating the potent antimicrobial effect of this bacteriocin. The difference in antimicrobial activity and release on solid media and in liquid broth gives an indication that there is a big difference in release profile on solid media and in a buffer/liquid medium. During release studies in a buffer medium, a high concentration gradient is created (such as in the case of the method used for determining release) by replacing the old buffer with new buffer at each sampling point. This can increase the rate of diffusion of the peptide from the nanofiber scaffold to the buffer medium. The detection limit of the protein assay is also in µg/ml, whereas the bacteriocins could still be active at lower concentrations of ng/ml. However, the concentration gradient can be much lower on solid media, resulting in lower release profiles and thus leads to increased periods of antimicrobial activity.

Bacteriocins remained active after electrospinning into nanofiber scaffolds. This technique provides the option to electrospin high concentrations of antimicrobial peptides into nanofibers. The rate at which these peptides are released is controlled by selection of the correct combination of polymers. The degradation rate and mechanical properties of PDLLA can be improved by blending with a hydrophilic polymer such as PEO. Antimicrobial peptides electrospun into nanofibers may have potential applications in the pharmaceutical and food industries to control microbial growth.

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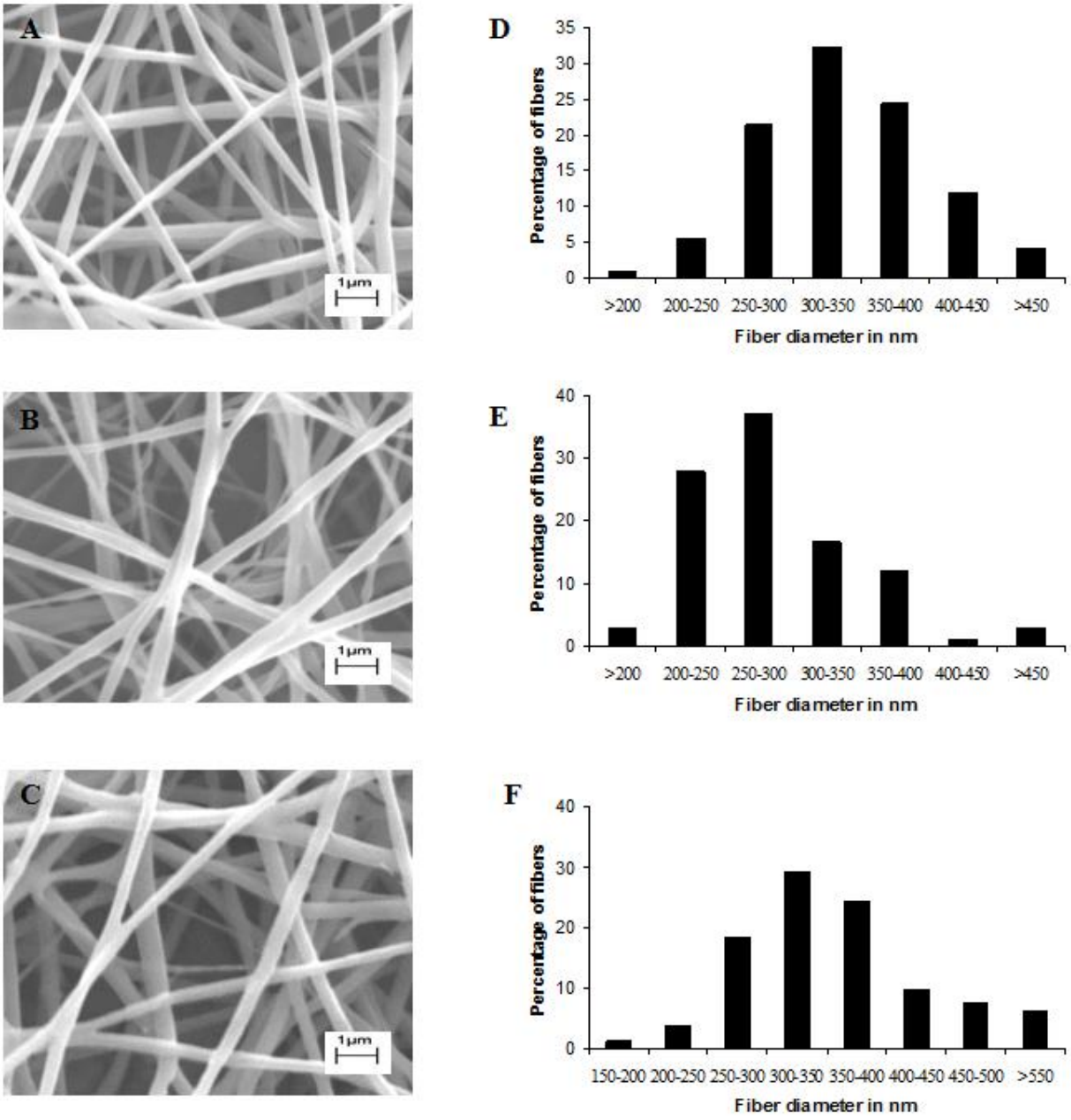

Fig. 1 SEM images of electrospun nanofibers containing plantaricin 423 (**A-C**) and fiber diameter distributions of the electrospun nanofibers (**D-F**). (**A and D**) PEO 50:PDLLA 50 (**B** and **E**) PEO 70:PDLLA 30 (**C** and **F**) PEO 90:PDLLA 10.

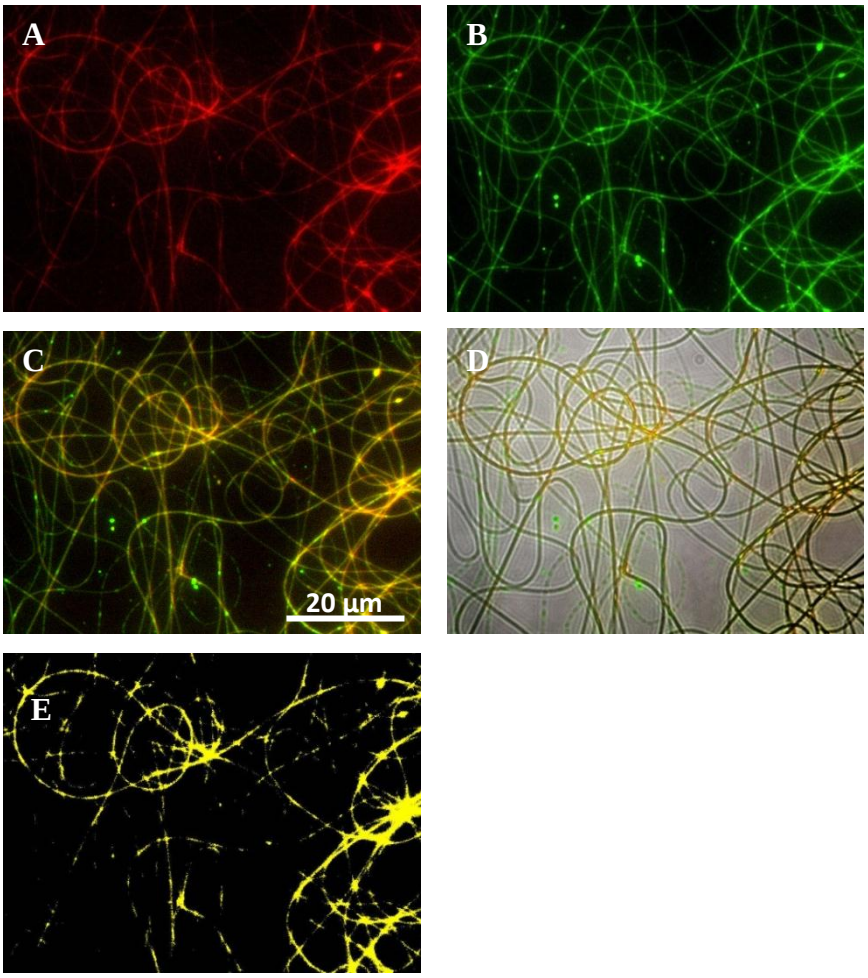

Fig. 2 Fluorescent images of PEO and PDLLA labeled with RBITC and FITC, respectively. (**A**) Fluorescent image of PEO, (**B**) fluorescent image of PDLLA, (**C**) overlay of the fluorescent signals, (**D**) optical image of nanofibers with fluorescent signals and (**E**) co-localization of the two signals, indicating miscibility between PEO and PDLLA.

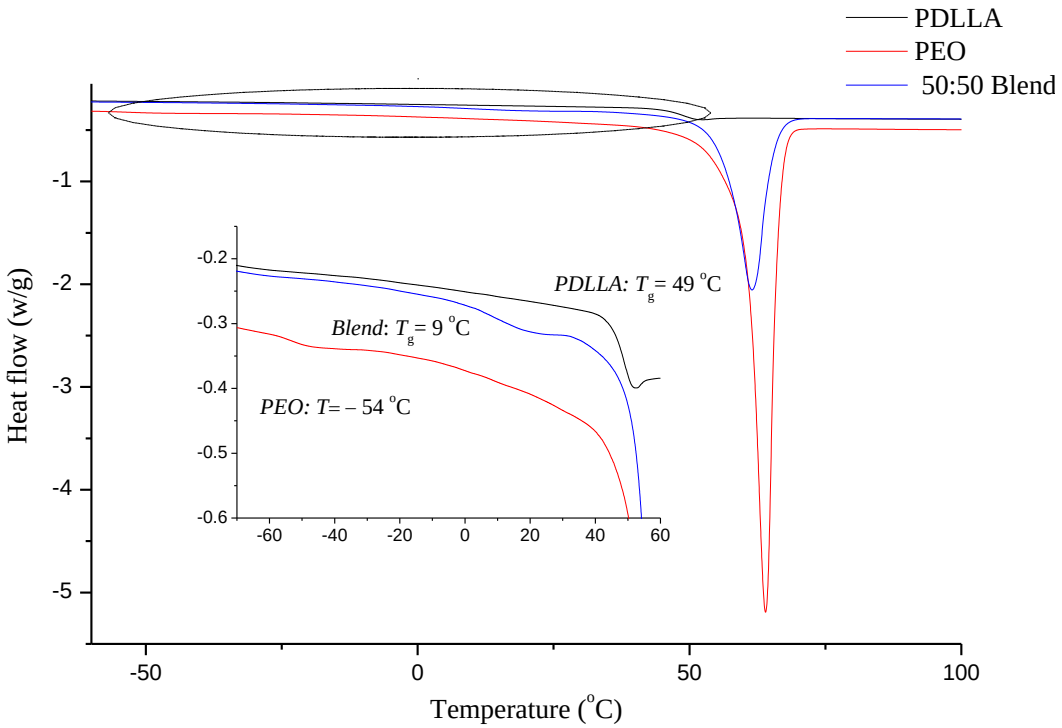

Fig. 3 Differential Scanning Calorimetric (DSC) thermogram of the PEO 50:PDLLA 50 blend, as well as the component polymers, PEO and PDLLA.

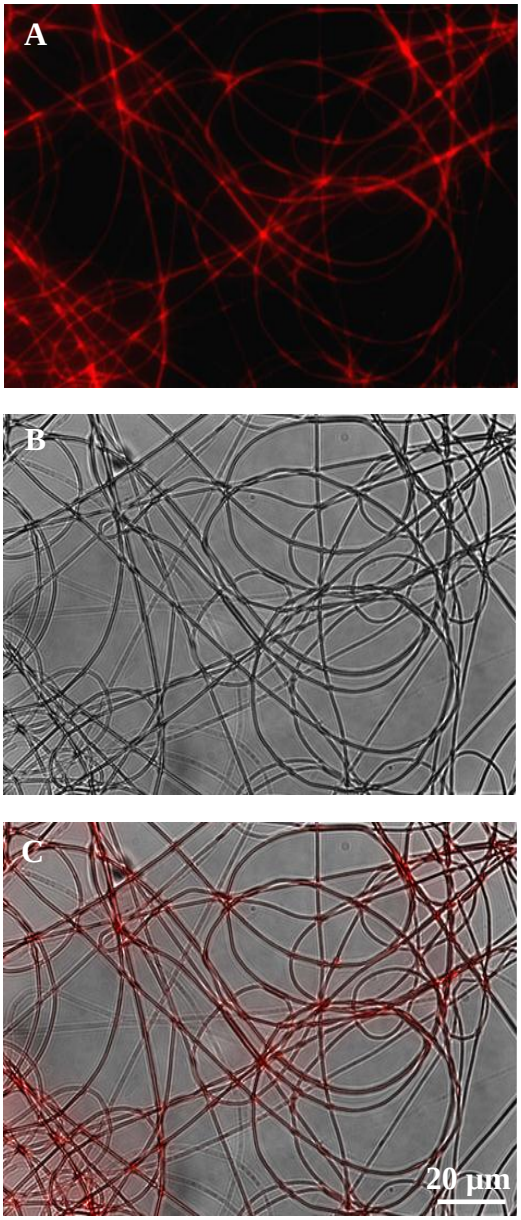

Fig. 4 Fluorescent images of plantaricin 423 labeled with RBITC and electrospun into nanofibers consisting of PEO 50:PDLLA 50. (**A**) Fluorescent image of plantaricin 423 labeled with RBITC, (**B**) optical image of nanofibers and (**C**) optical image of nanofibers with fluorescent signal.

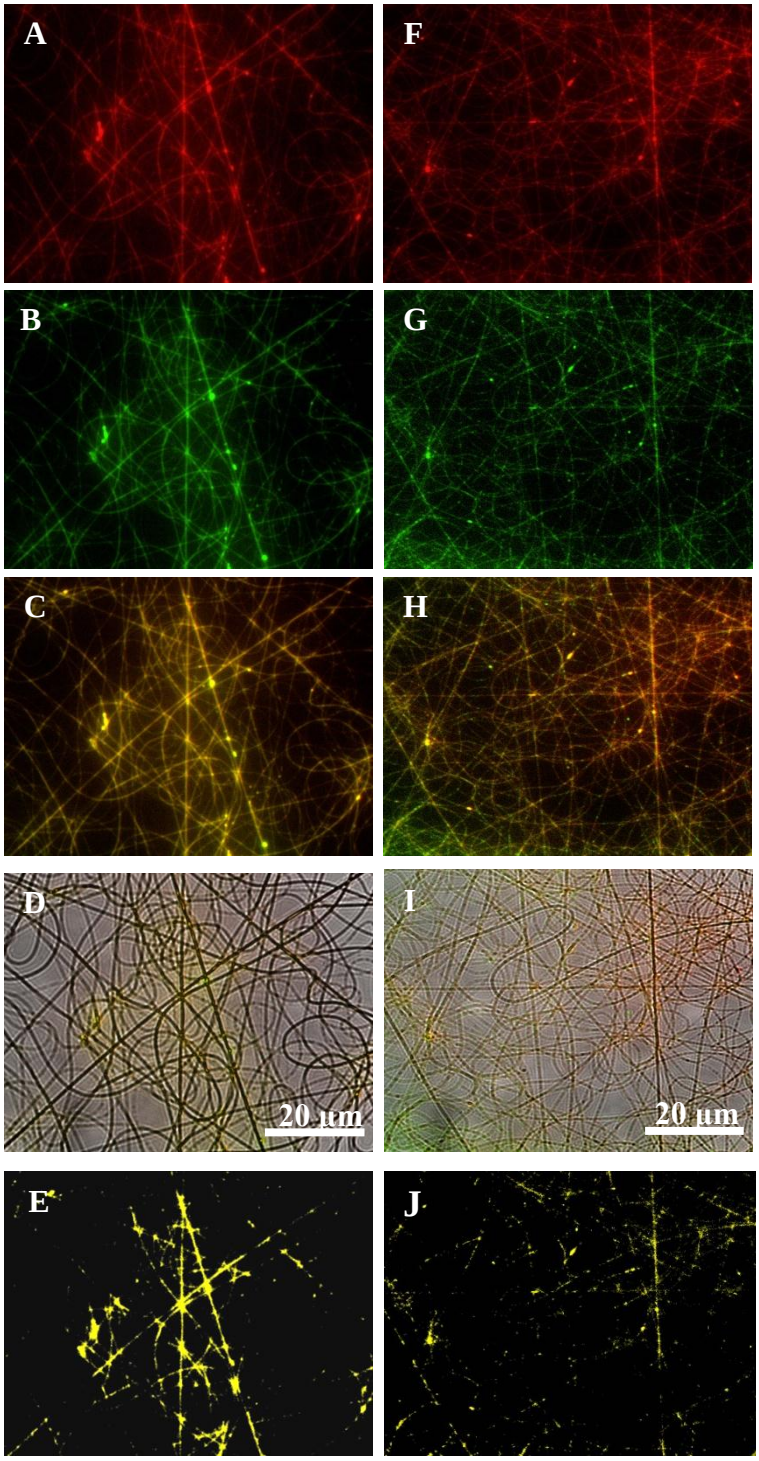

Fig. 5 Fluorescent images of labeled plantaricin 423 and polymers electrospun into nanofibers consisting of PEO 50:PDLLA 50. (**A**) Fluorescent image of PEO labeled with RBITC, (**B**) fluorescent image of the bacteriocin labeled with FITC, (**C**) overlay of the fluorescent images, (**D**) optical image of nanofibers with fluorescent signals, (**E**) co-localization of the signals, (**F**) fluorescent image of bacteriocin labeled with RBITC, (**G**) fluorescent image of PDLLA labeled with FITC, (**H**) overlay of the two fluorescent images, (**I**) optical image of nanofibers with fluorescent signals and (**J**) co-localization of the two signals.

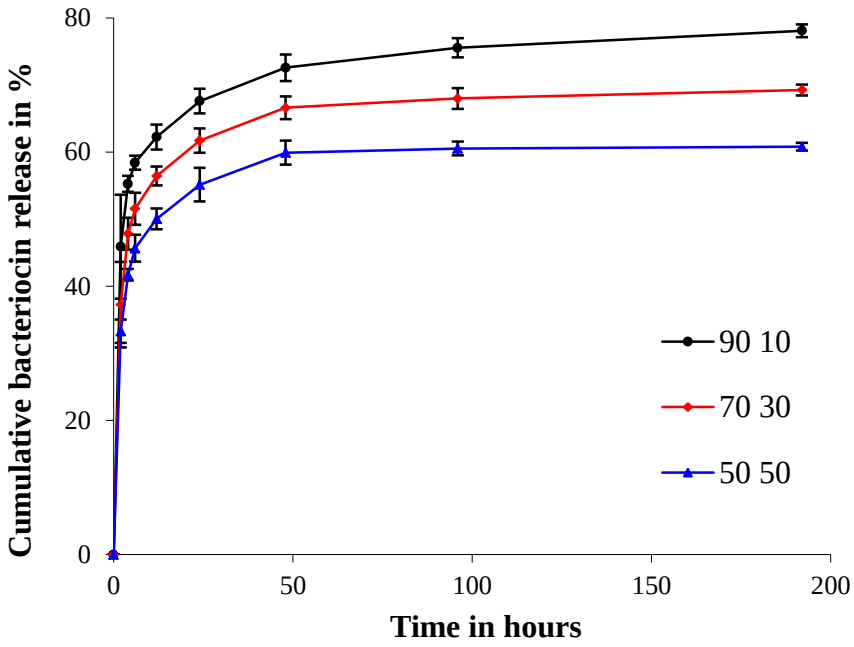

Fig. 6 Cumulative release of plantaricin 423 from electrospun PEO:PDLLA nanofiber blends. These nanofibers showed a high initial burst release and a more sustained release of bacteriocin over an 8-day period.

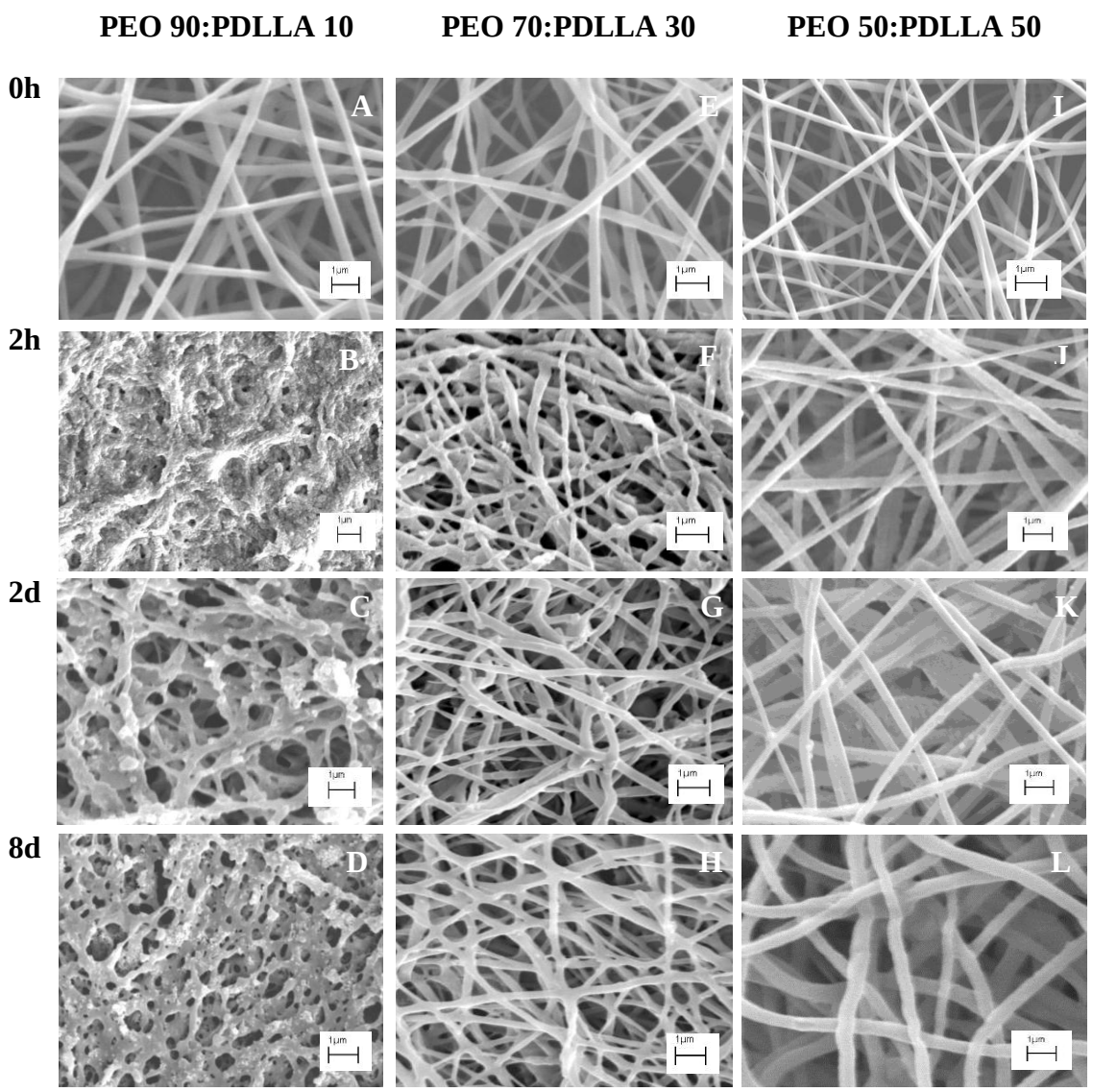

Fig. 7 SEM images of PEO:PDLLA blend nanofibers, containing plantaricin 423, incubated in PBS buffer pH 7.4 for different time intervals. Significant morphological changes are seen in the PEO 90:PDLLA 10 blend (**A-D**). The PEO 70:PDLLA 30 blend showed some morphological changes, however, the fiber structure stayed more or less intact (**E-H**). The PEO 50:PDLLA 50 blend showed very little morphological changes and the nanofiber structure stayed intact (**I-L**).

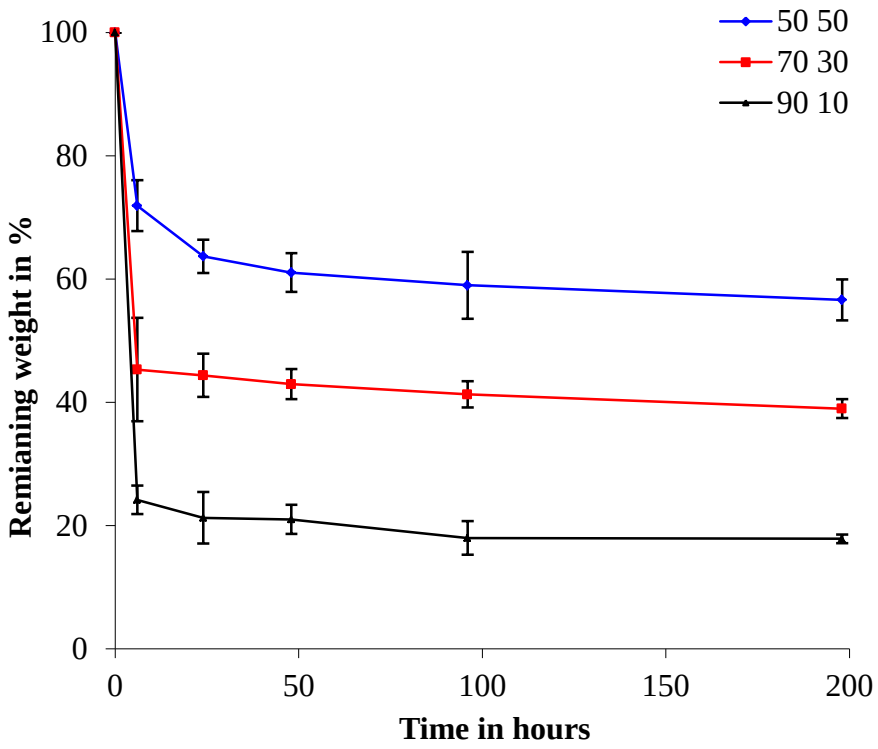

Fig. 8 Percentage of original weight of nanofibers remaining after incubation at 37 °C for 8 days.

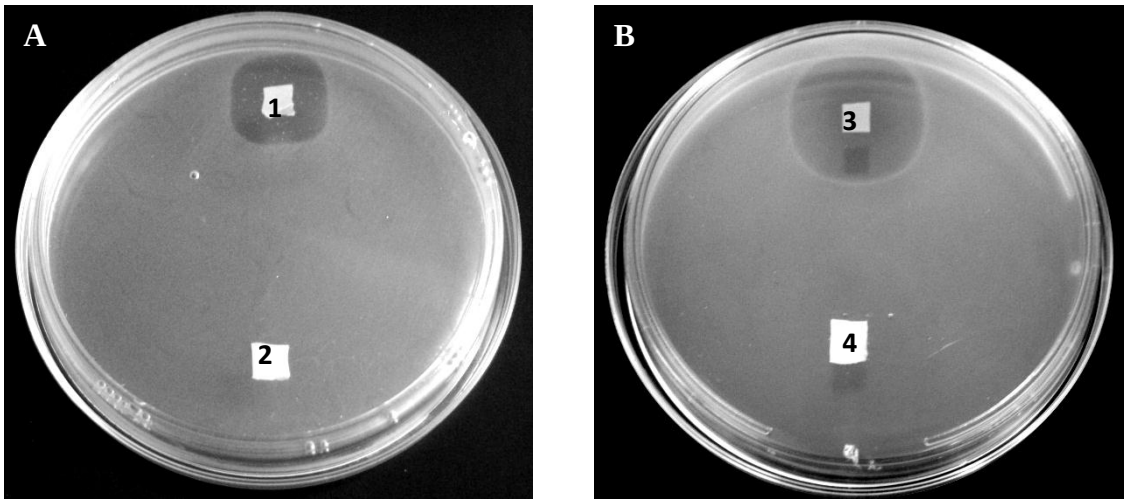

Fig. 9 Antimicrobial activity tests of PEO 50:PDLLA 50 blend nanofibers containing plantaricin 423 (**A**) and bacteriocin ST4SA (**B**). Modified Kirby Bauer tests were performed by placing approximately 1 cm^2 of fiber on plates seeded with target strains. *E. faecium* HKLHS and *L. monocytogenes* EGD-e served as target strains for plantaricin 423 and for bacteriocin ST4SA, respectively. A clear zone of inhibition can be seen surrounding the fibers containing bacteriocins after incubation. **1**-PEO 50:PDLLA 50 blend nanofibers containing plantaricin 423, **2**-PEO 50:PDLLA 50 blend nanofibers without plantaricin 423, **3**-PEO 50:PDLLA 50 blend nanofibers containing bacteriocin ST4SA and **4**- PEO 50:PDLLA 50 blend nanofibers without bacteriocin ST4SA.

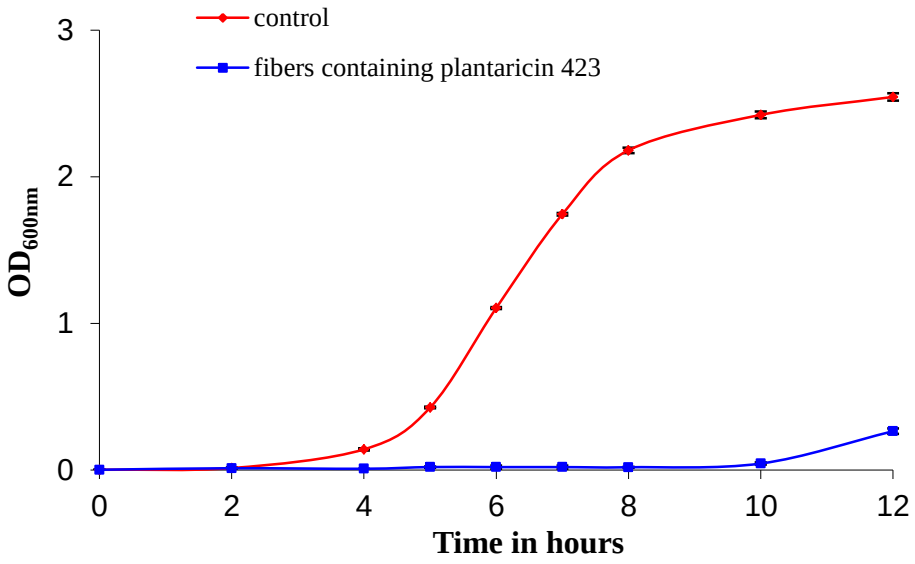

Fig. 10 Growth inhibition of *E. faecium* HKLHS by PEO 50:PDLLA 50 blend nanofibers releasing plantaricin 423.

CHAPTER 5

Evaluation of a Nisin-eluting Nanofiber Scaffold to Treat *S. aureus*-Induced Skin Infections in Mice

Abstract

Antibiotic resistance amongst pathogens is increasing and alternative treatments need to be explored. An antimicrobial nanofiber wound dressing was produced by co-electrospinning nisin into poly(ethylene oxide) and poly (D,L-lactide) (50:50) blend nanofibers. Active nisin diffused from the nanofiber wound dressings for at least 4 days *in vitro*, as shown with consecutive transfers onto plates seeded with methicillin-resistant strains of *Staphylococcus aureus.* The nisin-containing nanofiber wound dressing significantly reduced *S. aureus* Xen 36 bioluminescence and cell numbers in a murine skin infection model. Furthermore, the wound dressings stimulated wound closure of excisional wounds and no side effects could be observed. Nisin-containing nanofiber wound dressings have the potential to treat *S. aureus*-skin infections and accelerate the wound healing process.

Introduction

Injury to skin provides a perfect site for microbial invasion, leading to minor skin infections which can easily progress into life-threatening infections. With an infection, the processes normally associated with wound healing, i.e. homeostasis, inflammation, new tissue formation, remodelling and maturation, will be hampered (Guo and DiPeitro, 2010, Gurtner *et al.*, 2008, Nishio *et al.*, 2008). Gram-positive and Gram-negative bacteria, fungi and yeasts have been associated with skin infections (Church *et al.*, 2006, Vindenes and Bjerknes, 1995). *Staphylococcus aureus* is a virulent pathogen responsible for superficial and invasive skin and soft-tissue infections (SSTIs) (Baggett *et al.*, 2003, Daum, 2007, Fridkin *et al.*, 2005). Widespread use of antibiotics have led to an increase in antibiotic resistance in *S. aureus*, amongst others, with the emergence of health care-associated methicillin-resistant *S. aureus* (HA-MRSA) and community-acquired MRSA (CA-MRSA) (Kim, 2009, Naimi *et al.*, 2003). Vancomycin-resistant strains of *S. aureus* (VRSA) have also emerged, as well as the more recent linezolid-resistant *S. aureus* (LRSA) (Chang *et al.*, 2003, Endimiani *et al.*, 2011, Tsiodras *et al.*, 2001, Walsh, 1999). It is thus important to search for alternative antimicrobial compounds active against *S. aureus.*

Lantibiotics are extensively post-translationally modified bacteriocins and contain unusual amino acids as well as *meso*-lanthionine(s) and/or β-methyllanthionine(s) (Willey and van der Donk, 2007). Nisin A, produced by some strains of *Lactococcus lactis*, is the best studied lantibiotic and consists of 34 amino acids. During post-translational modification,

serine and threonine residues in the precursor peptide are dehydrated to form 2,3-didehydroalanine (Dha) and (Z)-2,3-didehydrobutyrine (Dhb), respectively. Subsequent cyclization results in the formation of one *meso*-lanthionine (Ala-S-Ala) and 4 β-methyllanthionines (Abu-S-Ala) in the mature peptide (Willey and van der Donk, 2007). Nisin has a dual mode of action by targeting the pyrophosphate moiety of the cell wall precursor lipid II and, by doing so, prevents polymerization of murein subunits during cell wall synthesis (Hsu *et al.*, 2004, Willey and van der Donk, 2007). Once bound to lipid II, nisin also forms pores in the cell membrane (Brötz *et al.*, 1998). Nisin is active against various Gram-positive bacteria, including MRSA, vancomycin-resistant enterococci (VRE), heterogeneous vancomycin-intermediate *S. aureus* (hVISA), *Streptococcus pneumoniae* and *Clostridium difficile* (Bartoloni *et al.*, 2004, Piper *et al.*, 2011, Severina *et al.*, 1998). Nisin also inhibits the outgrowth of *Bacillus* and *Clostridium* spp. endospores (Gut *et al.*, 2011, Rayman *et al.*, 1981).

Electrospinning is a versatile technique to produce fibers with very small diameters (in the nanometer to micrometer range), which have high oxygen permeability, variable pore size, a high surface area to volume ratio and are morphologically similar to the extracellular matrix (Li *et al.*, 2002, Smith and Ma, 2004, Yang *et al.*, 2005, Zhou *et al.*, 2008). Electrospun nanofibers are thus ideal as wound dressing materials. Antimicrobial compounds have been electrospun into nanofibers to generate antimicrobial materials for potential medical applications (Kim *et al.*, 2004, Rujitanaroj *et al.*, 2008). Electrospinning of bacteriocins into nanofibers and the use of the electrospun nanofibers as a drug delivery system for bacteriocins in the food and medical industries have only recently been reported (Dheraprasart *et al.*, 2009, Heunis *et al.*, 2010, 2011).

In this chapter we investigated the feasibility of co-electrospinning commercially available nisin A (Nisaplin®) into nanofibers consisting of a blend of poly(ethylene oxide) and poly(D,L-lactide). Wound dressing were evaluated for antimicrobial activity, their ability to treat *S. aureus* skin infections, as well as their effect on wound healing.

Materials and Methods

Materials

Poly(D,L-lactide) (PDLLA, 75 000-120 000 Da) and poly(ethylene oxide) (PEO, 200 000 Da) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Nisaplin® was obtained from (Danisco, Copenhagen, Denmark). All other reagents were of analytical grade. Protein

concentrations were determined by using the Micro-bicinchoninic Acid (BCA) kit (Pierce, Rockford, IL) and readings were recorded at 562 nm.

Electrospinning of nanofibers

Nisaplin® was suspended in *N*,*N*-dimethylformamide (DMF) to obtain 20 mg/ml nisin and centrifuged (8000g, 5 min). The supernatant, containing nisin, was used as solvent for 24% (w/v) PEO:PDLLA (50:50). The solution was heated to 40 °C on a hot plate and then electrospun using a gravity system as described by Heunis *et al.* (2010). A constant electric field of +10 kV was applied to the polymer solution and -5 kV to the collector. The collector was placed 15 cm from the polymer solution. The relative humidity was kept constant between 50 and 54% and the temperature between 28 and 30 °C. Nisin-containing nanofibers are further referred to as NF. Nanofibers electrospun without nisin served as control (CF).

Scanning electron microscopy of electrospun fibers

Images of the nanofibers were recorded with a Leo® 1430VP Scanning Electron Microscope (SEM). Samples were coated with a thin layer of gold to increase conductivity. Fiber diameters were determined using SEM Image Studio Software (version 10.1).

Release of nisin and in vitro *antimicrobial activity tests*

S. aureus Xen 29, 30, 31 and 36 (Caliper Life Sciences, Hopkinton, MA) was cultured overnight in Brain Hart Infusion (BHI) broth (Biolab Diagnostics, Midrand, South Africa), containing 200 µg/ml kanamycin to maintain the native plasmid containing the *lux* operon. Incubation was at 37 °C. Antimicrobial activity was assessed on BHI soft agar containing approximately 10^5 cfu/ml of one of these strains. Electrospun nanofibers (1 cm^2), containing approximately 768 µg/ml nisin, were placed on the surface of seeded agar plates and incubated overnight at 37 °C. Nanofibers that inhibited the growth of the *S. aureus* strains, i.e. with surrounding zones of growth inhibition, were transferred to a new plate seeded in the same way and incubated at 37 °C for 24 h. This process was repeated until no antimicrobial activity was recorded.

Animals

Approval to conduct research on mice was granted by the Ethics Committee of the University of Stellenbosch (Ref: 10 NM_DIC02). Adult male BALB/c mice (weighing 20 to 30g) were fed a standard rodent diet, housed in separate cages and kept under controlled environmental conditions (12 h light-dark cycles, 20-22 °C).

Skin injury and infection with S. aureus

The dorsal hair of mice was removed with Veet Hair Remove Lotion (Reckitt Benckiser, Elandsfontein, South Africa). The next day the mice were anaesthetized with 2% (v/v) isoflorane in O_2 (Isofor, Safe Saline Pharmaceuticals, Florida, South Africa) and full-thickness excisional wounds (5 mm in diameter) were made on the dorsal surface of each mouse, using a sterile biopsy punch (Stellenbosch Medical Suppliers, Stellenbosch, South Africa). Mice received buprenorphine (0.03 mg/kg of body weight, Temgesic, Schering-Plough (Pty) Ltd., Woodmead, South Africa) subcutaneously as analgesic directly before injury and for the first 4 days of the experiment. Each of the wounds was infected with 10 µl of 10^8 cfu/ml *S. aureus* Xen 36 directly after the punch biopsy.

Treatment of S. aureus *infection*

Treatment followed 1 h after infection. Wounds of mice (n=7) were covered with NF wound dressings of 1 cm^2 and were labeled the NF group (NFG). Wounds of control mice (n=7) were covered with CF wound dressings of the same size and were labeled the CF group (CFG). Micropore™ surgical tape (Alpha Pharm, Stellenbosch, South Africa) was used to keep the nanofiber dressings in contact with the wounds. Wound dressings were changed on the second and fourth day of the trial.

Monitoring of the bacterial infection

Signal strength of photons transmitted through the nanofiber dressings was determined prior to *in vivo* bioluminescent imaging. Two hundred µl (10^8 cfu/ml) of *S. aureus* Xen 36 cells was placed into a microtiter plate and the wells were covered with nanofibers, which was moistened with phosphate buffered saline pH 7.4 (PBS). Bioluminescent readings were recorded using the *In Vivo* Imaging System (IVIS® 100) of Caliper Life Sciences (Hopkinton, MA, USA). Image software® (version 3.0, Caliper Life Sciences, Hopkinton, MA) was used to quantify the photons emitted from regions of interest (ROIs). The values obtained were expressed as $\log_{10}$ photons per second per cm^2 per steradian (p s^{-1} cm^{-2} sr^{-1}). For *in vivo* bioluminescent imaging, mice were anaesthetized with 2% (v/v) isoflorane in O_2 and bioluminescent readings were recorded, every 24 h, through the bandages for 1 min using the IVIS® 100. Nanofiber wound dressings were moistened with 50µl PBS prior to bioluminescent imaging. Quantification of the photons emitted was achieved as described before and ROIs was set at 17.5 x 17.5 pixels.

Viable *S. aureus* Xen 36 cells adsorbed to the nanofiber wound dressings were determined by soaking the wound dressings in sterile saline, followed by serial dilution and plating on BHI plates, containing 200 µg/ml kanamycin, on day 2, 4 and 7. The plates were incubated at 37 °C for 24 h and viable cells determined. Wounds were digitally photographed daily, to investigate the influence of the wound dressings on wound closure, and images were analysed with ImageJ (Scion Corp., Frederick, MD, USA). Changes in wound size was expressed as a percentage of the original wound size, by using the equation D_N/D_0 x 100% (D_N = wound size on the day of measurement and D_0 = the wound size on day 0). On day 7 the mice were euthanized with an overdose of pentabarbitone sodium (Euthapent, Kyron Laboratories (Pty) Ltd., Benrose, South Africa) and wounds were excised to determine the number of viable *S. aureus* Xen 36 cells in the wounds. Excised wounds were homogenised, serially diluted in sterile saline and plated onto BHI plates, supplemented with 200 µg/ml kanamycin, and incubated at 37 °C for 24 h.

Effect of nanofiber wound dressings on closure of uninfected wounds

Punch biopsies were performed on mice as previously described. None of these mice were infected with *S. aureus.* The wounds of mice (n=3) were covered with NF wound dressings, as described before. Wounds in control groups were either covered with CF wound dressings (n=3) or only covered with gauze (n=3). Micropore™ surgical tape was used to keep all wound dressings in contact with the wounds. Wounds were digitally photographed daily and wound closure determined as described before. Wounds were excised for histological analysis on day 7 and fixed in 4% formaldehyde in 0.1M PBS buffer pH 6.5. Wound tissue samples were processed using automated procedures to impregnate and subsequently embed in paraffin wax, 5µm sections were prepared with a rotary microtome and manually stained with hematoxylin and eosin (H&E).

Statistical analysis

Statistical analyses of the data and comparison between groups were performed using one-way ANOVA and/or unpaired two-tailed t-tests. Statistica Data Analysis Software (version 10, StatSoft Inc) or GraphPad Prism Software (version 5.04, GraphPad Software Inc, California, USA) was used. A statistically significant difference was considered when $P < 0.05$.

Results

Nanofibers electrospun from a 24% (w/v) solution of PEO:PDLLA (50:50) had an average diameter of 466 ± 104 nm, whereas nanofibers co-electrospun with nisin had an average diameter of 330 ± 79 nm (Fig. 1).

Nisin remained active after electrospinning. Highest antimicrobial activity, based on diameter of inhibition zones, was recorded on day one against *S. aureus* Xen 29, MRSA Xen 30, MRSA Xen 31 and *S. aureus* Xen 36 (Fig. 2A). NF dressings inhibited the growth of *S. aureus* Xen 29 and MRSA Xen 30 for at least 4 days, as observed with the consecutive transfer to plates seeded with the same target organism (Fig. 2). Growth of Xen 31 was inhibited for 5 consecutive days (Fig. 2). The largest zone of growth inhibition (23 mm in diameter) was recorded against *S. aureus* Xen 36 after day one (Fig. 2). Growth inhibition of Xen 36 continued for 9 consecutive days (Fig. 2). Xen 36 was selected as target strain in further experiments. CF dressings did not inhibit the growth of any of the 4 target strains (Fig. 2). Nisin remained active in the nanofibers when stored at 4 °C for 8 months. Inhibition zones of 21 mm were observed after 24 h of incubation, when the nanofibers (stored at 4 °C for 8 months) were placed on plates seeded with *S. aureus* Xen 36.

The bioluminescent signal produced by *S. aureus* Xen 36 was transmitted through PEO:PDLLA nanofiber wound dressings, which would make *in vivo* imaging through the nanofiber wound dressings possible. Bioluminescent signal decreased from 2.0 x 10^6 to 1.6 x 10^6 p s^{-1} cm^{-2} sr^{-1} after transmission through moistened nanofiber dressings. NF wound dressings significantly reduced the bioluminescent signal emitted from *S. aureus* Xen 36 during 7 days of bioluminescent imaging (NFG) (Fig. 3). Mice receiving CF wound dressings (CFG), however, showed stable bioluminescence over the 7-day period.

The number of viable *S. aureus* cells present on NF wound dressings declined over the 7-day period. Average cell numbers per wound dressing of the NF wound dressings declined from 2.0 x 10^3 on day 2 to 1.7 x10^2 and <25 cfu/wound dressing on days 4 and 7, respectively. Only 20 cfu were detected on one wound dressing (on day 7) of a mouse that was treated with NF wound dressings. Cell numbers on CF wound dressings, on the other hand, declined from 6.9 x 10^7 on day 2 to 1.8 x 10^6 and 1.2 x 10^6 cfu/wound dressing on days 4 and 7, respectively.

The number of viable *S. aureus* cells was reduced in wounds covered with NF wound dressings and were significantly lower, compared to control wounds. The cell numbers of *S.*

aureus Xen 36 declined to 4.3 x 10^2 cfu/wound on day 7 when treated with NF dressings, compared to 2.2 x 10^7 in wounds treated with CF dressings (Fig. 4B). None of the mice in the NF group had bacterial counts higher than 10^4 cfu/wound, whereas all of the mice in the CF group had cell counts higher than 10^6 cfu/wound. No significant differences were observed in wound closure during 7 days of treatment with NF wound dressings or CF wound dressings. On average, wounds in the NF group showed an 84% reduction in wound size on day 7, whereas wounds treated with control nanofibers showed a 79% reduction in wound size.

A significant difference in wound area was observed in uninfected wounds covered with CF, compared to wounds covered with gauze during the 7 days. A significant difference was, however, only observed in wound closure of mice treated with NF wound dressings, compared to wounds that were covered with gauze, from day 4 until day 7 of the experiment (Fig. 6B). No difference was observed in wound closure between NF- and CF-treated wounds. Average wound sizes on day 7 ranged from 26.2 ± 3.9%, 8.7 ± 3.2% and 9.2 ± 4.6% of the original wound area for wounds covered with gauze, CF- and NF-wound dressings, respectively. Histological analysis revealed that wounds covered with gauze exhibited characteristics of re-epithelialization and neovascularization in the wound bed. However, tissues surrounding the wounds showed prolonged inflammatory infiltration, with (polymorphonuclear) neutrophils still visible on day 7. Wounds treated with CF wound dressings showed signs of re-epithelialization, keratinization and wound vascularization. Some signs of neovascularization could also be observed. Neutrophils were only present in one of three wounds on day 7. NF-treated wounds showed presence of connective tissue formation, re-epithelialization, keratinization and hair follicles could be observed. None of these wounds showed any signs of neutrophil infiltration on day 7. The NF-treated group is the only group in which clear fibrotic scar formation was evident, indicating that end stages of repair have been reached.

Discussion

Skin infections caused by antibiotic-resistant pathogens are problematic and delays the general wound healing process. Previously, PEO 50:PDLLA 50 nanofibers have been identified as the best scaffold for further analysis as potential wound dressing material for medical applications (Heunis *et al.,* 2011). PEO 50:PDLLA 50 nanofibers electrospun with nisin had a mean fiber diameter of 330 ± 79 nm, which corresponded well with that obtained previously (Heunis *et al.,* 2011, previous chapter). Nanofibers electrospun without nisin had a larger diameter. During electrospinning a high voltage is applied to a polymer solution or melt in a capillary and once the surface tension is overcome a polymer jet is ejected from the

capillary, which undergoes elongation and produces very thin fibers. An added advantage of electrospinning is the possibility to co-electrospin a variety of bioactive compounds into the nanofibers, by dissolving them directly in the solvent before electrospinning. Antibiotics, growth factors, antimicrobial peptides, silver nanoparticles, as well as living bacteria have been electrospun into a variety of natural and synthetic fibers, as well as blends thereof (Chew *et al.*, 2005, Heunis *et al.*, 2010, Kenawy *et al.*, 2002, Kim *et al.*, 2004, López-Rubio *et al.*, 2009, Luu *et al.*, 2003, Maretschek *et al.*, 2008, Rujitanaroj *et al.*, 2008). The addition of some molecules to a solution before electrospinning can result in a reduction of fiber diameter. PCLEEP (ε-caprolactone and ethyl ethylene phosphate copolymer) fibers showed a reduction in fiber diameter from 5.01 µm to 2.8 µm when electrospun with bovine serum albumin (BSA) (Chew *et al.*, 2005). The addition of Mefoxin® resulted in decreased fiber diameters (from 360 nm to 260 nm) when electrospun with PLGA/PLA/PEG-b-PLA(Kim *et al.*, 2004). Dheraprasart *et al.* (2009) however reported on a slight increase in fiber diameter when gelatin solutions were electrospun with nisin, however, the increase was not statistically significant. Addition of molecules to a solution before electrospinning may alter the conductivity, surface tension and possibly the viscosity of the solution and thus have an influence on the morphology and diameter of fibers formed during electrospinning.

In this study, antimicrobial activity was assessed on solid media to simulate topical conditions such as wounds. Nisin released from the PEO 50:PDLLA 50 nanofibers showed antimicrobial activity for a minimum of 4 days against *S. aureus* Xen 29 and MRSA Xen 30. The longest activity was observed against *S. aureus* Xen 36 for 9 days. An initial burst release with a sustained release of nisin was observed, which could be ideal to treat infections. Bacteria initially colonizing the wound could be killed or inhibited with the initial release of nisin, whereas the sustained release could help to prevent further infection. Similar results have been reported for bacteriocins released from PEO 50:PDLLA 50 blend nanofibers, with a high initial burst release followed by a sustained release (Heunis *et al.*, 2011). Bacteriocins released from the nanofibers inhibited the growth of a sensitive strain for up to 6 days when assessing antimicrobial activity on agar plates. Nisin released from electrospun gelatin nanofibers reduced *S. aureus* and *Listeria monocytogenes* numbers over 48 h in liquid medium, but was unable to reduce *Salmonella typhimurium* numbers (Dheraprasart *et al.*, 2009). Release of molecules/drugs from nanofibers, when mixed in the solution before electrospining, depends on various factors including solubility, polymer drug interaction, dissolution/degradation rate of the polymer/polymer blend nanofibers, as well as the

experimental conditions (Heunis *et al.,* 2011, Kenawy *et al.,* 2002, Kim *et al.,* 2007, Zeng *et al.,* 2003).

Survival rates of mice infected with *S. aureus* Xen 36 was 100% in both NF- and CF-wound dressing groups. This was ideal and offered the possibility to monitor the infection in real time as it progressed over the 7-day period with bioluminescent imaging, using the IVIS®. A statistically significant reduction in bacterial bioluminescence, cell numbers on the NF wound dressings, as well as cell numbers in infected wounds were observed when treated with NF wound dressings, as compared to CF wound dressing-treated groups. All of the mice, which were treated with NF wound dressings, had bacterial counts lower than 10^4 cfu/wound. Our results correlate well with previous published reports on the treatment of bacterial infected wounds and reduction of viable cell numbers *in vivo*. Chitosan acetate bandages significantly reduced bacterial bioluminescence of lux-tagged *P. aeruginosa* and *P. mirabilis* in a murine burn wound infection model (Dai *et al.,* 2009). Survival rates of mice were 73% and 66.7% when treated with chitosan acetated bandages, 27.3% and 62.5% when treated silver dressings and 13.3% and 0% in control groups for *P. aeruginosa* and *P. mirabilis*, respectivley (Dai *et al.,* 2009). Kumari *et al.* (2010) reported that animals with *K. pneumoniae* infected burn wounds, which were treated with either honey or aloe vera gel, had better survival rates than control animals over a 7-day period. Survival rates of 33.3% and 26.7% were reported for mice treated with honey and aloe vera gel, respectively, and 0% in control animals. Animals treated only with Knp 5 phage showed the highest survival rates and no additional beneficial effects was observed when Kpn5 phage was used in combination with aloe vera gel or honey (Kumari *et al.,* 2010). Nanoemulsion compound NB-201 decreased bacterial cells of *P. aeruginosa*-infected burn wounds from 7.9 x 10^7 to 6.5 x 10^4 in rats (Hemmila *et al.,* 2010). Nitric oxide nanoparticles showed similar results and caused a statistically significant reduction in MRSA counts in excisional wounds (Martinez *et al.,* 2009). A proline-rich synthetic peptide, designated A3-APO, showed potential as treatment for burn wound infections and decreased the bacterial load of wounds infected with different cell numbers of *Acinetobacter baumannii* (Ostorhazi *et al.,* 2010). Bacterial counts of mice, infected with 2 x 10^{11} cfu/wound, were in the range of 10^7 for control animals and below 10^3 for the majority of A3-APO-treated animals (Ostorhazi *et al.,* 2010). Further studies revealed that the peptide reduced MRSA counts in burn wounds from 10^7 to 10^4 cfu/mg tissue and increased survival of mice infected with a cocktail of *A. baumanni*, *K. pneumonia* and *P. mirabilis* (Ostorhazi *et al.,* 2011).

The optimal wound dressing should not just be able to treat infection, but also have a beneficial effect on wound healing and speed up the process to minimise recovery time. Autografts, xenografts and allografts have been used in the treatment of excisional wounds, however, some problems have been identified. These include limited availability of autografts in severely injured patients, whereas xenografts and allografts have problems associated with disease transmission and immune response (Kumbar *et al.*, 2008). Tissue engineering has resulted in the generation of various scaffolds to treat excisional wounds and promote wound healing. Chitosan have shown promise in the form of chitosan hydrogels, chitosan acetate bandages, as well as chitosan-(polyvinyl alcohol) nanofibers. Chitosan acetate bandages reduced inflammation and promoted accumulation of fibrous material in full-thickness excisional wounds (Burkatovskaya *et al.*, 2008). However, this did not have an added positive effect on wound closure in infected or uninfected wounds as compared to the control groups. Chitosan hydrogels containing DMEM/F12 (medium-Az-CH-LA) and a collagen sponge had similar wound healing profiles when applied to full-thickness wounds after dermal burns in rats (Kiyozumi *et al.*, 2007). Chitosan hydrogels containing DMEM/F12 (medium-Az-CH-LA) however did show more rapid neovascularization and the granulation tissue thickness was more prominent than in the collagen sponge treated groups (Kiyozumi *et al.*, 2007).

Although the main purpose of the study was to determine if NF wound dressings could treat *S. aureus*-induced infections, the effect of the NF- and CF-wound dressings on wound closure was also investigated. No difference in wound closure of infected wounds treated with NF- or CF-wound dressings was observed. Studies have actually revealed that *S. aureus*-infected wounds show improved wound closure over uninfected or infected and treated wounds (Burkatovskaya *et al.*, 2008). However, we did not notice this effect. Even though there was no significant difference in wound closure, the wounds that were treated with NF wound dressings had significantly less of a bacterial burden than CF wound dressing-treated wounds, as previously discussed. A statistically significant difference was, however, observed in wound closure of uninfected wounds treated with NF- or CF-wound dressings, as compared to control, gauze covered wounds. CF wound dressings, as well as NF wound dressings, showed a positive influence on wound healing, as determined by histological analysis. Hyaluronic acid nanofibers was shown to be effective in promoting wound healing of full-thickness excisional wounds in pigs and chitosan-poly(vinyl alcohol) nanofibers only showed a positive influence on wound healing when used in combination with R-Spondin I, a novel growth factor (Sundaramurthi *et al.*, 2012, Uppal *et al.*, 2011). Curcumin loaded poly(ε-caprolactone) (PCL) also showed improved wound healing from day 6 as compared to plain PCL over a 10

day period in a diabetic murine model (Merrell *et al.,* 2009). Other studies have also revealed that some nanofibers do not have an added positive influence over control groups on wound closure, however, histological analysis usually reveals that the wound healing process has been stimulated more in the nanofiber-treated groups (Lui *et al.,* 2010, Rho *et al.,* 2006). The addition of growth factors to the nanofibers usually accelerates wound closure as compared to control nanofiber treated groups, as would be expected (Choi *et al.,* 2008, Sundaramurthi *et al.,* 2012). The major factors of a nanofiber scaffold which positively influences wound healing thus seems to include hydrophobicity/hydrophilicity ratios of the nanofiber scaffolds which will influence attachment to the wound and prevent excessive fluid build-up, porosity influencing air and moisture permeability, as well as the benefit of bioactive molecules co-electrospun into or bound to the surface of the nanofibers (Lui *et al.,* 2012). Our nanofiber scaffolds have good wettability, good hydrophobicity/hydrophilicity ratios because of the blending of two hydrophobic/hydrophilic polymers before electrospinning, it retains its fibrous structure and its porosity upon incubation and thus have the potential as good overall scaffold to positively influence wound healing (Heunis *et al.,* 2011). This was, however, a preliminary study on wound healing of uninfected wounds and the exact influence of the nanofibers and/or the peptide on wound healing warrants further investigation. It can also not be ruled out that nisin can have an influence on wound healing, as cationic antimicrobial peptides play a role in the innate immune response (Barak *et al.,* 2005). Plantaricin A, a bacteriocin produced by *Lactobacillus plantarum*, has been shown to increase cell proliferation, enhance migration and influenced the expression of *TGF-β1*, *FGF7*, *VEGF-A* and *IL-8 in vitro* in human keratinocytes (Pinto *et al.,* 2011). Plantaricin A was further shown to promote antioxidant defences and increased the expression of *FLG*, *IVL* and *HBD-2* in NCTC 2544 keratinocytes (Marzani *et al.*, 2012). Decreased expression of TNF-α was also observed in plantaricin A treated cells. More recently, nisin Z was shown to have immunomodulatory activities and modulated the host immune response similar to natural host defense peptides (Kindrachuk *et al.*, 2012). Nisin Z mediated host protective immunity and conferred protection against Gram-positive and Gram-negative bacterial infections.

In conclusion, nisin was successfully co-electrospun into PEO 50:PDLLA 50 nanofibers and released nisin was able to inhibit the growth of *S. aureus* strains over a prolonged period of time. NF wound dressings were able to significantly reduce the bacterial burden of *S. aureus*-induced skin infections in a murine model. NF- and CF-wound dressings was able to significantly reduce wound closure of excisional wounds over 7 days in mice and showed no negative side effects on wound healing in a preliminary study. Nisin-containing

PEO:PDLLA nanofibers are thus suitable wound dressing materials to not only reduce the bacterial burden of *S. aureus* infected wounds, but also potentially decrease healing time of excisional wounds. As far as we could determine this is the first report of the *in vivo* evaluation of an antimicrobial peptide electrospun into nanofibers to treat skin infections induced by bacteria. Future research should include the combination of growth factors, anti-inflammatory agents, as well as antimicrobials to generate an optimal wound dressing which can treat infections and reduce time needed for wounds to heal.

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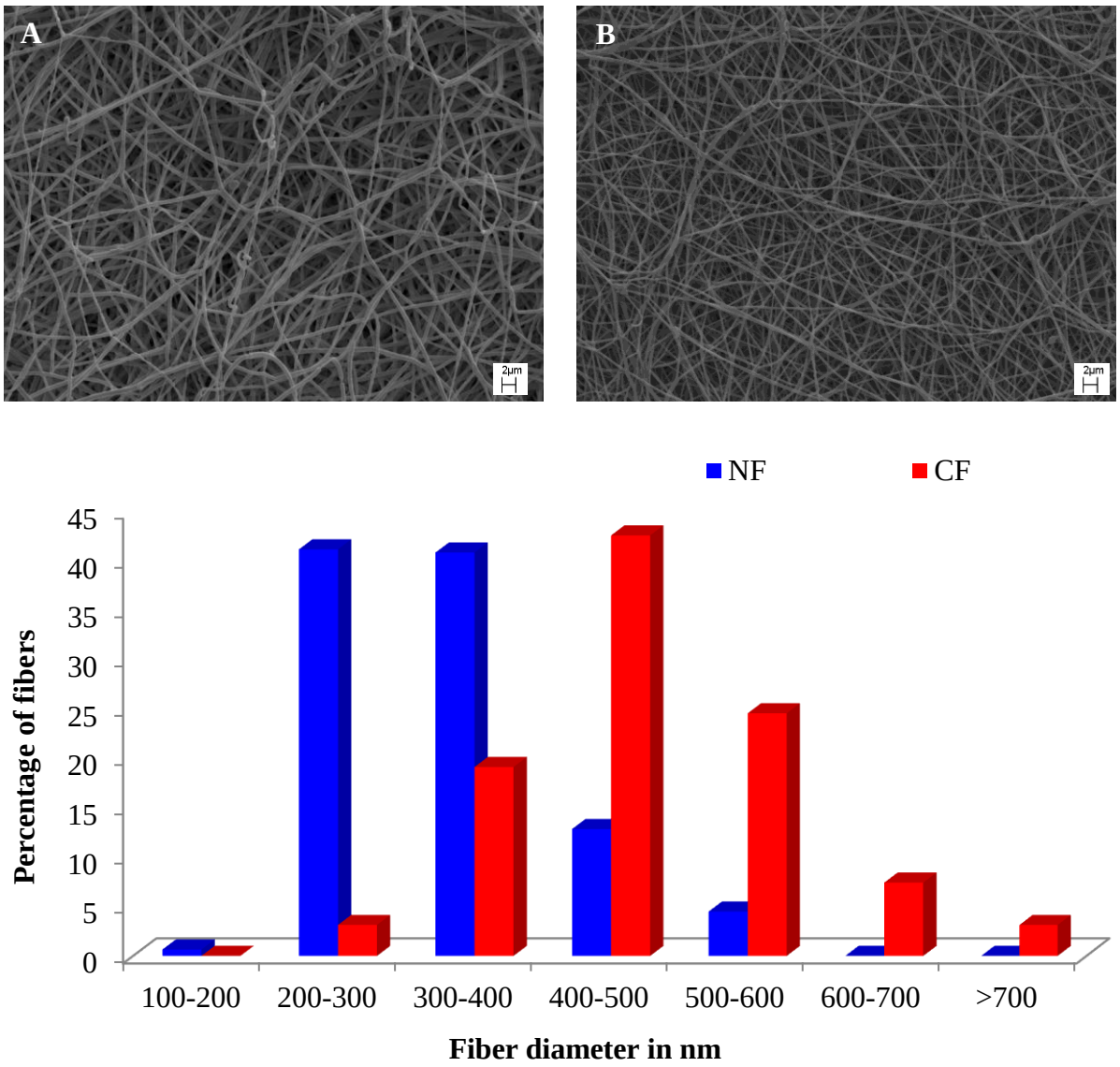

Fig. 1 Scanning electron microscopy (SEM) images of PEO 50:PDLLA 50 nanofibers electrospun **(A)** without and **(B)** co-electrospun with nisin. Distribution of fiber diameters in samples electrospun without nisin (CF) and with nisin (NF) are shown in **(C)**.

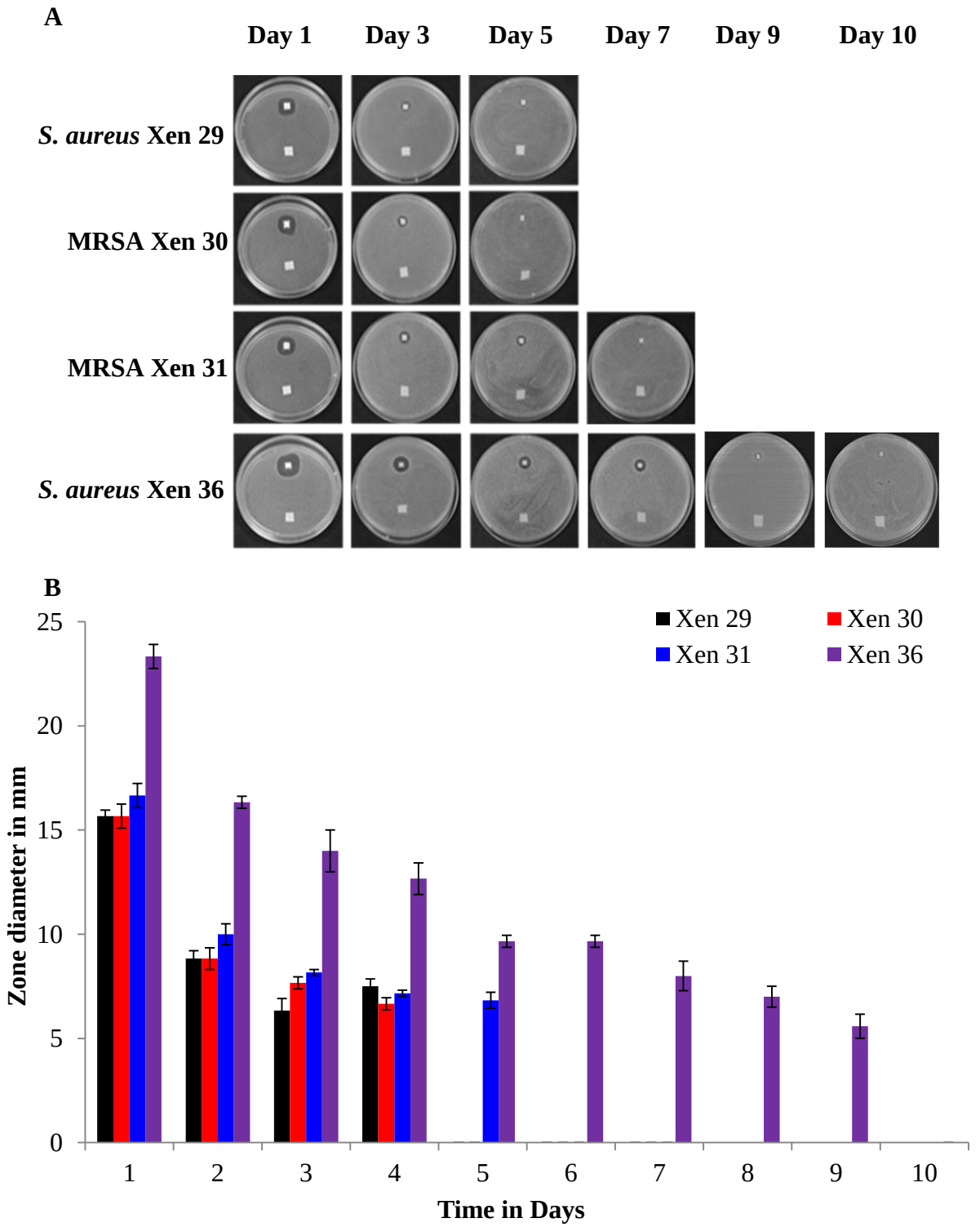

Fig. 2 Antimicrobial activity of PEO 50:PDLLA 50 nanofibers (**A**). The nanofiber sample placed in the top part of each petri-dish contained nisin (**NF**), whereas the sample placed below had no nisin (**CF**). The target strains of *S. aureus* were obtained from Caliper Life Sciences, Hopkinton, MA. Growth inhibition, depicted in zone diamemeter, as monitored over 10 days in NF is shown in (**B**).

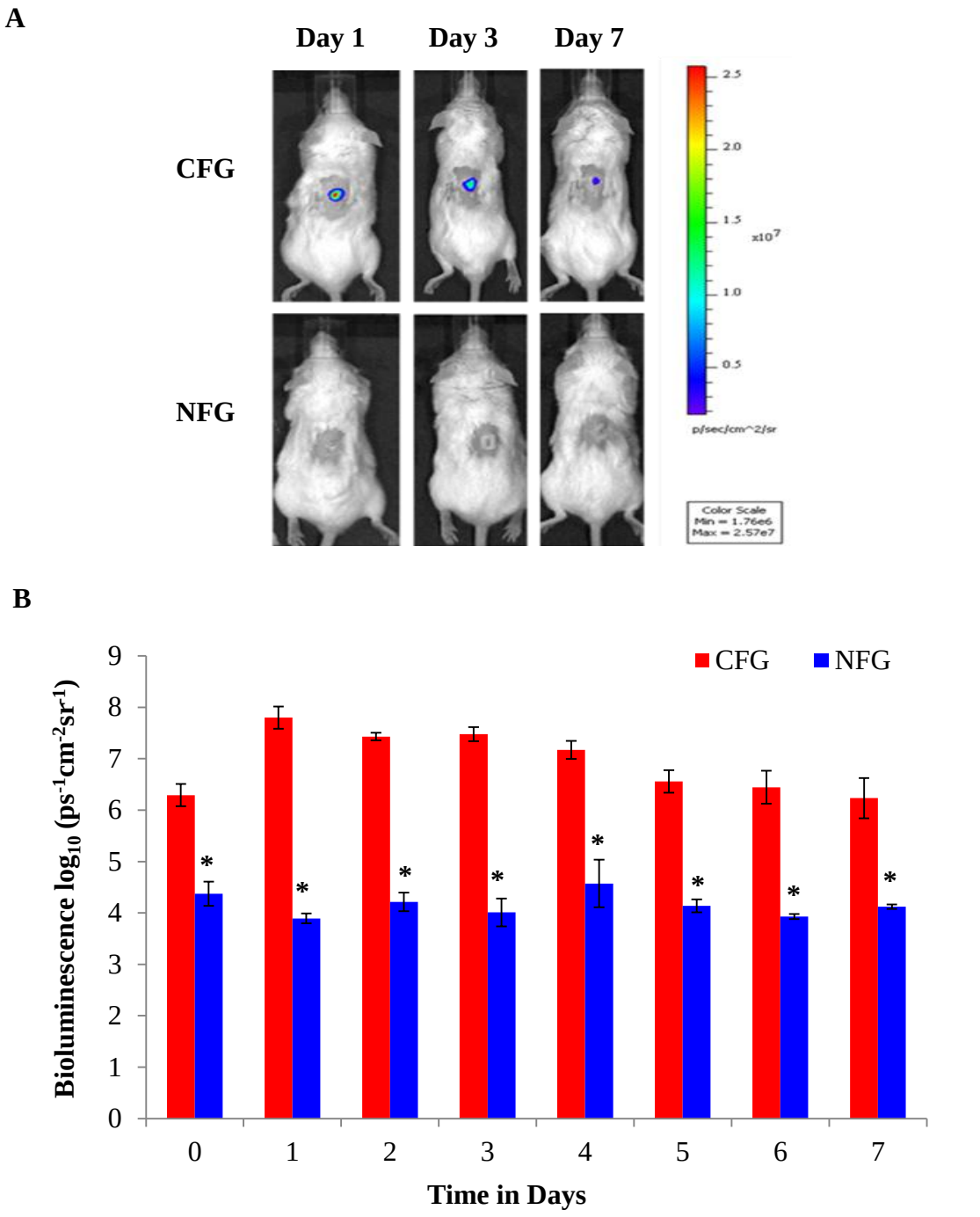

Fig. 3 Bioluminsecent images **(A)** and bioluminescent measurements **(B)** of mice infected with 10 µl of 10^8 cfu/ml *S. aureus* Xen 36 and treated with nisin-containing PEO 50:PDLLA 50 nanofiber wound dressings (**NFG**-nisin fiber group) and control nanofiber wound dressings (**CFG**-control fiber group). ***** $p<0.0001$ as compared to CFG.

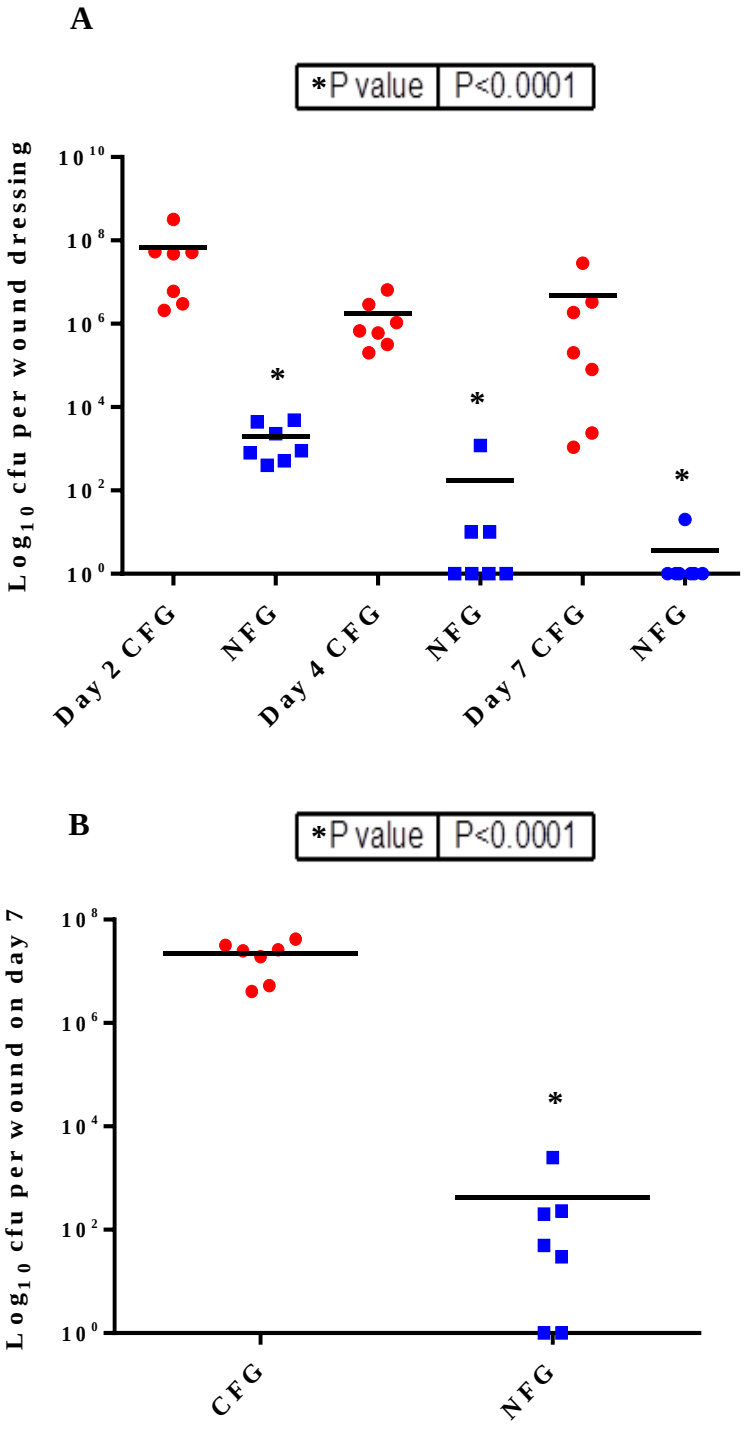

Fig. 4 **(A)** Colony forming units of *S. aureus* Xen 36 recovered from PEO 50:PDLLA 50 nanofiber wound dressings on day 2, 4 and day 7 of the experiment and **(B)** viable *S. aureus* Xen 36 in excised wounds on day 7. (**NFG**-nisin fiber group, **CFG**-control fiber group). * $p<0.0001$ as compared to CFG.

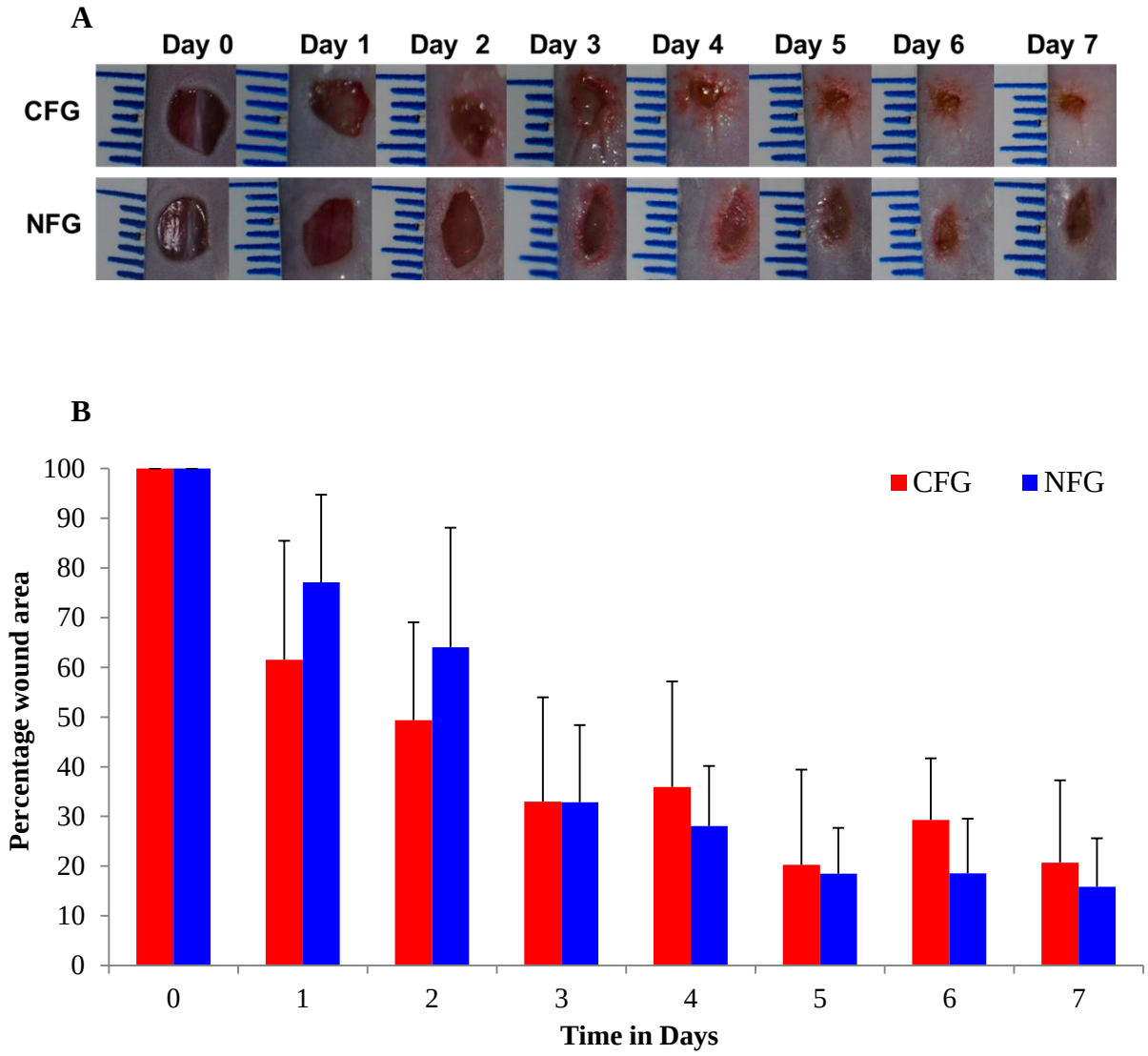

Fig. 5 Images of *S. aureus* Xen 36-infected wounds on the dorsal surface of mice treated with control PEO 50:PDLLA 50 nanofibers or nisin-containing nanofibers **(A)** and wound closure, expressed as a percentage, over 7 days **(B)**. (**CFG**-control fiber group and **NFG**-nisin fiber group). Distance between blue lines indicates 1mm.

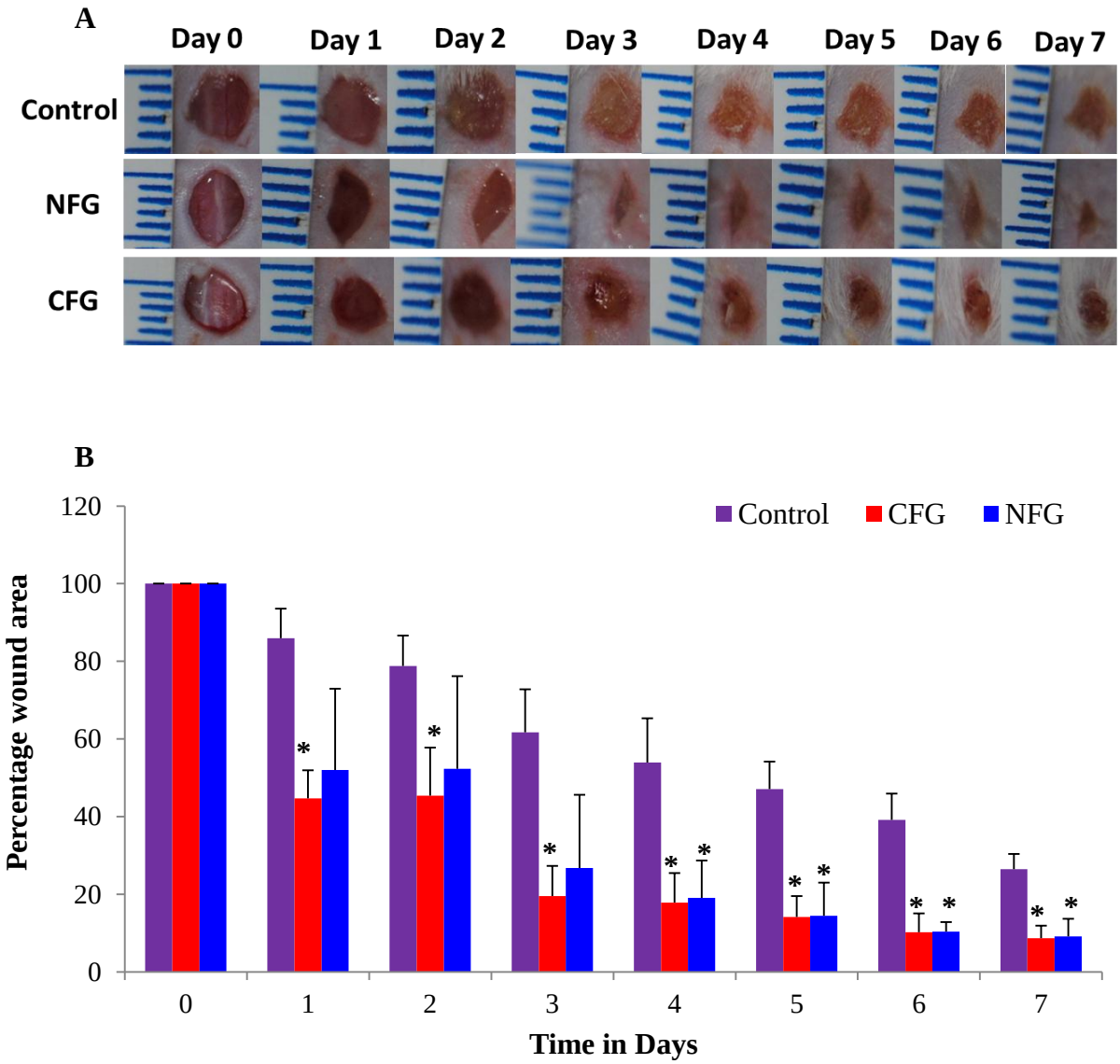

Fig. 6 Images of uninfected wounds, on the dorsal surface of mice, that received gauze (control), control PEO 50:PDLLA 50 nanofibers (**CFG**-control fiber group) or nisin-containing nanofibers (**NFG**-nisin fiber group) as wound dressings (**A**). Wound closure of wounds that received gauze, control nanofibers or nisin-containing nanofibers as wound dressings are shown in (**B**). * $p<0.05$ as compared to wounds covered with gauze. Distance between blue lines indicates 1mm.

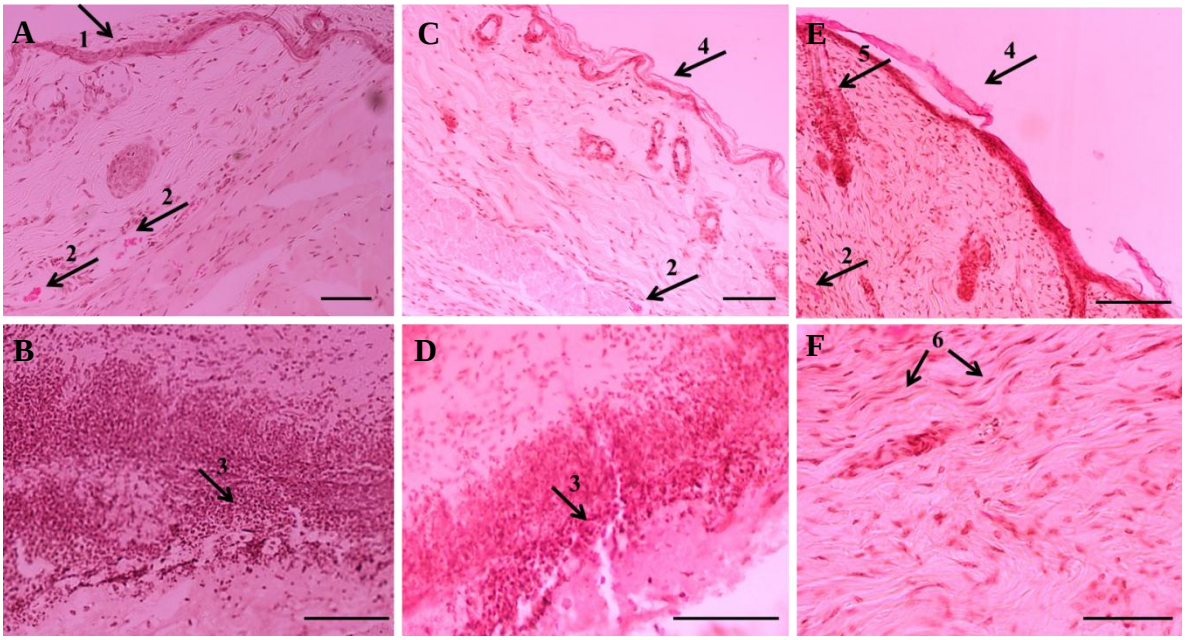

Fig. 7 Skin sections (stained with H&E) of excisional wounds covered with gauze **(A**-wound bed and **B**-tissue adjacent to wound bed**)**, control PEO 50:PDLLA 50 nanofibers **(C**-wound bed and **D**-tissue adjacent to wound bed**)** and nisin-containing nanofibers **(E** and **F,** both of wound bed**)** on day 7. **1**-re-epithelialization, **2**-neovascularization, **3**-neutrophils, **4**-keratinization, **5**-hair follicle and **6**-fibrosis (fibrotic scar formation). Scale bar = 20 µm.

CHAPTER 6

Nisin-containing Nanofiber Scaffolds as Potential Wound Dressing Material for Burn Wound Infections: Trial and Error

Abstract

Bacteriocins show great potential as alternatives to conventional antibiotics, especially with the increase in antibiotic-resistant pathogens. Antimicrobial nanofibers were prepared by co-electospinning nisin F into nanofibers consisting of poly(D,L-lactide) and poly(vinyl alcohol). These nanofibers could inhibit the growth of *S. aureus* for 2 days on agar plates. However, the nisin F-containing nanofibers were unable to reduce *S. aureus* bioluminescence in burn wound infections in mice. A nisin F solution could reduce *S. aureus* bioluminescence in infected burn wounds, however, did not reduce *S. aureus* viable cells on the same wounds. Nisin A was successfully co-electrospun into nanofibers consisting of poly(ethylene oxide) and poly (D,L-lactide) and retained activity after electrospinning. Nisin A-containing nanofibers were only able to reduce *S. aureus* bioluminescence once the burn wounds were debrided. Necrotic skin forms a barrier which seems to prevent the nisin from treating the infection. Burn wound debridement is thus important for successful treatment of bacterial burn wound infections with nisin.

Introduction

Thermal injury generates wounds that are perfect niches for microbial colonization, as it is a protein rich area which will sustain microbial growth (Altoparlak *et al.,* 2004). It also destroys the protective skin barrier, which can lead to infection (Church *et al.,* 2006). In the United States the cost involved in hospitalized fire and burn injuries amounts to more than 7.5 billion US dollars per annum and represents 2% of the total cost of injuries (CDC, 2011). From an estimated 1.1 million patients submitted to hospitals in the USA with burn injuries, 10 000 die due to infections (CDC, 2006). In South Africa 1223 patients with burn injuries have been admitted to the Red Cross War Memorial children's hospital trauma unit in 2011. This represented 14.7% of all reported injuries (Red Cross Trauma Unit, 2011).

Burn wound infections are difficult to treat, even with the advances in modern medicine (Dai *et al.,* 2009). Initial infection of a burn wound is usually caused by Gram-positive bacteria present in sweat glands and hair follicles (Altoparlak *et al.,* 2004, Nasser *et al.,* 2003). Secondary infection, which usually occurs within the first 5 to 7 days, is caused by bacteria, fungi and yeasts form the gastrointestinal- and upper respiratory-tract of the patient or can be acquired from the hospital environment (Altoparlak *et al.,* 2004, Church *et al.,*

2006, Erol *et al.*, 2004, Vindenes and Bjerknes, 1995). Burn victims are thus in need of specialized care to prevent infection, especially in cases where wounds cover most of the body surface area. *Staphylococcus aureus*, normally present on skin, is commonly isolated from infected burn wounds (Guggenheim *et al.*, 2009, Santucci *et al.*, 2003). *Pseudomonas aeruginosa*, *Acinetobacter* spp., coagulase-negative staphylococci, *Candida* spp., *Escherichia coli*, *Enterococcus* spp. and *Klebsiella pneumoniae* have also been isolated from infected burn wounds (Chim *et al.*, 2007, Guggenheim *et al.*, 2009, Santucci *et al.*, 2003). Antibiotic resistance in bacterial pathogens is on the increase and alternative treatments need to be explored (Neu, 1992). Various alternative treatments have been investigated to treat burn wound infections and include chitosan acetate bandages, chitosan hydrogels, natural antimicrobial agents (honey and aloe vera gel), *Klebsiella* bacteriophage Kpn 5 suspended in hydroxy propyl methyl cellulose hydrogels (E464, or HPMC) and nanoemulsions (Burkatovskaya *et al.*, 2008, Dai *et al.*, 2009, Hemmila *et al.*, 2010, Kumari *et al.*, 2010, Ribeiro *et al.*, 2009).

The potential biomedical applications of bacteriocins have recently been reviewed (Dicks *et al.*, 2011). Lantibiotics (lanthionine-containing bacteriocins) have great potential, especially as anti-staphylococcal agents. Nisin A is the best studied of all lantibiotics and is commercially available as Nisaplin® and Novasin™. Nisin targets the pyrophosphate moiety of lipid II and inhibits bacterial cell wall synthesis (Hsu *et al.*, 2004, Willey and van der Donk, 2007). It also form pores in cells, which is facilitated by first binding to lipid II (Brötz *et al.*, 1998). Nisin F, a natural nisin variant, was isolated from *Lactococcus lactis* spp. *lactis* F10 and differs from nisin Z by having one amino acid substitution (isoleucine to valine at position 30) (de Kwaadsteniet *et al.*, 2008). Of all nisin variants tested, nisin F was the most active against strains of *S. aureus*, methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant enterococci (VRE) and vancomycin-intermediate *S. aureus* (VISA) (Piper *et al.*, 2011). Nisin F repressed the growth of *S. aureus* in the upper respiratory tract of immunocompromised rats (de Kwaadsteniet *et al.*, 2009) and prevented growth of *S. aureus* *in vivo* when incorporated into brushite bone cement (van Staden *et al.*, 2012). Nisin F was, however, ineffective in the treatment of subcutaneous (de Kwaadsteniet *et al.*, 2010) and intraperitoneal (Brand *et al.*, 2010) *S. aureus* infections in mice.

Recently, the co-electrospinning of bacteriocins into nanofibers has been reported with potential biomedical and food applications (Dheraprasart *et al.*, 2009, Heunis and Dicks, 2010, Heunis *et al.*, 2010, 2011). Nisin A, co-electrospun into PEO:PDLLA nanofibers,

significantly reduced the cell numbers of *S. aureus* in full-thickness excisional wounds in mice (as shown in the previous chapter).

In this chapter wound dressings were prepared by co-electrospinning nisin F and nisin A into nanofibers. The ability of the wound dressings to control *S. aureus* infection in burn wounds was studied using a murine model.

Materials and Methods

Isolation and partial purification of nisin F

L. lactis spp. *lactis* F10 was cultured in MRS broth (Biolab, Biolab Diagnostics, Midrand, South Africa) at 30 °C for 24 h. Cells were harvested (8000g, 10 min, 4 °C), the pH of the cell-free supernatant adjusted to between pH 6.5-7.0 with 10M NaOH and then heated at 80 °C for 10 min to inactivate proteolytic enzymes. Peptides were precipitated from the cell-free supernatant with 70% saturated ammonium sulphate, desalted against distilled water by using a 1000 Da cut-off dialysis membrane (Spectrum Inc., CA, USA) and freeze-dried. Peptides were stored at -20 °C.

Electrospinning of nanofibers

Poly (D,L-lactide) (PDLLA, 75 000-120 000 Da) and partially hydrolyzed poly(vinyl alcohol) (PVA, 87-89% hydrolysed, 146 000-186 000 Da) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade. PVA solutions, 10-20% (w/v), were prepared in dimethylsulfoxide (DMSO). The PVA was dissolved by heating the solution to 40 °C. Freeze-dried crude nisin F (50 mg/ml, 12 800 AU/ml as determined against *S. aureus* Xen 36) was dissolved in *N*,*N*-dimethylformamide (DMF) and centrifuged (6000g, 1 min) to obtain the supernatant, which was used as solvent for the PDLLA (10-20%, w/v). The PVA in DMSO was mixed with the PDLLA solution in a ratio of (30:70) and co-electrospun as described by Heunis *et al.* (2010). These fibers were designated NFNF (nisin F-containing nanofibers). The controls contained no nisin F (NFCF). The voltage applied at the anode was +10kV and at the cathode (collector) -5kV. The distance between the anode and cathode was 15 cm and the relative humidity was controlled at 20-30%.

In another experiment nisin A (Nisaplin®) was dissolved in DMF to obtain 20 mg/ml of nisin in the solution and centrifuged (6000g, 2 min). The supernatant served as solvent for 24% (w/v) PDLLA blended with PEO (50:50). Solutions were heated to 40 °C on a hot plate and then electrospun as before. The relative humidity was kept constant between 50-55% and

the temperature between 27-30 °C. These nanofibers were designated NANF (nisin A-containing nanofibers). Control nanofibers contained no nisin A and were designated NACF.

Scanning electron microscopy of electrospun fibers

Images of the nanofibers were recorded with a Leo® 1430VP Scanning Electron Microscope (SEM). Samples were coated with a thin layer of gold to increase conductivity. Fiber diameter was determined by using the SEM Image Studio Software (version 10.1).

Release of antimicrobial peptides and in vitro *antimicrobial activity tests*

S. aureus Xen 36 was cultured in BHI broth (Biolab Diagnostics, Midrand, South Africa), supplemented with 200 µg/ml kanamycin for selection, and incubated at 37 °C for 24 h. The NFNF was tested for antimicrobial activity by placing 1.5 cm^2 of fibers on an agar plate seeded with *S. aureus* Xen 36. The plates were incubated for at 37 °C for 24 h, after which they were inspected for the formation of inhibition zones. The fibers were then placed on a new plate seeded with *S. aureus* Xen 36 and incubated for another at 37 °C for 24 h, after which they were once again inspected for the formation of inhibition zones. This was done repeatedly until no antimicrobial activity (inhibition zones) was observed. NANF were tested for antimicrobial activity by placing 1 cm^2 of fibers on an agar plate seeded with *S. aureus* Xen 36 and incubated for at 37 °C for 24h.

Preliminary in vivo *experiments*

Animals

Approval to conduct research on mice was granted by the Ethics Committee of the University of Stellenbosch (Ref: 10 NM_DIC02). Adult male BALB/c mice (weighing 20 to 30g) were fed a standard rodent diet, individually housed in animal cages and kept under controlled environmental conditions (12 h light-dark cycles, 20-22 °C).

Burn injury and infection

The dorsal hair of mice was removed with Veet Hair Remove Lotion (Reckitt Benckiser, Elandsfontein, South Africa).The following day, the mice were anesthetized with 2% (v/v) isoflorane in O_2 (Isofor, Safe Saline Pharmaceuticals, Florida, South Africa) and a burn was induced on the hairless dorsal surface of each mouse (n=3) with a brass rod (1 cm in diameter), pre-heated to 94 °C. Mice received buprenorphine (Temgesic, 0.03 mg/kg of body weight, Schering-Plough (Pty) Ltd., Woodmead, South Africa) subcutaneously as analgesic

directly before the burn and until day 4 of the experiment. A burn induced for 60 s resulted in reproducible burns and was used in subsequent experiments. Mice (n=3) were infected with 10 µl of 10^8-10^{10} cfu/ml *S. aureus* Xen 36, 1 h after the burns were induced.

Treatment of burn wounds

A NFNF wound dressing, of approximately 1.5 cm^2, was placed over the *S. aureus*-infected burn wound area 5 min after the infection. Nanofiber wound dressings without nisin (NFCF) served as control. Micropore™ surgical tape (Alpha Pharm, Stellenbosch, South Africa) was used to keep the nanofibers in contact with the wound area until the dressings were changed on days 2 and 4 of the experiment. In another experiment, burn wounds of mice (n=3) were treated with 10 µl nisin F (12 800 AU/ml, suspended in sterile distilled water) 5 min after infection and on days 2, 3 and 4 of the experiment. Mice (n=3) in control groups received sterile saline on the same days. Mice were euthanized with an overdose of pentabarbitone sodium (Euthapent, Kyron Laboratories (Pty) Ltd., Benrose, South Africa) on day 5 and the burn wounds excised to determine *S. aureus* cell numbers. The burn wounds were homogenised, the cell suspension serially diluted and plated onto BHI agar supplemented with 200 µg/ml kanamycin. Plates were incubated for at 37 °C for 24 h.

In another experiment, burn wounds was induced, as previously described, and some of the wounds were debrided after burn injury with scissors and forceps. Debrided and non-debrided wounds (n=2) were infected with 10 µl of 10^8 cfu/ml *S. aureus* Xen 36 and left for 1 h for the infection to establish. Wounds were subsequently treated once with NANF.

Bioluminescent imaging

Signal strength of photons transmitted through the nanofiber dressings was determined prior to *in vivo* bioluminescent imaging. *S. aureus* Xen 36 cells (10^7 cfu/ml) was placed into a microtiter plate and the wells were covered with nanofibers, which was moistened with phosphate buffered saline pH 7.4 (PBS). Bioluminescent readings were recorded using the *In Vivo* Imaging System (IVIS® 100) of Caliper Life Sciences (Hopkinton, MA, USA). Image software® (version 3.0, Caliper Life Sciences, Hopkinton, MA) was used to quantify the photons emitted from regions of interest (ROIs). The values obtained were expressed as $\log_{10}$ photons per second per cm^2 per steradian (p s^{-1} cm^{-2} sr^{-1}). For the *in vivo* experiment, mice were anaesthetized with 2% (v/v) isoflorane in O_2 and wound dressings were moistened with PBS buffer prior to bioluminescent imaging. Readings were recorded, every 24 h, through the wound dressings using the IVIS® 100. Micropore™ tape was removed before bioluminescent

imaging from mice that were treated with 10 µl of nisin F or sterile saline, as well as mice that were treated with NANF wound dressings. Living Image software® (version 3.0, Caliper Life Sciences) was used to quantify the photons emitted from regions of interest (ROIs) pre-set at 23.6 x 23.6 pixels. The values obtained were expressed as $\log_{10}$ photons per second per cm^2 per steradian (p s^{-1} cm^{-2} sr^{-1}).

Results

Nanofibers electrospun from a 10% (w/v) PDLLA 70:PVA 30 solution with and without nisin F (50 mg/ml, 12 800 AU/ml) had a bead-on-string morphology (Fig. 1). Uniform nanofibers with a smooth surface morphology were obtained when the PDLLA 70:PVA 30 concentration was increased to 20% (w/v) (Fig. 2A, C). The diameter of nanofibers without nisin F ranged from 100 nm to 350 nm, with a mean diameter of 186 ± 37 nm (Fig. 2B). The diameter of nanofibers containing nisin F ranged from 200 nm to 500 nm, with a mean diameter of 321 ± 54 nm (Fig. 2D).

Nisin F released from the nanofibers inhibited the growth of *S. aureus* Xen 36 for 2 days on agar plates. The size of the inhibition zones were more-or-less the same over the first 2 days (21.75 mm on day 1 and 18.25 mm on day 2). No inhibition zones were recorded on day 3 (Fig. 3), indicating that nisin F was released from the nanofibers with a high initial burst during the first 2 days of incubation.

Burn wounds induced at 94 °C for 10 s did not result in severe burn damage. However, after 25 s much more damage to the epidermis was visible (Fig. 5B). Burns at 94 °C for 60 s induced severe damage to the epidermis and could potentially create an optimal area for microbial colonization (Fig. 5C). Burn wounds were easily infected by *S. aureus* Xen 36 when 10 µl of 10^8-10^{10} cfu/ml of bacteria was applied to the wounds and infections remained stable for 7 days (Fig. 6).

The bioluminescent signal decreased from 6.9 x 10^6 to 1.9 x 10^6 p s^{-1} cm^{-2} sr^{-1} when covered with nanofibers. No significant differences in *in vivo* bioluminescent signals were observed over 5 days between burn wounds that were treated with NFNF- and NFCF-wound dressings (Fig. 7 and 8).

Further optimization was done to determine if burn wound infections can be treated with a nisin F solution or nisin A-containing nanofibers. The following are result obtained from further optimization experiments. Firstly, mice (n=3) were infected and treated with nisin F, as previously described. A slight decrease in bioluminescent signal was recorded

from day 3 after treatment with nisin F (Fig. 9B). Bioluminescent signal from treated groups was lower than control groups until the end of the trial (day 5). No significant difference was, however, observed in cell counts between treated and control groups (Fig. 10). The colony forming units per wound were 2.0 $x10^7$ and 5.2 x 10^7 for the control and treated group, respectively.

NANF wound dressings inhibited the growth of *S. aureus* Xen 36 *in vitro* (Fig. 12). *S. aureus* Xen 36 bioluminescence was reduced *in vivo*, but only when the burn wounds were debrided before infection and treatment with NANF (Fig. 13).

Discussion

All of the fibers electrospun in this study were in the nanometer range, with the NFCF having slightly smaller fiber diameters than the NFNF (Fig. 2). Increase in polymer concentration resulted in nanofibers with more uniformity. Various factors influence the morphology of the fibers that are formed during electrospinning which can include, viscosity, elasticity, conductivity and surface tension of the solution, strength of the electric field, distance between the needle tip and collector, humidity as well as temperature (Deitzel *et al.,* 2001, Huang *et al.,* 2003). Polymer concentration and molecular weight is also very important. It has been shown that PLLA of 1% (w/w) produces a bead-on-string morphology when electrospun, whereas 3% (w/w) PLLA forms nanofibers with a much more smooth structure (Maretschek *et al.,* 2008). PEO electrospun at 4wt% in water also had more of a beaded structure as compared to fibers electrospun from a 10wt% solution (Deitzel *et al.,* 2001).

NFNF inhibited the growth of *S. aureus* Xen 36 for only 2 consecutive days. The area of the fibers decreased dramatically after one day of incubation and a slight decrease was seen on day 2, as compared to day one. The high burst release, within the first 2 days, could be ascribed to the dissolution of the PVA, as this polymer is highly soluble in aqueous environments. Nisin F on the surface could also easily be released by diffusion from the fibers. These results suggests that PDLLA 70:PVA 30 nanofibers can serve as a delivery system for nisin F, if only for a short time.

Burn wounds, as well as bacterial numbers needed for stable infection, were optimized before the nanofibers could be investigated as wound dressing in an *in vivo* murine model. Burn wounds with severe epidermal damage could be induced in mice, which could easily be infected with *S. aureus* Xen 36 (Figs. 5 and 6). A 60 s burn at 94 °C produced an optimal wound for infection. All the mice survived the burn injury and infection over the 7-day

period. This made bioluminescent imaging and thus real time monitoring of the infection possible in the same animal over the course of the experiment. Dai *et al.* (2009) induced burn wounds successfully in mice with a heated brass bar at (92-95 °C). Fatal infections were induced by applying 10^7 cfu *P. aeruginosa* to a 10 s burn, as well as applying 10^8 cfu of *P. mirabilis* to a 30 s burn. In this study we wanted to compare bioluminescent measurements of control groups to treated groups and thus needed our control group to survive, i.e. for comparative purposes.

The NFNF wound dressings were unable to treat the *S. aureus*-induced burn wound infections (the bioluminescent signals did not differ between treated groups). This could be due to the inability of the nisin F to reach the *S. aureus* cells and the peptide could thus not exert its antimicrobial activity. It could also be that the concentration of nisin F released from the fibers was not high enough to inhibit *S. aureus* growth *in vivo*, as the burn wound is relatively dry as compared to human burn wounds (Dai *et al.*, 2009). However, the wound dressings were moistened daily before readings were taken to facilitate nisin F release and to maximize transmission of the bioluminescent signal through the bandages. When a nisin F solution was applied to the burn wound, it reduced the bioluminescent signal of *S. aureus* Xen 36. However, nisin F was unable to decrease *S. aureus* cells in the burn wound. Various factors influence the bioluminescent signal emitted by lux-tagged pathogens, including oxygen availability, adenosine-5'-triphosphate (ATP) and tissue depth (Andreu *et al.*, 2010). Oxygen and adenosine-5'-triphosphate (ATP) is essential as they are needed by the enzyme luciferase to oxidize the luciferin and reduced flavin mononucleotide (FMNH2) (Meighen, 1991). Tissue depth and oxygen could not play a role in the bioluminescent reaction, as the infection was topical. The nisin F thus seemed to have more of an effect on the metabolic state of *S. aureus* Xen 36 *in vivo*, rather than a bactericidal effect.

NANF wound dressings reduced the bioluminescent signal in debrided burn wounds. The wound dressing was, however, unable to reduce bacterial bioluminescence on non-debrided burn wounds. It would thus seem that the damaged tissue formed during burn injury hampers the efficacy of the peptide to penetrate the wound and interact with the *S. aureus* cells. Further investigation of NANF wound dressings is needed on open (excisional) wounds as this was a preliminary optimization trial with only two mice (this was addressed in the previous chapter).

Burn wounds have successfully been induced in mice and stable *S. aureus*-induced burn wound infections was achieved. Nisin F was released from the fibers in the active form

and inhibited the growth of *S. aureus* Xen 36 *in vitro*. However, it was unable to *treat S. aureus*-induced burn wound infections *in vivo*. A nisin F solution was shown to be ineffective in treating and/or repressing the growth of *S. aureus* Xen 36 *in vivo* in burn wounds. NANF wound dressings could only treat debrided burn wounds. This would indicate that these fibers would be better at treating open excisional wounds instead of wounds were damaged (necrotic) tissue could prevent antimicrobial penetration of the wound, unless wound debridement is performed.

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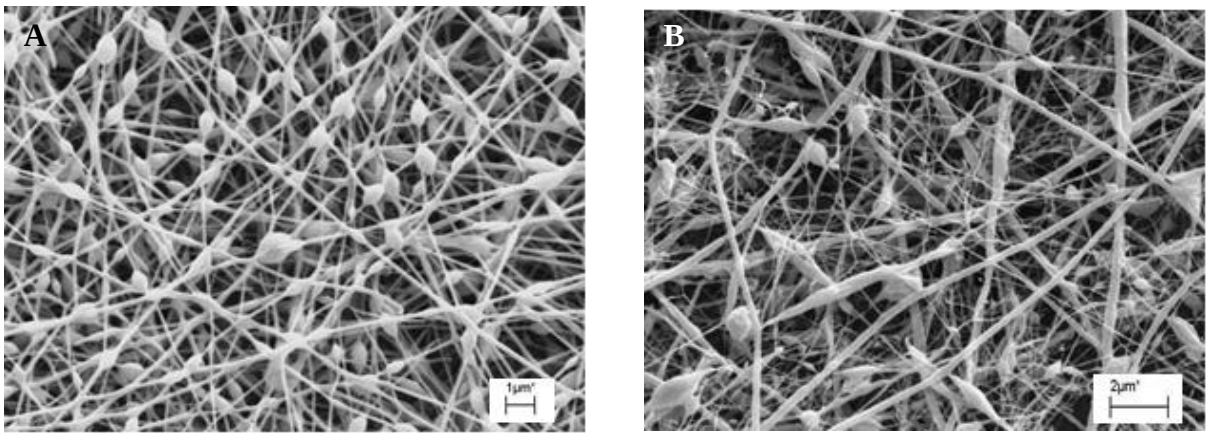

Fig. 1 SEM images of electrospun nanofibers at a concentration of 10% (w/v) PDLLA 70: PVA 30, with (**A**) and without (**B**) nisin F.

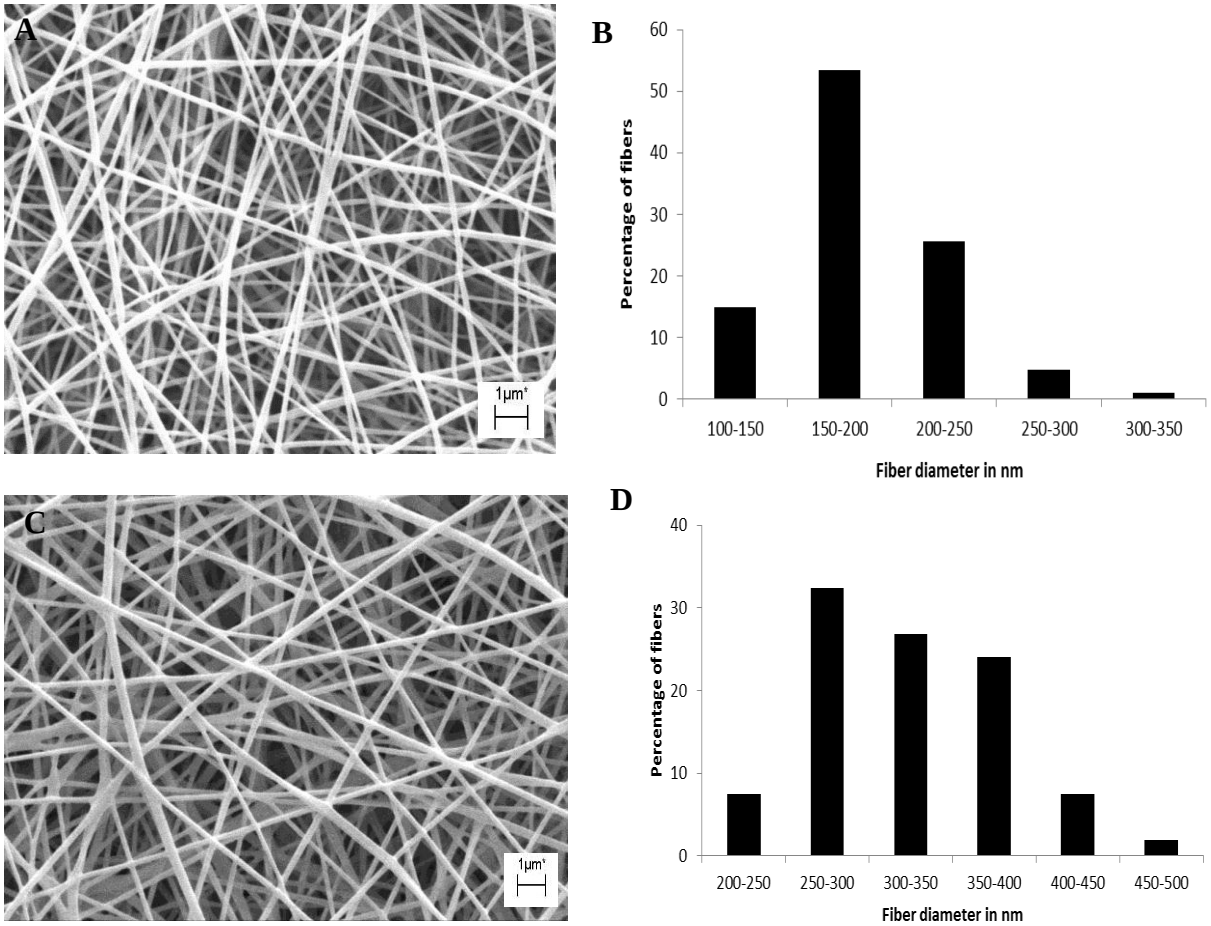

Fig. 2 SEM images of electrospun nanofibers with the distribution in fiber diameters. (**A**) 20% (w/v) PDLLA 70:PVA 30 fibers without nisin F, (**B**) fiber diameter distribution of 20% (w/v) PDLLA 70:PVA 30 fibers without nisin F (mean = 186 ± 37 nm), (**C**) 20% (w/v) PDLLA 70:PVA 30 fibers with nisin F and (**D**) fiber diameter distribution of 20% (w/v) PDLLA 70:PVA 30 fibers with nisin F (mean = 321 ± 54 nm).

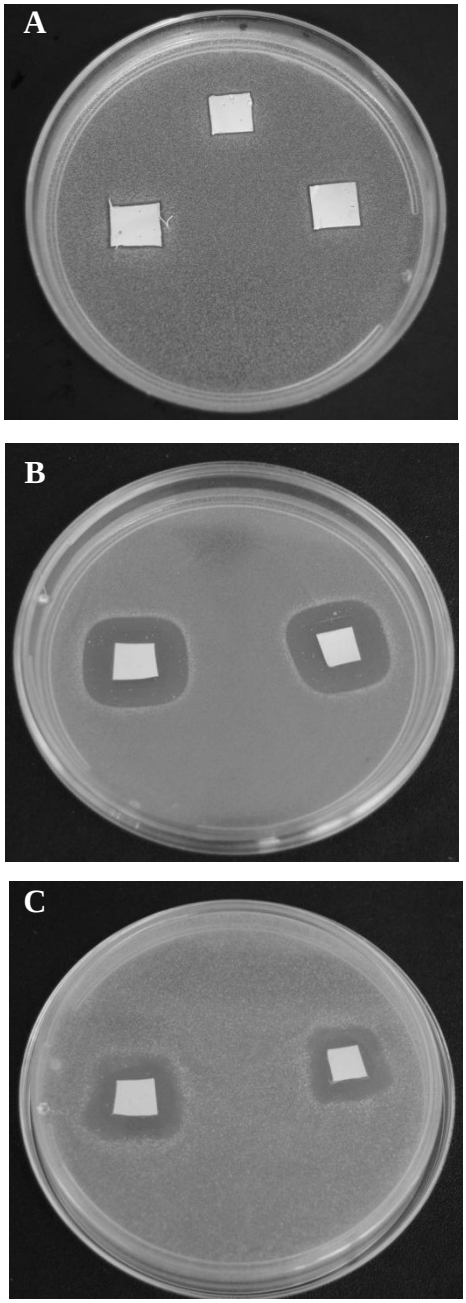

Fig. 3 Antimicrobial activity of electrospun 20% (w/v) PDLLA 70:PVA 30 nanofibers against *S. aureus* Xen 36 (**A**) Control fibers, without nisin F, (**B**) antimicrobial activity after 1 day of incubation at 37 °C and (**C**) antimicrobial activity after day 2 of incubation at 37 °C.

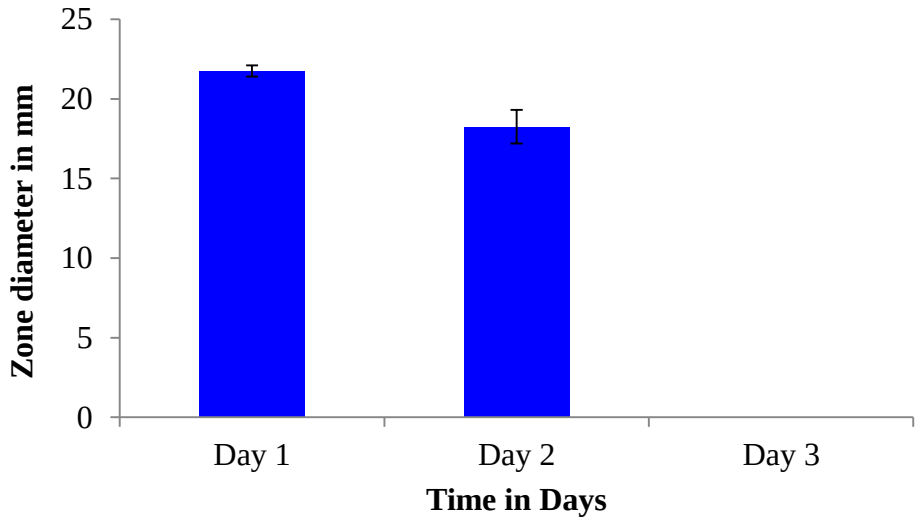

Fig. 4 Diameter of inhibition zones formed surrounding nisin F-containing PDLLA 70:PVA 30 nanofibers.

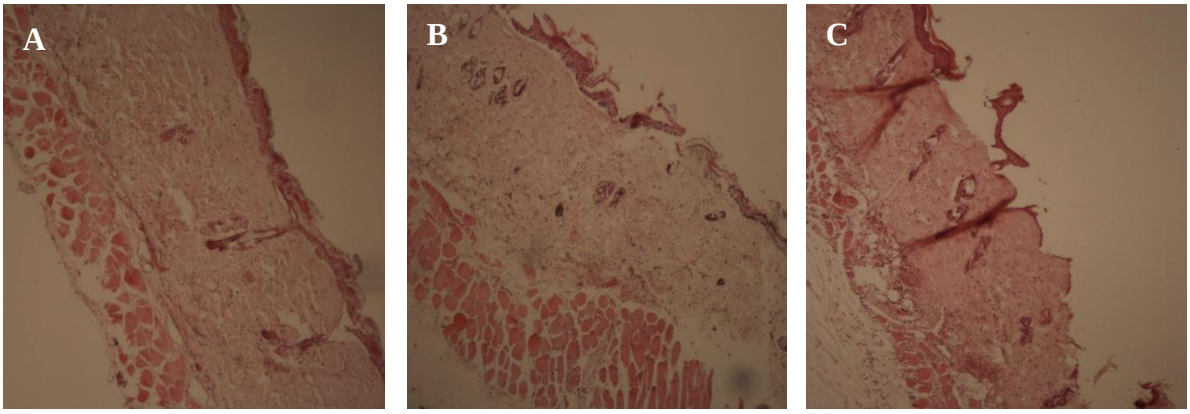

Fig. 5 Skin sections of mice burnt with a brass rod heated to 94 °C (**A**) control/unburnt skin, (**B**) burns induced for 25 s and (**C**) burns induced for 60 s.

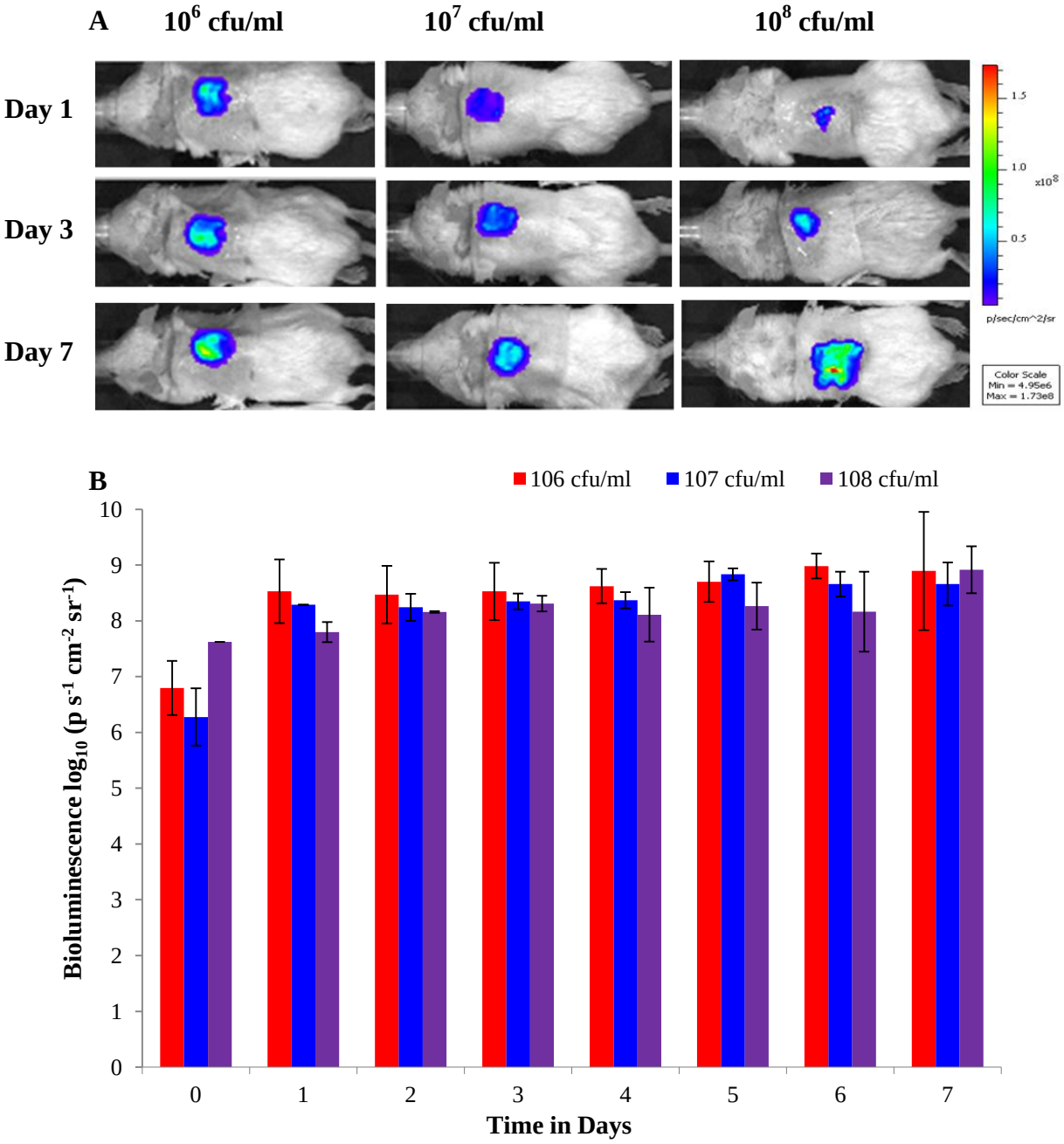

Fig. 6 **(A)** Bioluminescent images and **(B)** bioluminescence $\log_{10}$ values recorded from mice infected with 10 µl of 10^8-10^{10} cfu/ml *S. aureus* Xen 36, inoculated onto a burn wound that was induced by placing a brass rod (94 °C) for 60 s on the dorsal surface of the mice.

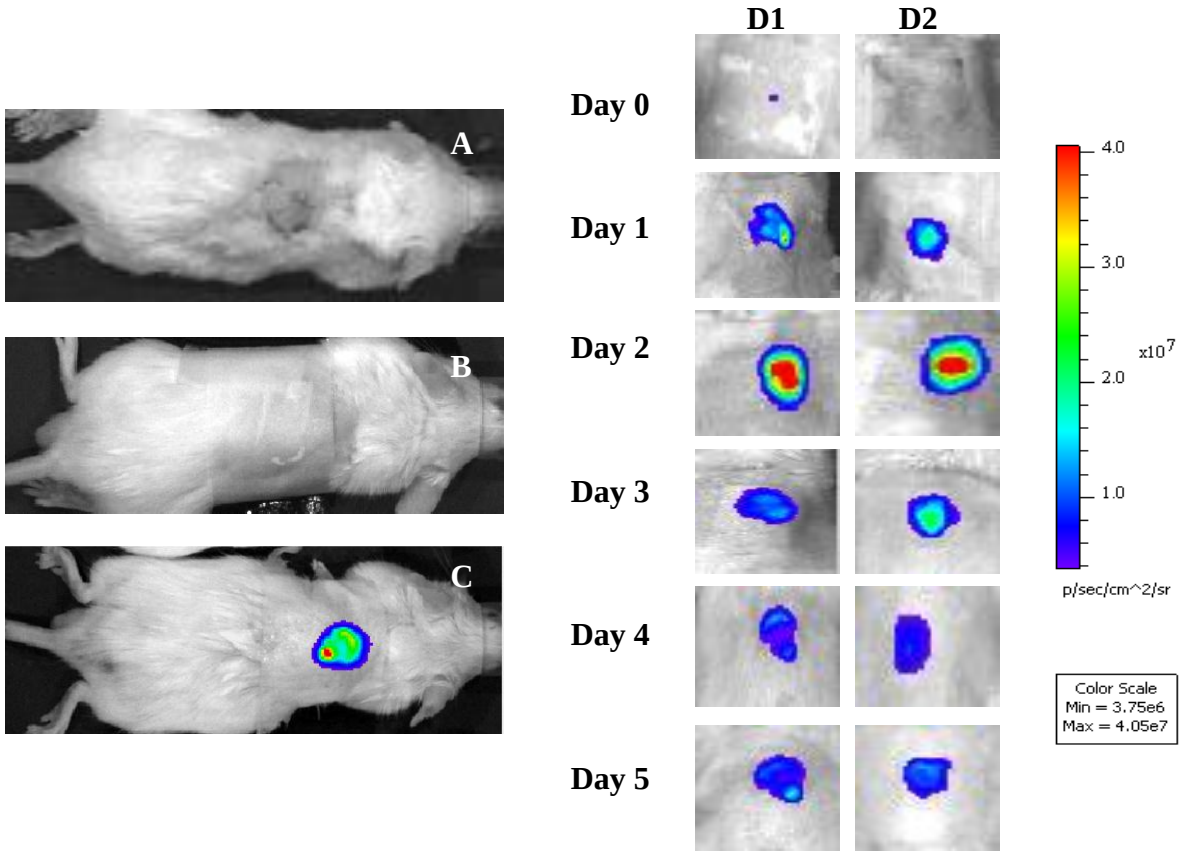

Fig. 7 Images and bioluminescent images from mice with a *S. aureus* Xen 36 (10 µl of 10^8 cfu/ml) induced burn wound infection. (**A**) Image of a burn wound without wound dressing, (**B**) image of a nanofiber wound dressing covered with micropore tape, (**C**) image showing bioluminescent signal through the moistened nanofiber wound dressing and (**D**) bioluminescent images of mice infected with *S. aureus* Xen 36 (10^6 cfu/ml) over a 5 day period. (**D1**) PDLLA 70:PVA 30 nanofibers without nisin F (NFCF) and (**D2**) PDLLA 70:PVA 30 nanofibers with nisin F (NFNF).

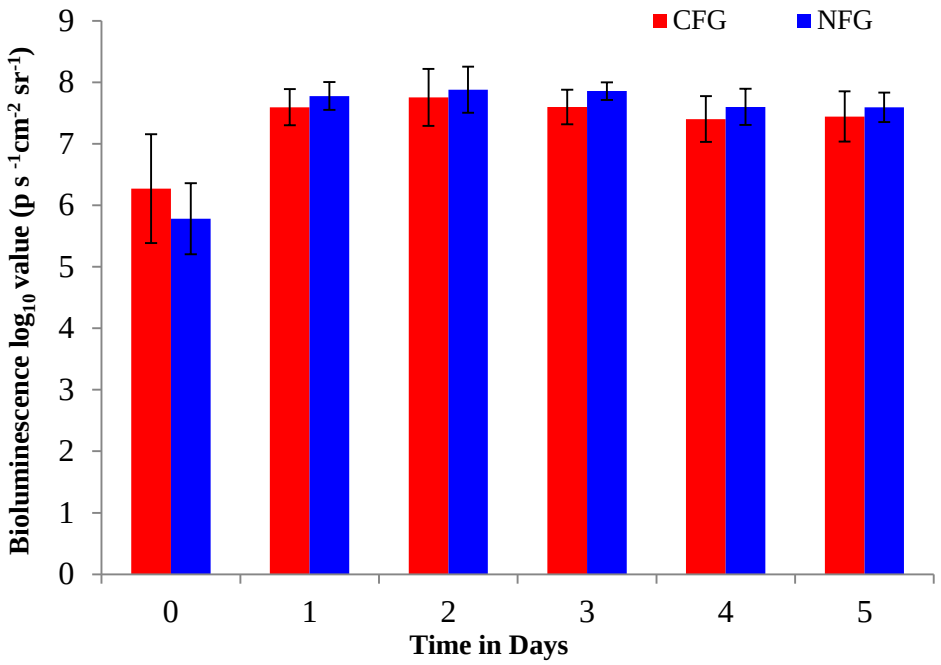

Fig. 8 Bioluminescence $\log_{10}$ values from mice with an *S. aureus* Xen 36 (10 µl of 10^8 cfu/ml cfu/ml) induced burn wound infection, treated with PDLLA 70:PVA 30 nanofibers without nisin F (**CFG**-control fiber group) and with nisin F (**NFG-**nisin F fiber group).

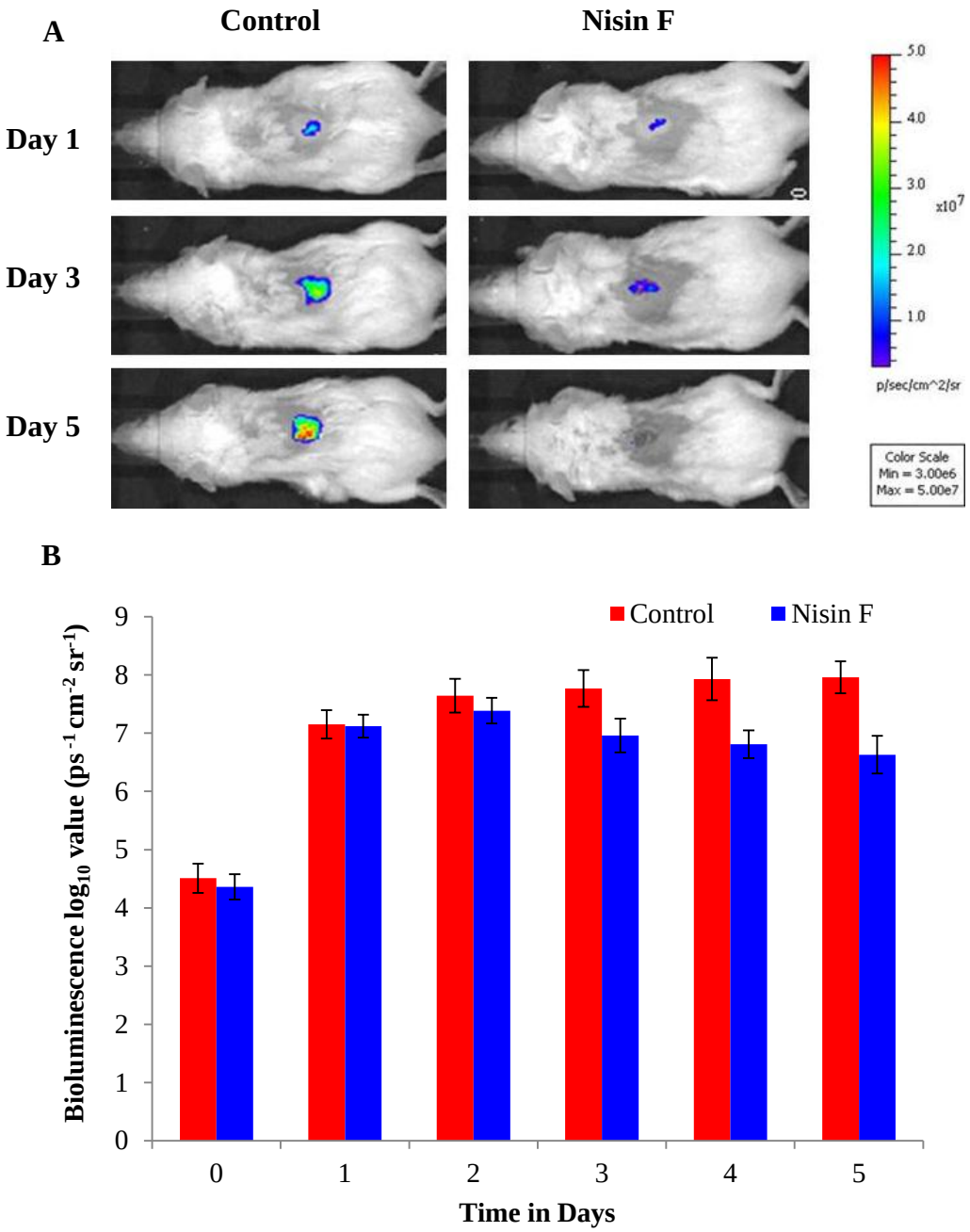

Fig. 9 **(A)** Bioluminescent images and **(B)** bioluminescence $\log_{10}$ values from mice infected with 10 µl of 10^8 cfu/ml *S. aureus* Xen 36 inoculated onto a burn wound that was induced by placing a brass rod (94 °C) for 60 s on the dorsal surface of the mice. Mice were treated with 10 µl nisin F 5 min after infection and on days 2, 3 and 4.

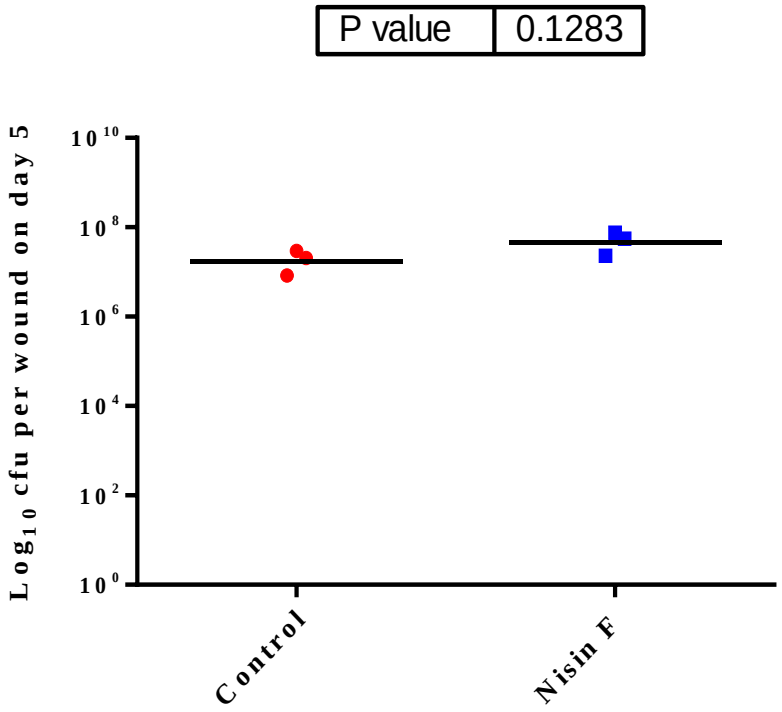

Fig. 10 Viable cells in burn wounds of mice infected with 10 µl 10^8 cfu/ml *S. aureus* Xen 36, on day 5. Wounds were treated with either 10 µl nisin F or 10 µl saline 5 min after infection, and on days 2, 3 and 4.

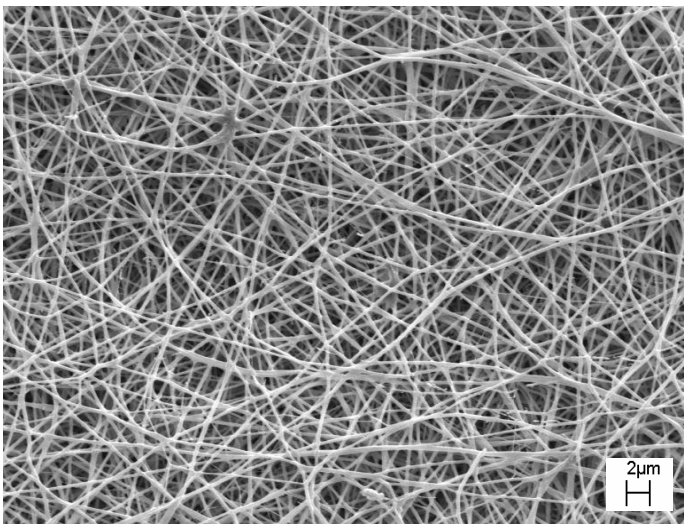

Fig. 11 SEM image of electrospun PEO: PDLLA nanofibers containing nisin A (NANF).

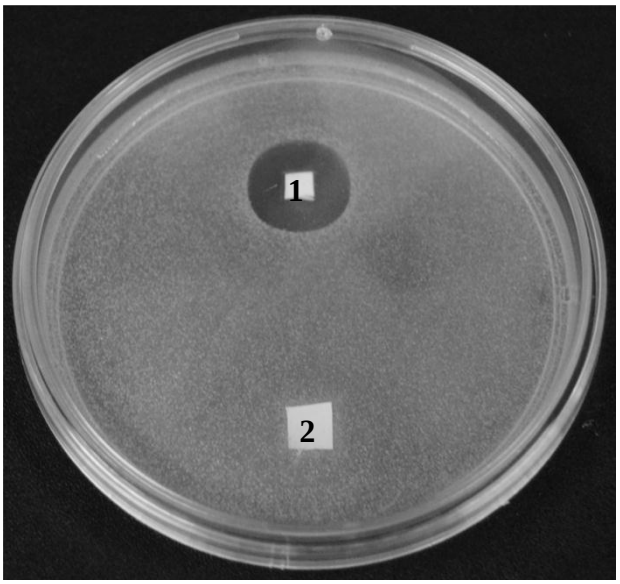

Fig. 12 Antimicrobial activity of nisin A-containing PEO:PDLLA nanofibers against *S. aureus* Xen 36. PEO:PDLLA nanofibers with (**1**) and without (**2**) nisin A.

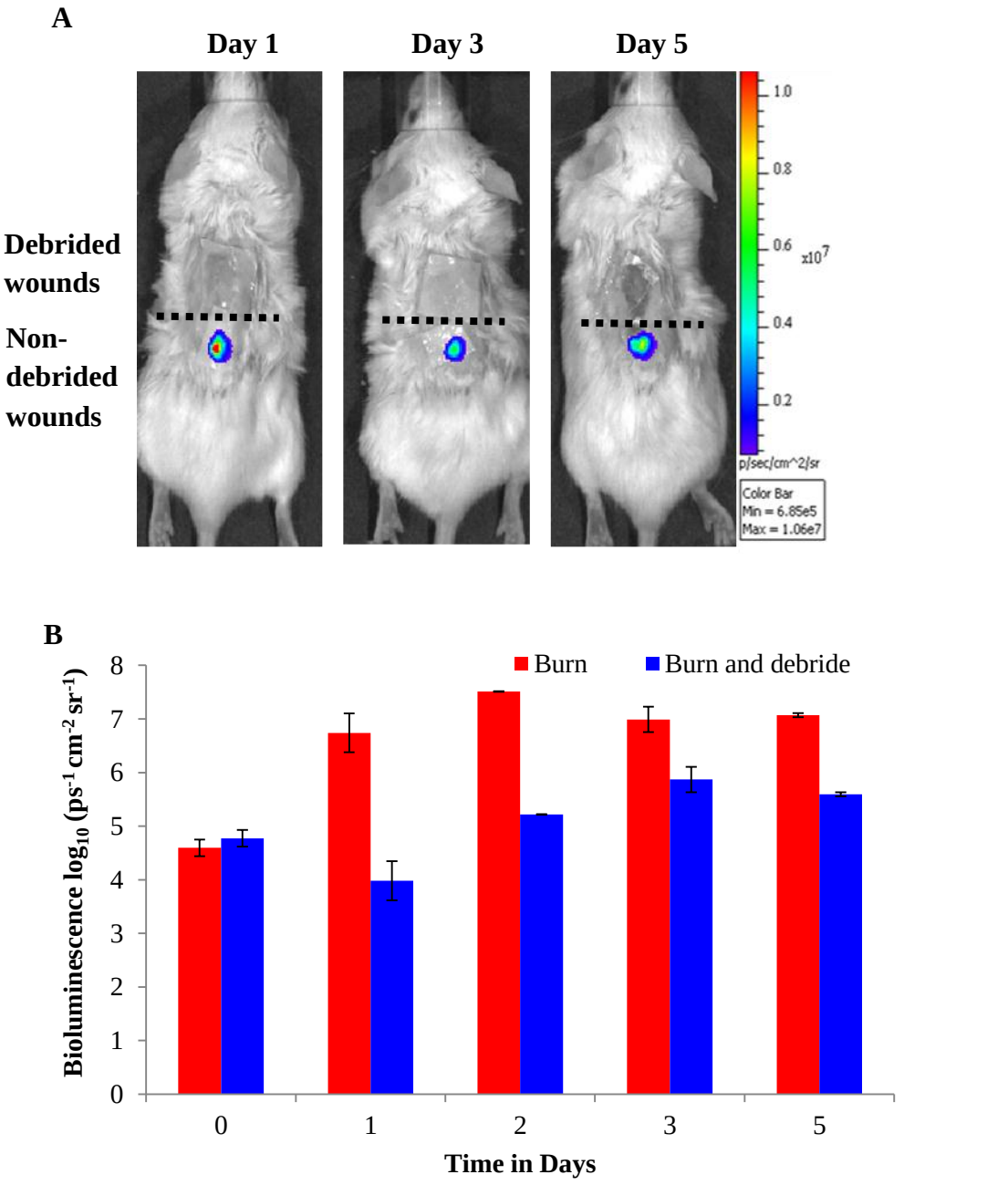

Fig. 13 (**A**) Bioluminescent images and (**B**) bioluminescence $\log_{10}$ values from mice infected with 10 µl of 10^8 cfu/ml *S. aureus* Xen 36 inoculated onto a burn wound, which was either debrided or non-debrided and treated with PEO:PDLLA nanofibers, containing nisin A.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

Probiotics are living organisms with health promoting properties and bacteriocin production can be considered as a very important probiotic trait (Dobson *et al.,* 2012). Electrospinning is a versatile technique to incorporate a variety of bioactive compounds into polymeric nanofibers to facilitate release thereof, or to encapsulate living organisms including bacteria, viruses and even mammalian cells (Agarwal *et al.,* 2008). In this study, the probiotic *Lactobacillus plantarum* 423 was successfully electrospun into poly(ethylene oxide) (PEO) nanofibers and only showed a slight reduction in viability, from 2.3 x 10^{10} to 4.7 x 10^{8} cfu/ml after electrospinning. *L. plantarum* 423 cells released from the nanofibers were still able to produce plantaricin 423. As far as we could determine this was the first report on the encapsulation of probiotic lactic acid bacteria (LAB) into electrospun nanofibers. Other studies have also demonstrated the feasibility of co-electrospinning viable bacterial cells into nanofibers. *Escherichia coli* and *Staphylococcus albus* also survived co-electrospinning into poly(vinyl alcohol) (PVA) nanofibers and remained viable for three months at -20 and -55 °C (Sulalha *et al.,* 2006). *Micrococcus luteus* showed better survival rates than *E. coli* when electrospun into PEO nanofibers with 74% surviving, where only 0.1% of the *E. coli* survived (Gensheimer *et al.,* 2007). *Bifidobacterium animalis* Bb12, was successfully encapsulated into PVA nanofibers by using co-axial electrospinning (López-Rubio *et al.,* 2009). *B. animalis* Bb12 remained viable for 40 days at room temperature and for 130 days at 4 °C in PVA nanofibers. Comparable survival rates to our study were obtained when *Lactobacillus acidophilus* FTDC 8933 was co-electrospun into nanofibers consisting of soluble dietary fiber and PVA (Fung *et al.,* 2011). These results thus indicate that electrospun nanofibers can serve as a successful delivery system for bacteria, including probiotic LAB.

The increase in antibiotic resistance has placed an enormous burden on health care and treatment of bacterial infections is becoming more problematic. Bacteriocins have traditionally found application in the preservation of food products by preventing the growth of spoilage organisms. However, recently bacteriocins have received increased attention as alternatives to antibiotics to treat bacterial infections. As knowledge on host-microbe interactions expands with next-generation sequencing technologies and the human microbiome project, it is becoming apparent that we need to steer away from broad-spectrum antimicrobials as they can disrupt commensal populations (Cotter *et al.,* 2012). This can also be related to the skin microbiota in which commensals, as those in the GIT, can protect the host from infection and prevent inflammatory conditions (Cogen *et al.,* 2008). The fact that

bacteriocins have a relatively narrow spectrum of activity, as compared to some antibiotics, and that they are active against antibiotic-resistant pathogens has increased their potential use as next-generation antimicrobials. It is thus not surprising that this is an active field of research. The feasibility of incorporating bacteriocins into nanofibers was shown in this study by co-electrospinning plantaricin 423 into PEO nanofibers. Plantaricin 423 retained its activity after electrospinning and could still inhibit the growth of *Enterococcus faecium* HKLHS and *Lactobacillus sakei* DSM 20017^{T}. Recently, nisin was shown to retain antimicrobial activity after co-electrospinning into gelatin nanofibers, with subsequent cross-linking with saturated glutaraldehyde vapour (Dheraprasart *et al*. 2009). These nanofibers were investigated for food preservation.

Plantaricin 423 and bacteriocin ST4SA were chosen as model bacteriocins to develop a nanofiber drug delivery system for bacteriocins, with the ultimate goal of generating an antimicrobial wound dressing. Plantaricin 423 showed low total release with an initial burst release from poly(D,L-lactide) (PDLLA) nanofibers, with negligible release thereafter. Plantaricin 423 release was only observed for 6 h. An initial burst release has also been reported for other molecules from electrospun nanofibers (Kenawy *et al.,* 2002, Kim *et al.,* 2004, Peng *et al.,* 2008). Core-shell structured nanofibers usually show a lower burst release profile (Maretschek *et al.,* 2008, Yang *et al.,* 2008). Hydrophilicity and hydrophobicity of the nanofiber scaffolds have been shown to influence the release profile of various drugs, as well as having an effect on cell attachment and proliferation in tissue engineering (Leung and Ko, 2011, Maretschek *et al.*, 2008). Polymer-drug interaction and solubility of molecules in the polymer solution will also influence the release profile of the drug (Kim *et al.*, 2007, Zeng *et al.*, 2003).

Subsequently, a blending technique was employed to modify the properties of our nanofiber scaffold and this was achieved by blending PEO and PDLLA in various ratios before electrospinning. Blending polymers has previously been shown to modify properties of electrospun fibers (Kim *et al.,* 2007, Maretschek *et al.,* 2008, Nijenhuis *et al.,* 1996). A PEO 50: PDLLA 50 blend showed lower levels of structural degradation and reduced weight loss. It also showed a lower release profile than the other blends, but was still able to release enough plantaricin 423 and bacteriocin ST4SA to inhibit the growth of *E. faecium* HKLHS and *Listeria monocytogenes* EGD-e, respectively. The PEO 50:PDLLA 50 nanofiber scaffold was thus identified as the best candidate for further analysis as potential wound dressing.

Nisin A was successfully co-electrospun into PEO 50:PDLLA 50 nanofibers to generate an antimicrobial wound dressing, which was able to inhibit the growth of *Staphylococcus aureus* on agar plates for an extended period of time. Nisin A-containing nanofiber wound dressings were able to significantly reduce *S. aureus* bioluminescence, as well as viable cells of *S. aureus* in infected full-thickness excisional wounds in mice. *S. aureus* Xen 36 cell numbers was 4.3 x 10^2 cfu/wound on day 7, when treated with nisin A-containing nanofiber wound dressings, whereas wounds treated with control nanofiber wound dressings had 2.2 x 10^7 cfu/wound. A 5 $\log_{10}$ reduction was thus obtained in the nisin A-containing nanofiber wound dressings treated groups, as compared to the control group. Increasing reports on *in vivo* antimicrobial efficacy of bacteriocins, especially lantibiotics, have emerged over the last few years (Bower *et al.*, 2002, Brand *et al.*, 2010, de Kwaadsteniet *et al.*, 2009, 2010, Piper *et al.*, 2012, van Staden *et al.*, 2012). The use of bacterocins in skin infections has also been proposed, although very few *in vivo* studies have been reported (Dawson *et al.*, 2009, Valenta *et al.*, 1996, Walsh *et al.*, 2003). De Kwaadsteniet *et al.* (2010), reported on the inability of nisin F to treat subcutaneous *S. aureus* skin infections in mice. A synthetic peptide, A3-APO, was able to reduce viable cells of *Acinetobacter baumannii* and MRSA in burn wound skin infection models in mice (Ostorhazi *et al.*, 2010, 2011). Other alternatives to antibiotics that have been investigated as treatment for skin infections include chitosan acetate bandages, natural antimicrobial agents (honey, aloe vera gel), *Klebsiella* bacteriophage Kpn 5 suspended in hydroxy propyl methyl cellulose hydrogels (E464, or HPMC), nanoemulsions and nitric oxide nanoparticles (Dai *et al.*, 2009, Hemmila *et al.*,2010, Kumari *et al.*, 2010, Martinez *et al.*, 2009). As far as we could determine this is the first *in vivo* study evaluating the efficacy of nisin-containing nanofibers as antimicrobial wound dressing to treat bacterial infections in full-thickness excisional wounds.

Nisin A-containing nanofiber wound dressings, as well as control wound dressings, improved wound healing of uninfected full-thickness excisional wounds in mice. Wounds covered with Nisin A-containing nanofiber wound dressings showed signs of re-epithelialization, keratinization and formation of connective tissue. No neutrophils were observed in this group. However, neutrophils could be observed in all samples of control groups (covered with gauze) and one sample that received control wound dressings as treatment. Nanofibers have been extensively investigated as they have shown potential in tissue engineering and as wound dressings, where they promoted wound healing *in vitro* and *in vivo* (Chen *et al.*, 2008, Choi *et al.*, 2008, Dubský *et al.*, 2012, Sundaramurthi *et al.*, 2012, Tchemtchoua *et al.*, 2011). It has also been shown that plantaricin A increased cell

proliferation, enhanced migration and influenced gene expression of genes in human keratinocytes (Pinto *et al.,* 2011). More recently, nisin Z was shown to have immunomodulatory activities and modulated the host immune response (Kindrachuk *et al.,* 2012). The exact role of nisin, or other bacteriocins, in *in vivo* wound healing warrants further investigation to elucidate the mechanisms involved.

Nisin F was shown to be unable to treat *S. aureus*-induced burn wound infections in mice, when released from PDLLA 70:poly(vinyl alcohol) (PVA) 30 nanofibers, as well as in solution. Nisin A-containing PEO 50:PDLLA 50 nanofiber wound dressings were only able to treat *S. aureus*-induced burn wound infections in mice when the wounds were debrided before infection. This indicates that the necrotic tissue forms a barrier, preventing the bacteriocins from reaching bacterial cells and thus negatively affects treatment. Debridement is performed in severely infected wounds or when wound healing is compromised, to expose healthy tissue. Debridement has been used in animal models when studying skin infections, especially those generated by deep dermal burns (Hemmila *et al.,* 2010, Kiyozumi *et al.,* 2007, Ribeiro *et al.,* 2009).

In conclusion, nanofibers were shown to be a suitable drug delivery system for living probiotic LAB as well as for bacteriocins. Bacteriocins were successfully co-electrospun into nanofibers to generate an antimicrobial wound dressing. The bacteriocins retained their activity after electrospinning and were able to inhibit the growth of a sensitive strain once released from the nanofibers. Blending was shown to be an effective way to modify the release of bacteriocins from nanofibers and improved the overall properties of the wound dressings. Nisin-containing nanofiber wound dressings were able to treat *S. aureus*-induced full-thickness excisional skin infections in mice and improved wound healing of uninfected wounds. *S. aureus*-induced burn wound infections were more difficult to treat and debridement was necessary. Bacteriocin-eluting nanofiber scaffolds can thus serve as potential wound dressing to treat infections, as well as to improve healing of skin wounds. This work can be expanded with a comparative study, to compare the efficacy of the nisin-eluting nanofiber wound dressings generated in this study with commercially available wound dressings. Future research should also include the combination of bacteriocins with growth factors, anti-inflammatory agents as well as other antimicrobials to generate an optimal wound dressing which can treat infections and reduce time needed for wounds to heal.

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ADDENDUM

Release of Plantaricin 423 from Electrospun Poly(D,L-lactide) Nanofibers

Introduction

Electospinning is a versatile technique to produce fibers with very small diameters (Heunis *et al.*, 2010, Kim *et al.*, 2007, Maretschek *et al.*, 2008, Zeng *et al.*, 2003). Electrospun nanofibers can potentially serve as drug delivery systems for bacteriocins from lactic acid bacteria, with potential food and medical applications. Electrospinning of plantaricin 423 into poly(ethylene oxide) nanofibers (PEO) generated an antimicrobial nanofiber scaffold (Heunis *et al.*, 2010). The PEO nanofibers are, however, highly soluble in water and would thus not be suitable as drug delivery system on its own. Here we investigate a more hydrophobic polymer, poly(D,L-lactide) as drug delivery system for plantaricin 423.

Materials and methods

Materials

Poly (D,L-lactide) (PDLLA, 75 000-120 000 Da) was purchased from Sigma-Aldrich (St. Louis, MO, USA). A Micro-bicinchoninic acid (BCA) assay kit was purchased from Pierce (Rockford, IL) for protein concentration determination. All other chemicals used were of analytical grade.

Production and isolation of plantaricin 423

Lactobacillus plantarum 423 was cultured in MRS broth (Biolab Diagnostics, Midrand, South Africa) at 37 °C for 24 h. Cells were harvested (8000g, 10 min, 4 °C), the pH of the cell-free supernatant adjusted between pH 6.5-7.0 with 10M NaOH and then heated at 80 °C for 10 min. Plantaricin 423 was precipitated from the cell-free supernatant with 70% saturated ammonium sulfate (Sambrook *et al.*, 1989) and desalted against distilled water by using a 1000 Da cutoff dialysis membrane (Spectrum Inc., CA, USA). The sample was freeze-dried and stored at -20 ^{o}C.

Electrospinning of nanofibers

Freeze-dried plantaricin 423 (20 mg/ml) was dissolved in *N*,*N*-dimethylformamide (DMF) and centrifuged (6000g, 1 min). The supernatant was used to dissolve 43% (v/v) PDLLA and the solution was electrospun using a setup previously described (Heunis *et al.*, 2010). During electrospinning a constant electric field of +10 kV was applied to the polymer solution and -5

kV to the collector. The distance between collector and polymer solution was 15 cm. The relative humidity was kept constant at 50-55%.

SEM imaging of electrospun fibers

Imaging of the nanofibers was accomplished using a Leo® 1430VP Scanning Electron Microscope (SEM). Prior to imaging the samples were coated with a thin layer of gold in order to make the sample surface conductive. The fiber diameter was determined by using the SEM Image Studio Software (version 10.1).

Release of plantaricin 423 form PDLLA nanofibers

Release studies were carried out in phosphate buffered saline (PBS buffer), pH 7.4. Nanofibers were collected and 25 mg were placed in sterile glass bottles containing 2 ml PBS buffer pH 7.4. The glass bottles, containing nanofibers, were incubated at 37 °C with constant agitation at 30 rpm. At pre-determined time intervals 2 ml PBS buffer pH 7.4 was removed from the glass bottles and replaced with fresh buffer. The protein concentration in the samples was determined by using the BCA protein assay kit by measuring the absorbance at 562 nm.

Degradations studies

Degradation studies were carried out by placing 25 mg of nanofibers in 2 ml sterile PBS buffer pH 7.4 in glass bottles. The glass bottles, containing nanofibers, were incubated at 37 °C with constant agitation at 30 rpm. At pre-determined time intervals 2 ml PBS buffer pH 7.4 was removed from the glass bottles. Nanofibers were freeze dried and weight loss in the sample was measured. Samples were subsequently visualized by SEM to investigate morphological changes.

Labeling of plantaricin 423

Plantaricin 423 was labeled with Rhodamine B isothiocyanate (RBITC). For labeling, 20 mg/ml of RBITC was added to a plantaricin 423 solution in DMF, which was left to react for 48 h at 4 °C with constant stirring in the dark. The reaction product was dialyzed for three weeks against distilled water by using a 1000 Da cut off dialysis membrane (Spectrum Inc., CA, USA) to remove unbound RBITC. The sample was then freeze-dried and stored at -20 °C. Labeled plantaricin 423 was electrospun into nanofibers as previously described and visualized with fluorescent microscopy. Nanofibers were excited at 572 nm by using a Xenon-Arc burner light source (Olympus Biosystems GMBH). Fluorescent images were captured with an Olympus Cell^R system attached to an IX-81 inverted fluorescent

microscope. An Olympus Plan Apo N 60×/1.4 Oil objective was used. Images were taken with an F-view-II cooled CCD camera (Soft Imaging Systems) and the background subtracted using the Cell^R imaging software.

Results

The nanofibers had a mean fiber diameter of 182 ± 25 nm, with most of the fibers in the 100-250 nm range (Fig. 1A, B). Release studies from the PDLLA nanofibers revealed that there was an initial burst release that released ±18.0% of the total amount of plantaricin 423 within the first 2 h (Fig. 2 A, B). A release of bacteriocin was seen for up to 6 h, where after the release was negligible. The PDLLA nanofibers appeared to swell as the incubation time increased (Fig. 3 A-D). No significant morphological changes in the incubated PDLLA nanofibers were seen and the mesh retained its fibrous structure for 7 days of incubation. PDLLA nanofibers showed a slight decrease in weight during the first few hours of incubation, where after no significant decrease in weight was seen (Fig. 4 A, B). Up to 92% of the original weight remained in the samples after 7 days of incubation. Fluorescent images of RBITC-labeled plantaricin 423 showed that the bacteriocin was evenly distribution in the PDLLA nanofibers (Fig. 5).

Discussion

Electospinning of a 43% (w/v) solution of PDLLA, containing 20 mg/ml plantaricin 423, in DMF resulted in fibers in the nanometer range. Fiber diameters ranged from 65 nm to 375 nm, with most of the fibers in the 150-200 nm range. The fast release of plantaricin 423 in the first 2 h could be ascribed to the fact some of the bacteriocin could be close to the surface of the fiber or that the bacteriocin was not completely entrapped within the fiber structure. These bacteriocins could then easily, by means of diffusion, be released into the buffer medium. The low percentage of bacteriocin release (27.5%) could also be ascribed to the slow degradation of PDLLA. This is supported by the fact that bacteriocin release was only seen up to 6 h and could thus not be as a result of nanofiber degradation, as little or no degradation was seen with SEM images and low weight loss was recorded. Other studies have also revealed that poly(lactic acid) on its own, is not very suitable for the controlled release of molecules because of its hydrophobicity. (Maretschek *et al.,* 2008, Zeng *et al.,* 2003). The PDLLA fibers appeared to swell as the incubation time increased (Fig. 5 A-D). No significant morphological changes in the incubated PDLLA nanofibers were seen and the mesh retained its fibrous structure for 7 days of incubation. The swelling could also have helped to facilitate the initial

release of bacteriocin so that bacteriocins, which had not been encapsulated or close to the surface of the fibers, could be released.

PDLLA nanofibers showed a slight decrease in weight during the first few hours of incubation, where after no significant decrease in weight was seen (Fig. 7 A, B). This could also explain the initial release of bacteriocin from this mesh during the first few hours of incubation. After 6 h no significant release of bacteriocin from this scaffold, or weight loss was recorded.

There are different ways to change the hydrophobicity of fibers. One method is to use emulsion electrospinning, where an aqueous solution containing a hydrophilic polymer is emulsified into a polymer solution in an organic phase. This emulsion can then be electrospun to form core-shell structured fibers. The higher the concentration of hydrophilic polymer in the aqueous solution, the lower the hydrophobicity will be and this will lead to a higher total amount of released molecule from the fibers. Cytochrome C has been encapsulated in nanofibers by emulsion electrospinning. (Maretschek *et al.,* 2008) This was done by emulsifying an aqueous solution of cytochrome C in a chloroform solution containing PLLA. A low level of cytochrome C was released. Controlled release was obtained when PLLA was blended with poly(L-lysine) (PLL) and poly(ethylene imine) (PEI), hydrophilic polymers. A blend containing 50% PLL released most of the protein (75%) with a high initial burst release. Another method is to use co-axial electrospinning. During co-axial electrospinning two polymers in different solvents, where the one can be an organic solvent and the other one an aqueous solution, are electrospun with a specialized needle system to create core shell structured nanofibers (Jiang *et al.,* 2005). Fibers electrospun with the co-axial setup are similar in structure to the emulsion spun fibers. These methods however have drawbacks, i.e. the emulsification step to prepare stable emulsions needed before emulsion electrospinning, as well as the specialized needle systems needed for co-axial electrospinning.

Blending polymers directly in a suitable solvent before electrospinning, is a much easier and less time consuming way to change the properties of the fibers formed by electrospinning. If phase separation occurs during fiber formation, it is possible that hydrophilic and hydrophobic domains will form in the fibers. The hydrophilic domains will dissolve faster than the hydrophobic domains in the fiber structure, generating pores that will help facilitate the release of the encapsulated molecule (Kim *et al.,* 2007). Blending can easily be used to change the hydrophobicity, thermal stability and mechanical properties of the fibers (Nijenhuis *et al.,* 1996). This method was chosen to improve the hydrophobicity of the

electrospun PDLLA fibers and to help facilitate the release of the bacteriocin from the electrospun nanofibers, as described in chapter 4.

Plantaricin 423 was successfully electrospun into PDLLA nanofibers. Release of plantaricin 423 was seen, however only for up to a few hours, after which no significant release was seen. PDLLA fibers were able to retain their fibrous structure and original weight over a 7-day period. PDLLA nanofibers were shown to be unsuitable as delivery system for bacteriocins. Blending polymers with PDLLA could provide for a better delivery system for bacteriocins.

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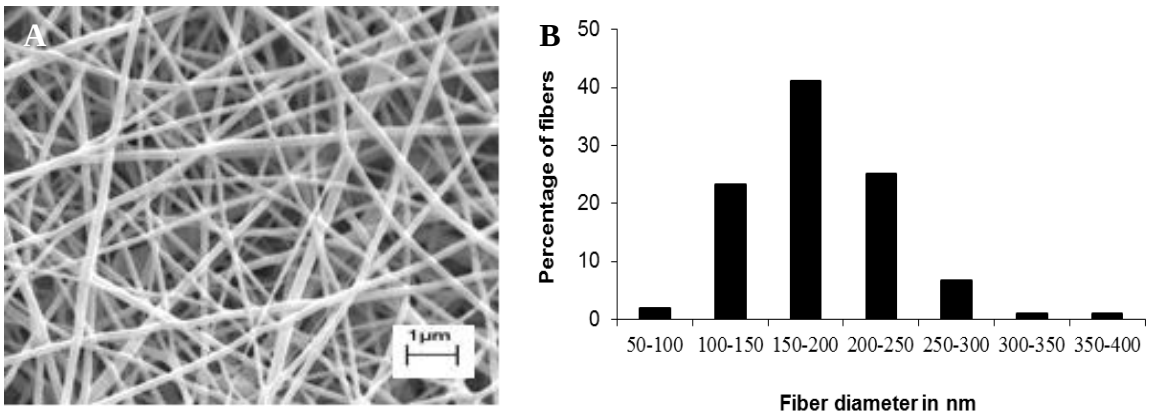

Fig. 1 SEM image of electrospun PDLLA nanofibers containing plantaricin 423 (**A**) and fiber diameter distribution of the electrospun nanofibers (**B**).

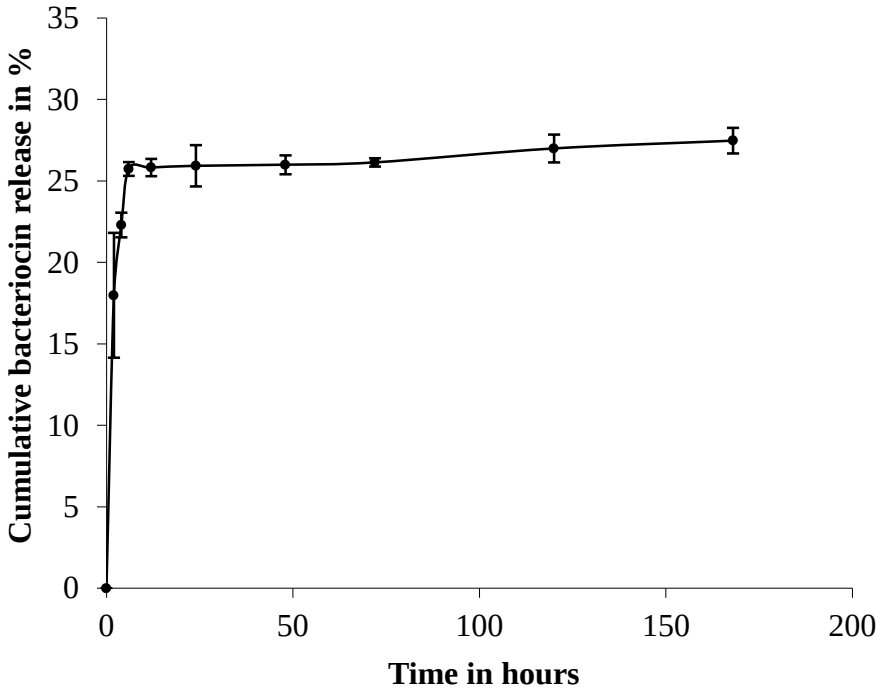

Fig. 2 Cumulative release of plantaricin 423 from electrospun PDLLAnanofibers.

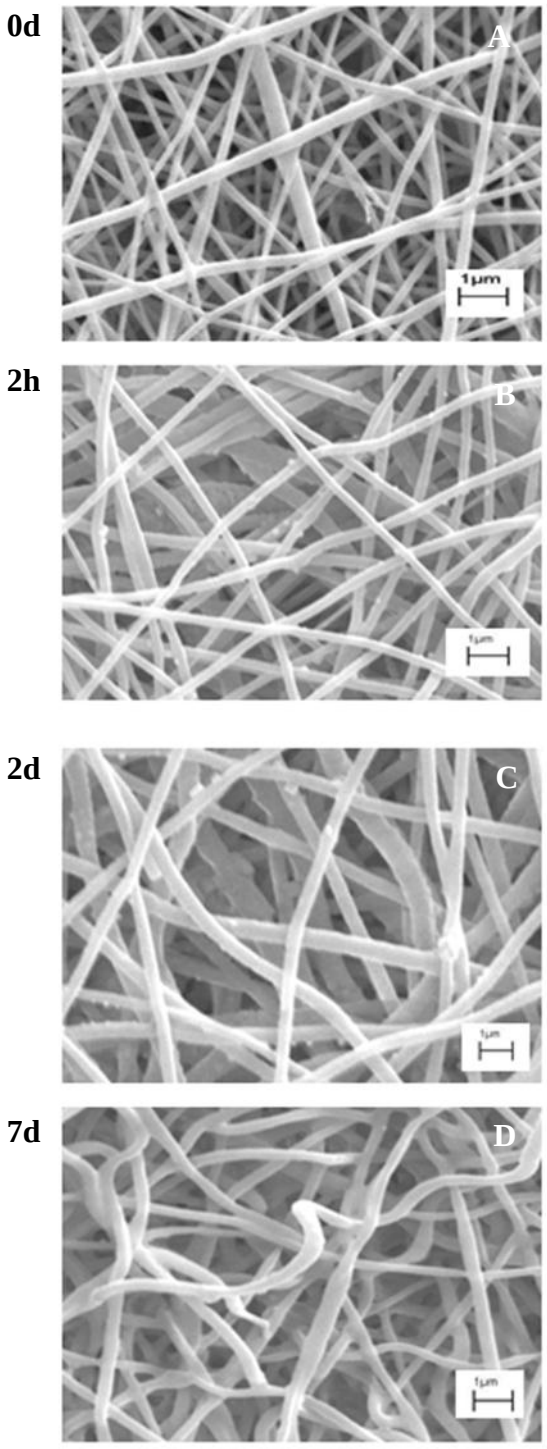

Fig. 3 SEM images of PDLLA incubated in PBS buffer pH 7.4 for different time intervals. No significant morphological changes are seen, except that the nanofiber appears more swollen as the incubation time increased.

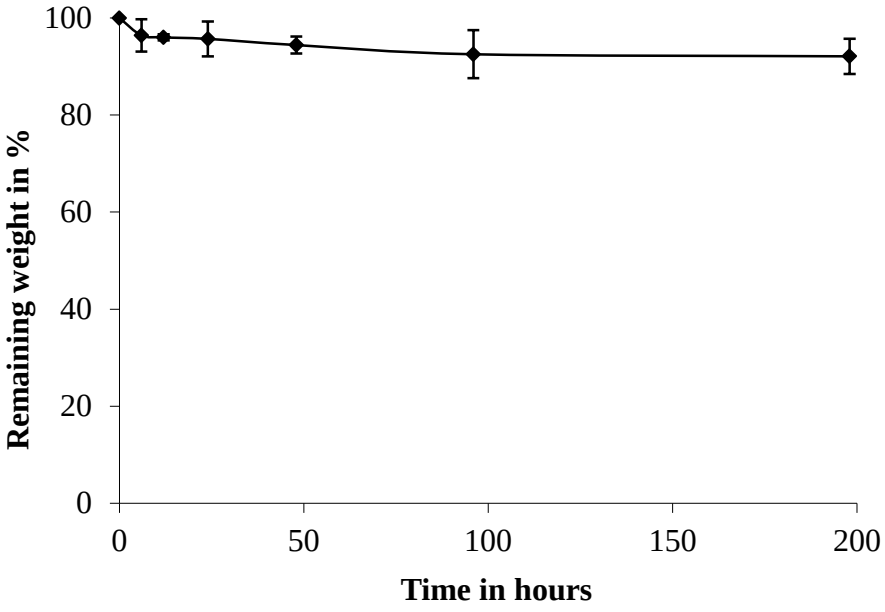

Fig. 4 Percentage original weight of PDLLA nanofibers remaining after incubation at 37 °C for 7 days.

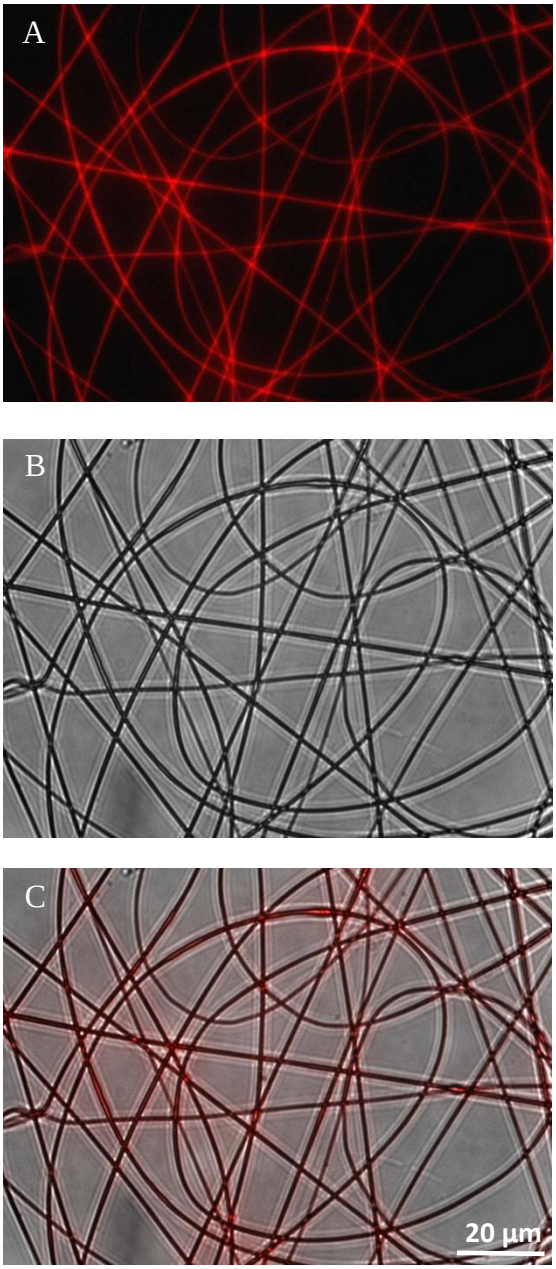

Fig. 5 Images of RBITC-labeled plantaricin 423 electrospun into PDLLA nanofibers. (**A**) Flourescent image of RBITC-labeled plantaricin 423, (**B**) optical image of PDLLA nanofibes and (**C**) overlay of optical and fluorescent images.