Developing bone cement implants impregnated with bacteriocins for prevention of infections

by
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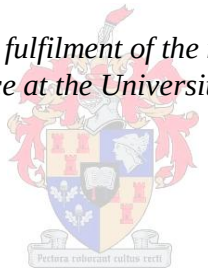

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Summary

Infection is one of the major causes of increased morbidity and the escalating costs associated with orthopedic surgery. The areas that are infected are often difficult to reach and thus difficult to treat. In some surgeries antibiotic-loaded bone cements are used to control infection. Polymethylmethacrylate (PMMA) and calcium phosphate-based bone cements (CPC) are usually used as bone fillers. CPC are bioresorbable and biocompatible (unlike PMMA cements), but can only be used in non- or low-load bearing areas and are thus more applicable in cranio-and maxilla-facial surgeries. Several *in vitro* and *in vivo* trials have been conducted on the incorporation of antibiotics and other therapeutic agents into CPC and the release of these agents. As with any solid matrix, release is defined by specific parameters, i.e. matrix porosity, solubility of the drug and interaction of the drug with the cement.

The increase in antibiotic-resistant pathogens, mainly as a result of overuse of antibiotics, has a major impact on the choice of antibiotics that are used in the treatment of bacterial infections. The search for alternative antimicrobial compounds that are active against resistant pathogens, is thus of utmost importance.

Antimicrobial peptides (bacteriocins) produced by lactic acid bacteria may pose a possible alternative to antibiotics. Some of these peptides are active against antibiotic-resistant pathogens. Bacteriocins are small cationic, hydrophobic, or amphiphilic peptides active against a narrow range of target organisms. Most of these peptides are active in the nanomolar range. It may then be advantageous to incorporate bacteriocins into CPC to evaluate if they may be used as an alternative to antibiotics.

The aim of the project was to evaluate if bacteriocins could be successfully incorporated into self seting brushite bone cement and remain effective *in vivo* without altering basic cement characteristics. Incorporation of bacteriocins into CPC is a novel concept. The low setting temperature and pH of CPC renders it the ideal matrix for incorporation of antimicrobial peptides. In this study, peptide ST4SA, a class IIa broad-spectrum bacteriocin, has been incorporated into brushite bone cement and characterized *in vitro.* Incorporation of the peptide did not have a significant effect on the crystal entanglement or setting reaction of the cement. Peptide ST4SA was rapidly released and inhibited the growth of the target strain effectively. In another experiment, peptide ST4SA was suspended in poly (lactide-*co*-glycolide) and electrosprayed to form micro particles that were entrapped in brushite cement. Association of the peptide with microparticles resulted in a delayed release from the cement, followed by a constant release.

Nisin F, a class Ia bacteriocin was also incorporated into brushite cement and its activity studied *in vitro* and *in vivo*. Similar results were observed *in vitro* as recorded with peptide ST4SA incorporated into brushite cement. Small cylinders of brushite cement loaded with nisin F were implanted into subcutaneous pockets in mice and each pocket infected with a bioluminescent strain of *Staphylococcus aureus* (Xen 36). Nisin F in the bone cement prevented the growth of *S. aureus* in the wound and controlled infection.

With this study we have shown that antimicrobial peptides that differ in structure (classes I and II) could be incorporated into bone cement and control the growth of *S. aureus in vivo* and *in vitro*. The mode of action of these peptides differs from antibiotics in that they form a permanent pore in the cell membrane of the target organism. This minimizes the chance of a strain becoming resistant to the peptide. Incorporation of antimicrobial peptides into bone cement may be a possible alternative to antibiotics in the control of bacterial infections associated with implants.

Opsomming

Infeksie is een van die grootste bydraende faktore tot sterftes en verhoogde kostes in ortopediese chirurgie. Geinfekteerde areas is dikwels moeilik bereikbaar en dus ook moeilik om te behandel. In sommige operasies word antibiotika-gelaaide beensement gebruik om infeksie te beheer. Polymetielmetakrilaat (PMMS) en kalsium fosfaat gebaseerde beensement (KFS) word gebruik as been vullers. KFS is biover enigbaar en bio-absorberend (in teenstelling met PMMS), maar kan slegs in geen- of liggewig-draende areas gebruik word en is dus van groter toepassing in skedel-, kaak- gesig- en mondchirurgie. Verskeie *in vitro* en *in vivo* toetse is al gedoen op die inkorporering van antibiotika en ander terapeutiese middels in KFS en die vrystelling daarvan uit die matriks. Soos met enige soliede matriks is vrylating van die geïnkorporeerde bestanddeel afhanklik van sekere parameters, onder andere porositeit, oplosbaarheid van die middel, en die interaksie van die middel met beensement.

Die toename in antibiotika-weerstandbiedende patogene plaas geweldige druk op die keuse van antibiotika wat gebruik word in die beheer van bakteriese infeksie. Die soeke na alternatiewe antimikrobiese middels aktief teen bestande patogene is dus van kardinale belang.

Antimikrobiese peptiede (bakteriosiene) gepproduseer deur melksuur bakterieë mag dalk ’n alternatief tot antibiotika wees. Sommige van hierdie peptiede is aktief teen verskeie weerstandbiedende patogene. Bakteriosiene is kationiese, hidrofobiese of amfifiliese peptiede wat naverwante bakterieë inhibeer of doodmaak. Die meeste van hierdie peptiede is aktief op nanoskaal vlak. Dit mag dalk dus voordelig wees om bakteriosiene in been sement te evalueer as moontlike alternatiewe tot antibiotika.

Die doel van die proejek was om te evaleer of bakteriosiene suksesfol in “brushite” sement geinkorporeer kan word en steeds effektief *in vivo* bly sonder om die basiese einskappe van die sement te verander. Inkorporasie van bakteriosiene in KFS is ’n nuwe konsep. Die lae stollingstemperatuur en pH van KFS maak dit moontlik om bakteriosiene daarin te inkorporeer. In hierdie studie is peptied ST4SA, ’n klas IIa wye-spektrum bakteriosien, in “brushite” sement geïnkorporeer en *in vitro* bestudeer. Die toevoeging van die peptied het nie ’n beduidende effek op die stolreaksie of kristal verstrikking van die sement gehad nie. Peptied ST4SA is effektief vrygelaat en het die groei van die teikenorganisme suksesvol onderdruk. In ‘n ander eksperiment is peptied ST4SA in poli (D,L-laktied-ko-glikolied) gesuspendeer en met behulp van elektrosproeiing tot mikropartikels omvorm en is in “brushite” sement geïnkorporeer. Assosiasie van die peptied met mikropartikels het die inisiële vrylating van die peptied vertraag, gevolg deur ‘n konstante vrylating.

Nisien F, ’n klas Ia lantibiotikum, is ook in “brushite” sement geïnkorporeer en die aktiwiteit daarvan *in vitro* en *in vivo* bestudeer. Die *in vitro* eienskappe is soortgelyk aan die eienskappe wat vir peptied ST4SA-gelaaide sement waargeneem is. Klein stafies “brushite” sement, waarin nisien F geïnkoproreer is, is in onderhuidse sakkies in muise geplaas en die area met ’n bio-liggewende bakterie (*S. aureus* Xen 36) geïnfekteer. Nisien F in die beensement het die groei van *S. aureus* in die wond onderdruk en infeksie beheer.

Met hierdie studie het ons bewys dat bakteriosiene wat struktureel van mekaar verskil (klasse I en II) in beensement geïnkorporeer kan word en die groei van *S. aureus in vitro* en *in vivo* kon beheer. Die wyse waarop hierdie peptiede die groei van sensitiewe organismes inhibeer verskil van dié van antibiotika deurdat dit porieë in die selmembraan vorm. Die moontlikheid dat organismes weerstandbiedend raak tot die peptied is dus heelwat skraler. Die insluit van antimikrobiese peptiede in beensement mag dalk ’n alternatief tot antibiotika wees in die voorkoming van bakteriële infeksie geassosieer met ortopediese chirurgie.

This thesis is dedicated to friend and mentor Peter Marais

Biographical sketch

Anton Du Preez van Staden was born in Windhoek, Namibia on the 7th of March, 1987. He matriculated at Windhoek High School, Namibia, in 2005. In 2006 he enrolled as B.Sc. student in Molecular Biology and Biotechnology degree at the University of Stellenbosch and obtained the degree in 2008. In 2009 he obtained his B.Sc (Hons) in Microbiology, also at the University of Stellenbosch. In 2010 he enrolled as M.Sc. student in Microbiology.

Preface

This thesis is presented as a compilation of manuscripts. Each chapter is introduced separately and is written according to the style of the respective journal. One article has been published from the current research and two manuscripts have been submitted for publication. The addendums contain additional information not presented in the main chapters. All other sections and chapters are written according to the instructions for Journal of Applied Microbiology.

Chapter 2.1, "Calcium orthophosphate-based bone cements (CPCs): Applications, antibiotic release and alternatives to antibiotics" has been submitted to Journal of Biomaterials and Biomechanics.

Chapter 3, "Release *of Enterococcus mundtii* bacteriocin ST4SA from self-setting brushite bone cement" has been published in Probiotics and Antimicrobial Proteins, vol. 3, pp. 119-124 (2011).

Chapter 5, "Nisin F-loaded brushite bone cement prevented the growth of *Staphylococcus aureus in vivo"* has been previsionally accepted for publication in Journal of Applied Microbiology (November 2011).

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Contents

Chapter 1

Introduction*

*This chapter is written according to the instructions of Journal of Applied Microbiology.

Introduction

Infections associated with orthopedic surgery are a major cause of morbidity and cost (Cosgrove and Carmeli 2003; Zimmerli 2006). Bone as a solid matrix does not expand and contract as readily as other tissue, resulting in antibiotics being less effective when taken intravenously or orally (Frommelt 2006). Antibiotic-loaded bone cements have been used as a prophylactic measure to prevent infection (Jiranek *et al.* 2006). Polymethylmethacrylate (PMMA) cement is the standard mixture used in most orthopedic surgeries. The polymer is non-biodegradable, i.e. it cannot be resorbed by the body under natural conditions (van Landuyt *et al.* 1999; Van de Belt *et al.* 2001). Calcium phosphate-based bone cements (CPC) have also been extensively investigated, due to their bioresorbable and biocompatible properties. These cements have lower mechanical strength and are usually only used in non- or low-load bearing areas, and are thus more applicable in cranio- and maxillo-facial surgeries (Ambard and Mueninghoff 2006). CPC have shown promise clinically and antibiotic-loaded CPC have been investigated extensively *in vitro* and *in vivo* (Miyamoto *et al.* 1995; Ohura *et al.* 1996; Kurashina *et al.* 1997; Otsuka *et al.* 1997; Petruzzelli and Stankiewicz 2002; Kasperk *et al.* 2005; Ruhe *et al.* 2005; Schnieders *et al.* 2006; Ginebra *et al.* 2006; Alkhraisat *et al.* 2010; Fullana *et al.* 2010; Neovius and Engstrand 2010). The abuse of antibiotics over the past few decades has resulted in an increase in antibiotic-resistant pathogens (Neu 1992). Infection with an antibiotic-resistant pathogen is one of the major concerns in hospitals across the world. The low yield in novel and effective antibiotic discovery has limited the last defence antibiotics to only a few (Fischbach and Walsh 2009). The search for alternatives to antibiotics is therefore important if antibiotic-resistant pathogens are to be kept under control (Joerger 2003).

Bacteriocins produced by Gram-positive bacteria may provide a possible alternative to antibiotics. Bacteriocins have been around as long as antibiotics, but have not yet been established as a viable alternative (Nes 2011). Bacteriocins are antimicrobial peptides with activity against a narrow range of bacteria, unlike most antibiotics that have a broad range of activity. The advantage of bacteriocins over antibiotics is that they are active in nanomolar concentrations, and usually only against specific species (Morgan *et al.* 2005; Nissen-Meyer *et al.* 2009). Bacteriocins from lactic acid bacteria (LAB) have received the most attention, mainly due to importance of these bacteria in food fermentations. Bacteriocins are divided into three main groups, i.e. the post-transcriptionally modified heat-stable lantibiotics (class I) and the non-modified heat-stable bacteriocins (class II). The main classes are further subdivided into subclasses. The third group (formerly class III) consists of heat-liable bacteriolysins (Rea *et al.* 2011). Bacteriocins have shown antimicrobial activity against antibiotic-resistant pathogens such

as methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci (Piper *et al.* 2010). The spectrum of antiomicrobial activity of bacteriocins may be broadened when used in combination with other therapeutic agents (Ghiselli *et al.* 2004). The possible use of bacteriocins in association with prosthetic implants and other medical devices have been investigated *in vitro* and *in vivo* (Bower *et al.* 2002; Ghiselli *et al.* 2004; Van Staden *et al.* 2011). These studies revealed promising results and are paving the road to the development of alternative antimicrobial therapy.

This study investigated the possibility to incorporate peptide ST4SA, a class II bacteriocin produced by *Enterococcus mundtii* ST4SA, and nisin F, a class I bacteriocin produced by *Lactococcus lactis* subsp. *lactis* F10 into CPC. The structural, chemical and antimicrobial properties of CPC, impregnated with peptide ST4SA, nisin F and PLGA-associated peptide ST4SA, have been studied. The *in vivo* activity of nisin F-loaded brushite cement was studied in mice that have been infected with *S. aureus* Xen 36.

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Chapter 2

Literature Review

Chapter 2.1: Calcium Orthophosphate-Based Bone Cements (CPCs): Applications, Antibiotic Release and Alternatives to Antibiotics*

Chapter 2.2: Bacteriocins and Micro or Nanoparticle Polymeric Protein Delivery Systems**

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Chapter 2.1

Calcium Orthophosphate-Based Bone Cements (CPCs): Applications, Antibiotic Release and Alternatives to Antibiotics

Running head: Calcium Orthophosphate-based Bone Cements Reviewed

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Abstract: *Calcium orthophosphate bone cements (CPCs) are widely used in orthopedic surgery. Implants are highly susceptible to infection and often lead to the formation of microbial biofilms. Antibiotics are often incorporated into bone cement. The increase in number of microorganisms acquiring or developing resistance to antibiotics, such as methicillin resistant* Staphylococcus aureus *(MRSA) and vancomycin resistant enterococci (VRE), is a major concern. Bacteriocins (antimicrobial peptides) offer an alternative to antibiotics. Their mode of activity involves permanent destabilization of the plasma membrane of target cells, rendering little chance of developing resistance. A number of broad-spectrum bacteriocins produced by lactic acid bacteria and* Bacillus *spp. have recently been described. In this review the major characteristics of calcium phosphate bone cements, prosthetic joint associated infections and treatment of these infections is discussed. The role of antimicrobial agents in CPCs is discussed and the possibility of incorporating bacteriocins in prosthetic devices is investigated.*

KEY WORDS: Calcium phosphate bone cement; prosthetic joint infection; nano/microparticles; bacteriocins

INTRODUCTION

Calcium orthophosphate based cements (CPCs) are widely used in orthopedic and maxillofacial surgery and numerous papers have been published on the *in vitro/in vivo* properties of different CPC formulations (Table I) [1-11]. CPCs have excellent osteoconductive properties, i.e. low interference with bone function, are replaced by new bone tissue, are easily moulded and set *in situ* [5-9]. CPCs made from brushite and hydroxyapatite has the added advantage of being bioresorbable, which excludes the need for autografts and allografts[9].

Implants and bone replacement materials provide an ideal environment for infection and microbial biofilm formation [12]. Infections may lead to serious complications, often requiring debridement or complete removal of the prosthesis [13-15]. Infection is normally prevented by prophylactic treatment with antibiotics. However, blood flow surrounding areas of implants is usually poor, which results in little success achieved with antibiotics administered intravenously or orally [12]. Non-resorbable polymers such as poly-methylmethacrylate spheres (PMMA), combined with antibiotics have been used with some success. However, PMMA implants have to be removed, thus increasing the chance of contracting secondary infection [9]. Unlike PMMA, CPC implants can be resorbed by osteoclasts and do not need to be removed. A number of papers have been published describing the incorporation and release of antibiotics into CPCs [16-21].

This review discusses the major characteristics of CPCs and problems encountered with orthopedic and maxillofacial infections. The role of antimicrobial agents in CPCs is discussed and the idea of including bacteriocins (antibacterial peptides) in bone cements is investigated.

Characteristics of CPCs

Several compositions of CPCs are used in orthopedic and maxillofacial constructions. CPCs are biocompatible, osteogenic and osteoconductive, rendering them the ideal substitute for bone [4, 21-28]. CPCs are produced by mixing a reactive calcium phosphate with a liquid solvent to produce a rapidly setting paste [21]. The aqueous phase allows for the dissolution of the initial calcium orthophosphates, supplying calcium and orthophosphate ions to the solution. The ions interact and precipitate to form either the end products or precursor phases [29, 30]. The chemical reactions that take place during setting of CPCs depends on their composition, however there are only two chemical types of setting reactions. Reactions may be either acid-base (two-component cements) reactions (interaction) or the transformation of a metastable phase into a more stable phase (one-component cements).

The first chemical type forms due to an acid-base interaction between a relatively neutral/basic calcium orthophosphate and an acidic calcium orthophosphate [29, 30]. In the reaction below (eq. 1) tetracalcium phosphate (basic) reacts with anhydrous dicalcium phosphate (neutral) to produce a slightly basic hydroxyapatite.

$$2Ca_2(PO_4)_2O + 2CaHPO_4 \rightarrow Ca_{10}(PO_4)_6(OH)_2 \quad (1)$$

In the formulation used by Lemaitre and co-workers [31], β-tricalcium phosphate (close to pH 7.0) reacts with acidic monohydrate monocalcium phosphate (eq. 2) to form slightly acidic dicalcium phosphate dihydrate (DCPD), also known as brushite.

$$\beta\text{-}Ca_3(PO_4)_2 + Ca(H_2PO_4)_2 \cdot H_2O + 7\ H_2O \rightarrow 4CaHPO_4 \cdot 2H_2O \quad (2)$$

The second chemical type is the transformation (hydrolysis) of a calcium orthophosphate from a metastable phase into a more stable phase [21, 30]. After mixing, the calcium phosphate (CaP) precipitates and forms crystals with specific mechanical properties [29]. Only one calcium orthophosphate takes part in the reaction, producing a stable calcium:phosphate ionic ratio [30]. A typical example of such a reaction is shown in eq. 3, where α/β-tricalcium phosphate, nanocrystalline TTCP or ACP dissolved in water forms calcium-deficient hydroxyapatite (CDHA) crystals [29, 30]. A single-phase cement powder, consisting of K- and Na-containing CDHA, has been proposed by Tas and Aldinger [32].

$$3\ (\alpha\text{-}/\beta\text{-})Ca_3(PO_4)_2 + H_2O \rightarrow Ca_9(HPO_4)(PO_4)_5(OH) \quad (3)$$

Apatite cements

Apatite cements are usually highly viscous and easily moldable and set at or above physiological pH, depending on the solvent [10, 29]. Water is not a reactant in the setting reaction of most apatite cements and only allows for the dissolution of the initial calcium phosphates [30]. Therefore, the amount of water needed in apatite cements is small. In brushite cements water always takes part in the transformation reaction and is required for DCPD formation. Brushite cements are therefore known as hydraulic cements, whereas apatite cements usually do not carry this label [30, 34]. The setting reaction results in poorly crystalline hydroxyapatite (pHA) or CDHA, with possible traces of non-reacting starting material [20, 23-29]. If carbonates are present in the initial mixture, non-stoichiometric carbonate apatite ($Ca_{8.8}(HPO_4)_{0.7}(PO_4)_{4.5}\ CO_3)_{0.7}\ (OH)_{1.3}$) may form as final product [35-36]. CDHA and carbonate apatite have a low crystallinity, similar to that found

in biological apatite (bones and teeth). CDHA has good *in vivo* resorption characteristics [23, 37] and is ideal to use as bone replacement material.

The setting rate of apatite cements is longer compared to brushite cements and may thus cause complications in operations [10, 29, 38]. To decrease the setting reaction, phosphoric acid, MCPM, soluble orthophosphates or precipitated HA particles may be added [10-11, 20, 27, 37]. These additives reduce the setting time to 10-15 min, which is acceptable in a clinical environment with an ideal setting time; shorter than 15 min [30]. Setting time is also influenced by particle size and volume of the solvent [10, 38].

Mixing with water dissolves the initial calcium orthophosphates and crystals form [23]. Mechanical strength of apatite cements is influenced by particle (powder) size, powder to liquid ratio and type of solvent used [33, 39]. Porosity plays a major role in mechanical properties. A higher powder to liquid ratio decreases porosity, which results in an increase in mechanical strength [10, 29]. However, decreased porosity may result in decreased osteoconductivity. High compressive strength does not, however, mean the cement is capable of withstanding shear forces *in vivo.* Tensile strength is an important parameter in developing a formulation for *in vivo* applications [29, 40]. The compressive- and tensile-strengths of most CPCs are diverse, ranging from 10-100 MPa for compressive strength and 1-10 MPa for tensile strength, depending on the formulation [29]. Apatite cements usually fall in the higher end of this scale, but are used in combination with metal implants or in low-load bearing areas [10-11].

Apatite cements compare well with biological apatite, rendering them biocompatible [26, 30, 41]. The forces linking the crystals are weak, which allow them to easily detach *in vivo* and be ingested by osteoclasts and macrophages. This is followed by replacement of bone by osteoblasts [8, 27, 42]. The biocompatibility and resorbability of apatite cements have been illustrated with *in vivo* experiments [2, 20, 26-27, 41].

Brushite cements

Unlike most apatite cements, brushite cements are hydraulic, i.e. water always participates in the chemical transformations involved in DCPD formation [30]. The setting of brushite cements is based on acid base reaction/interaction (eq. 2) and precipitates to DCPD (mineral; brushite) at pH<6.0 [11, 43], resulting in an acidic paste. As with apatite cements, several formulations are used for brushite cements, including βTCP + MCPM (eq. 2), βTCP + H_3PO_4, nanocrystalline HA + H_3PO_4, and tetracalcium phosphate + MCPM [43-44]. Unlike apatite cements, brushite cements may start out as a liquid but rapidly sets into hard cement [29, 45-46]. Setting times may be as short

as 30-60 seconds, necessitating the use of retardants such as trisodium citrate, citric acid or pyrophosphates. These additives inhibit the formation of DCPD crystals, which may have an influence on mechanical properties [11, 45-46]. Some additives increase the setting time of brushite from 30-60 sec to 5-10 min [11, 47].

Brushite cements are biocompatible and bioresorbable, and due to higher solubility of DCPD compared to CDHA, they are degraded faster *in vivo* compared to apatite cements [48]. The fast degradation results in a loss of mechanical strength, but as the resorbed cement is replaced by bone cells, the mechanical strength of the defected site increases [7, 49-50]. Low mechanical strength (ranging from 10 MPa to 60 MPa) and high degradation of brushite cements make them slightly inferior to apatite cements [51]. The high degradation of brushite may result in the cement being resorbed faster than bone replacement takes place, resulting in the formation of immature bone or gaps between cement and bone [25, 48]. Brushite cements are not only resorbed by osteoclasts, but also by dissolution, resulting in high degradation rates [29]. Addition of a bone anchor, such as βTCP granules, promotes formation of mature bone [7, 29, 52-53]. Incorporation of growth factors into the cement increases tissue response (i.e. bone formation) to brushite cement [25]. The high resorption properties of brushite can however provide an edge over the stable apatite cements with longer resorption characteristics.

Inflammation caused by brushite cements may be due to the release of orthophosphoric acid when DCPD is partially converted to CDHA [54, 55]. This can be prevented by the addition of magnesium ions (*in vitro*), or by the implantation of preset cements [56, 57].

CPCs in orthopedic surgery

The use of CPCs in orthopedic surgery is limited due to their mechanical prosperities. Positive results have been reported with the use of CPCs in the treatment of fractures, with decrease in patient pain and a reduced risk of losing fracture reduction when compared with autogenous bone graft [58-60]. Cement augmentation after hardware removal have positive results, including increase failure strength which reduces the chances of refracture and allows for earlier weight bearing [61]. CPCs can also be used to coat prosthetic implants for improved fixation.

The use of CPCs has also been investigated for use in vertebroplasty and kyphoplasty [30]. Using a canine model, Turner and co-workers showed that CPCs are viable alternatives to PMMA cements in the treatment of large vertebral defects [62]. If the vertebroplasty procedures are done correctly with the appropriated amount of CPC (correct powder to liquid ratio), positive results

are obtained without complications [62-64]. CPCs have been successfully used in kyphoplasty resulting in increased vertebral height, reduced pain and increased patient mobility [65].

Infection

Infections associated with orthopedic surgery are rare [13-14], but difficult to diagnose and treat. Orthopedic implants are highly susceptible to infection and provide a prime spot for biofilm producing bacteria to flourish [12, 66-67]. Improvements in health care have resulted in increased life expectancy, which in turn means the number of patients that require orthopedic implant surgery are on the increase, combined with an increase in the risk of acquiring an infection [68]. Risk of contracting an infection is also associated with nutritional status, history of pre-existing joint disease (e.g. arthritis), diabetes, obesity [69-71], prior infection of native bone, surgical site infection (unrelated to prosthesis) and HIV infection [66, 68, 71]. Hematogenous infection is spurred by bacteraemia, caused by skin, respiratory tract, dental and other systemic infections [72-73].

Microorganisms infect implants during surgery, through hematogeneous seeding caused by unrelated bacteraemia, or direct spreading from neighbouring lesions. Streptococci, *Staphylococcus aureus* and Gram-negative bacilli are usually associated with prosthetic and bone infections [69, 74-75].

CPCs in craniofacial and maxillofacial surgery

The use of CPCs in craniofacial (CF) and maxillofacial (MF) surgeries provides various advantages including the reduced use of autologous grafts and alloplastic implants [30]. The low mechanical stress associated with these areas means low mechanical properties of CPCs do not play such a huge role. The ability to mould the CPC paste *in situ* provides an additional clinical advantage [76]. Several authors have shown high biocompatibility and bioresorbability of CPCs, with little or no occurrence of inflammation [2, 6-7, 26-27, 41, 48, 57]. Implant stability is an important factor in CF and MF surgeries. Membranes and meshes are frequently used and it is important that they do not interfere with the implant. Losee and co-workers [77] showed that a PLA mesh did not interfere with the osteoconductivity, remodelling capacity and biocompatibility of cement. Tamimi and co-workers [78] have also showed that it is possible to obtain stable CPC implantation without reinforcement. Friedmann and co-workers [79] used hydroxyapatite cement for the reconstruction of frontal sinus and the frontofacial skeleton of 38 patients. They reported an 82% overall success rate for the reconstructions and suggested it's superiority over acrylic implants. Other studies have also reported high success rates when using CPCs in CF and MF surgeries [80-81].

Infection

Infection is the most common complication in CF and MF surgery and results in high morbidity [82]. Infection may result due to secondary surgery, resulting in extended hospital stay, additional procedures as well as increase chances of deformity [83-84]. The close proximity of these surgical sites to areas with a high bacterial flora (nasal and oral cavities) may also result in increase contamination [83].

Fialkov and co-workers [83] reported an infection rate of 8% during 349 CF surgeries. This is higher than for other orthopedic surgeries and may be due to contamination with nasal and/or nasal flora. Wong and co-workers [85] reported on infection rates of secondary CF surgeries involving hydroxyapatite cements. Of the 17 patients studied, 10 presented with infection, 9 patients required debridement, followed by delayed reconstruction. Their study shows a high rate of infection with Norian cement and is not the first report of mixed results using this cement [86]. Fearon and co-workers [82] reported a lower infection rate (2.5%) in 567 patients undergoing intracranial surgery, with 85% of infection occurring after secondary operations.

Antibiotic-loaded CPCs

Characteristics such as bio activity, bioresorbability, injectability and rapid *in situ* setting render CPCs attractive drug delivery vectors. Furthermore, CPCs set at low-temperatures which allow the incorporation of heat liable drugs or proteins [29]. Prophylactic antibiotics are usually taken orally or intravenously which limits their access to the effected bone site. Bone is a solid matrix and cannot expand. Thus, inflammation causes a reduction in blood flow, which restricts contact between the pathogen and the antibiotic [12, 87]. CPCs can access inaccessible bone sites and deliver drugs in a high concentration without being toxic to the rest of the body. Localized treatment is advantageous in the treatment of skeletal disorders (osteoporosis and osteoarthritis) and infections, which usually require long and painful treatments. Other drugs or proteins, including anti-cancer and anti-inflammatory agents, can also be incorporated into CPCs [9, 37, 88]. The advantage CPCs has over ceramics is that drugs can be added to any one of the two phases, whereas in ceramics the drugs have to be impregnated [9]. Several factors influence release of antimicrobial agents from CPCs. Parameters such as cement chemistry, drug cement interaction, cement porosity as well as use of polymeric drug delivery systems, may influence drug release.

Several studies have been performed to determine the drug loading capacity of CPCs (Table I). Bohner and co-workers [43] investigated the properties of gentamicin-loaded brushite cement. The antibiotic did not affect the physiochemical properties of CPCs and was released in an active

form. Compared to gentamicin-loaded PMMA beads [47], which are not resorbable, have very slow release rates and have to be surgically removed after a few months, gentamicin loaded brushite cement has a clear advantage. Young and co-workers [18] studied chlorhexidine (non-antibiotic) loaded CPCs. The results obtained were similar to the gentamicin loaded cement. Both cements followed Fickian diffusion, i.e. a high initial burst release, followed by a plateau until most of the drug is released. Both authors concluded that the released drug was suitable for the treatment of initial infection and that the incorporated drugs had no negative effects on the physiochemical properties of the cements. Hofmann and co-workers [38] experimented with the porosity of cement. The authors reported an almost linear drug release over 350 h. However, reducing the porosity of the cement has a negative effect on osteoconductive properties [89].

For CPCs from which antibiotics are rapidly released a polymeric drug delivery system may be incorporated. The use of nanotechnology to control drug release from biodegradable nanofibers and nano- or microparticles has been extensively studied over the past few years [90-94]. The release rate depends on several factors, including the interaction between the drug and polymer [95]. A hydrophobic, e.g. poly (L-lactic acid) (PLLA) or polycaprolactone (PCL) polymer provides a slow release and, in some cases, only degrades over several months. In contrast to this, a hydrophilic polymer such as poly (ethylene oxide) (PEO), releases the antibiotic at a rapid rate [93]. Co-polymers such as poly (lactide-co-glycolic acid) (PLGA) or blends of structurally different polymers (block polymers), have a slower release rate and offer an alternative to treatment over an extended period [93]. It is thus important to select a polymer with the correct release kinetics.

Gentamicin incorporated as microparticles into CPCs is slowly released [19]. Gentamicin incorporated into PLGA resulted in a linear release profile that lasted for 100 days. The minimum inhibitory concentration (MIC) of the antibiotic was released at each of the time points monitored. Bohner and co-workers [43] experimented with a double-delivery system, i.e. microparticles were first released from the CPCs, followed by release of the antibiotic from the microparticles. The authors claim to have achieved a more controlled release of the antibiotic, illustrating the effectiveness of using a double-delivery system. A study by Takechi and co-workers [96] showed the incorporation of flomoxef sodium into an anti washout apatite cement (containing chitosan). They reported a rapid release for the first 24 h followed by a less pronounced release for up to 72h, with a total of 49% release seen in the highest loaded samples. They also showed that it is possible to decrease the initial release as well as increase the amount of antibiotic released with the addition of chitosan to the cement mixture.

Stellmann and co-workers [2] reported a decrease *S. aureus* infection in a rabbit femur model when using an antimicrobial peptide (hLF1-11) loaded cement. They however also showed that using a gentamicin loaded cement resulted in a larger number of sterile femurs. Another *in vivo* study by Hamanishi and co-workers [20] investigated the release of vancomycin from cement when implanted in tibial condyles of rabbits. They found that the concentration of vancomycin in the bone marrow was above the MIC 3 weeks after implantation. High concentrations of the antibiotic did seem to interfere with initial bone ingrowth, resulting in fibrous tissue between cement and new bone.

CPCs can also be used to deliver anti-inflammatory agents, anti-cancer drugs, proteins and growth factors.

Although the incorporation of antibiotic-loaded cement with prosthesis is common practice, only a few *in vivo* studies and clinical trials have been done to evaluate its effectiveness. It cannot be assumed that the same release kinetics observed *in vitro* will be recorded in more complex *in vivo* circumstances.

Alternatives to antibiotics: Bacteriocins

The increase in number of microorganisms acquiring or developing resistance to antibiotics is a major concern [97]. Pulido and co-workers [13] identified 63 patients out of 9245 with prosthetic joint infections. In 91% of the cases the causative organism could be identified, of which 30% were resistant to methicillin. Incorporation of antibiotics in bone cement can also increases the potential for antibiotic resistance, as illustrated by Thornes and co-workers [97]. In this study, a higher percentage (78%) of antibiotic-resistant *Staphylococcus epidermis* has been recorded in gentamicin-loaded cement. Alternatives to antibiotics are therefore needed to reduce the emergence of antibiotic resistant pathogens [98].

Bacteriocins (antimicrobial peptides) produced by lactic acid bacteria are possible alternatives to antibiotics. They are small, ribosomally synthesized cationic, hydrophobic and amphiphilic peptides composed of 20-60 amino acid residues and are divided into three classes [99-100]. Bacteriocins show antimicrobial activity against closely related species and in several cases against antibiotic resistant microorganisms such as MRSA (methicillin resistant *Staphylococcus aureus*) and VRE (vancomycin resistant enterococci) [101-105]. Nisin F, produced by *Lactococcus lactis* F10, inhibits growth of clinical *S. aureus* strains *in vitro* and *in vivo*. An *in vivo* study by Brand and co-workers. [106] showed that Nisin F10 was able to control *S. aureus* Xen 36 infection for at least 15 min. The short activity is most likely due to degradation by proteolytic enzymes.

However, Nisin F has been indicated as the most promising natural nisin variant for clinical applications [(94)]. Mersacidin produced by *Bacillus* sp. HIL Y-85, has also been shown to eradicate MRSA *in vitro* and shows potential for clinical application [(105)].

The main drawback of using bacteriocins is their instability *in vivo* [(106)]. Instability may be overcome by encapsulating the bacteriocins with biocompatible polymers. Salmaso and co-workers [(107)] showed that poly-L-lactide nanoparticles containing nisin had prolonged antimicrobial activity (*in vitro*) compared to nisin not encapsulated. In addition to stability, polymeric delivery systems also provide controlled release [(107)]. Bacteriocins can be used in conjunction with antibiotics to reduce resistance and increase antimicrobial activity. Incorporation of bacteriocins into bone cements, directly or using a polymer based system, can provide a solution to the increase in antibiotic resistance and may thus increase the effectiveness of antibiotics.

Conclusion

Incorporation of growth factors, proteins, and anti-cancer and anti-inflammatory agents in CPCs is now common practice. Incorporation of antibiotics into CPCs proofed effective. However, special care has to be taken to avoid an increase in antibiotic resistance. The use of bacteriocins in combination with, or as an alternative to, antibiotics may be used in the fight against antibiotic resistant pathogens. Polymer nanotechnology shows great potential in controlling the release of drugs from cements and as a method to protect drugs against enzymatic degradation. The inclusion of antimicrobial peptides in nano- and microparticles incorporated into bone cements has to be investigated. This also requires more research on drug incorporation and drug release kinetics.

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TABLE I - EXAMPLES OF CALCIUM PHOSPHATE CEMENT MIXTURES AND INCORPORATED THERAPEUTIC AGENTS (WHERE INDICATED)

Cement formulation	Study	Incorporated	Substance type
TTCP[a] , DCP[b]	*In vivo*	hLF1-11 peptide	Antimicrobial peptide
TTCP, DCPD[c] (apatite cement)	*In vitro/ In vivo*	Vancomycin	Antibiotic
β-TCP[d], MCPM[d] (brushite cement)	*In vitro*	RANKL[h]	Bioactive molecule (protein)
α-TCP[d], DCPA[f], $CaCO_3$, HA	*In vivo*	Rh-BMP-2[i]	Growth factor (protein)
Sr-β-TCP, MCPM	*In vitro*	Doxycycline	Antibiotic
β-TCP, MCPM (brushite cement)	*In vitro*	Gentamicin	Antibiotic
β-TCP, MCPM, chitosan (brushite cement)	*In vivo*	VEGF[j], PDGF[k]	Growth factors (protein)
TTCP, DCPA	*In vitro*	N/A	N/A
Not specified	*In vivo*	Gentamicin	Antibiotic
TTCP, DCPD	*In vitro*	Mercatopurine	Anticancer drug
TTCP, DCPD, HA[g]	*In vitro/ In vivo*	Indomethacin,	Anti-inflammatory agent
TTCP, DCPA	*In vitro*	N/A	N/A
β-TCP, MCPM (brushite cement)	*In vitro*	Vancomycin & ciprofloxacin	Antibiotics
β-TCP, MCPM (brushite cement)	*In vitro*	Chlorohexidine	Antimicrobial agent
TTCP, DCPA[c], Chitosan	*In vitro*	Flomoxef sodium	Antibiotic
α-TCP, TTCP, DCPD, PLLA[l]	*In vivo*	Albekacin sulfate	Antibiotic

[a] Tetratricalcium phosphate; [b] Dicalcium phosphate; [c] Dicalcium phosphate anhydrous/dehydrate; [d] α/β-tricalcium phosphate; [e] Monocalcium phosphate monohydrate; [f] Dicalcium phosphate anhydrous; [g] Hydroxyapatite; [h] Receptor activator for nuclear factor κ B ligand; [i] Recombinant human bone morphogenetic protein-2; [j] Vascular endothelial growth factor; [k] Platelet derived growth factor; [l] Poly-L-lactic acid.

[2, 5, 10, 18, 20, 25, 37, 38, 43, 88, 96-97, 108-111]

Chapter 2.2

Bacteriocins and Micro- or Nanoparticle Polymeric Protein Delivery Systems

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Introduction

Increase in multi-drug resistant pathogens (MDRP) has substantial social and economic implications (Lister 2002; Cosgrove and Carmeli 2003). The frequent use of antibiotics has accelerated the incidence of resistance among pathogens. In 1941 almost all strains of *Staphylococcus aureus* were susceptible to penicillin G. However, by 1944 strains of *S. aureus* capable of producing β-lactamase to inactivate penicillin G have been reported (Neu 1992). The excessive use of ceftazidime is another example of the association between antibiotic misuse and spread of microbial resistance (Rice *et al.* 1990). A decrease in the discovery of novel and effective antibiotics serves as motivation to develop alternative approaches in antimicrobial therapy (Walsh 2003; Fischbach and Walsh 2009).

Bacteriocins are separated from antibiotics by several factors, including mode of action and binding sites (Brötz *et al.* 1998; Shai 2002; Hsu *et al.* 2004). Bacteriocins are usually active against specific species, often those closely related to the producer strain, whereas antibiotics have a broad range of activity. Bacteriocins are, however, active at nanomolar concentrations, whereas antibiotics are required at much higher concentrations (Brötz *et al.* 1998). Bacteriocins from Gram-positive bacteria, especially lactic acid bacteria (LAB), have generated the most attention over the past years. The GRAS (generally regarded as safe) status of these organisms, their application in the food industry and as probiotics, has led to a vast increase in research activities, also on the antimicrobial peptides they produce. Some bacteriocins have activity against MDRP, such as methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE) (Kruszewska *et al.* 2004; Piper *et al.* 2010). In addition to MDRP, some bacteriocins have also indicated activity against other clinical strains (Knoetze *et al.* 2008; De Kwaadsteniet *et al.* 2009).

Bacteriocins are grouped into three main classes (Klaenhammer 1993; Chen and Hoover 2003; Cotter *et al.* 2005). Class I bacteriocins are defined as lantibiotics and are divided into subgroups A and B based on structure and mode of action (Cotter *et al.* 2005). Class II bacteriocins are heat-stable, non-lanthionine-containing peptides and are separated into four subgroups (Bauer and Dicks 2005). Pediocin-like bacteriocins are grouped into class IIa, bacteriocins that require two peptide for activity are grouped in class IIb, cyclic peptides in class IIc and single non-pediocin like peptides in class IId (Van Reenen *et al.* 2003; Cotter *et al.* 2005; Albano *et al.* 2007). Class III contains the bacteriolysins (Cotter *et al.* 2005).

The use of bacteriocins is therefore a possible alternative to antibiotics. There are, however, several issues that have to be addressed if bacteriocins are to be used *in vivo* for the treatment of infection. Bacteriocins are sensitive to degradation by proteases and other degrading enzymes (Gupta and Batish 1992; Joerger 2003). Therefore, it is important to protect them from possible degradation. Another problem is the mechanism by which the bacteriocins are delivered. One possible solution to this problem is the use of polymeric delivery systems (Bilati *et al.* 2005; Yoshida *et al.* 2006; Kanczler *et al.* 2007; Heunis *et al.* 2010; Heunis *et al.* 2011). Microparticles are able to prolong the activity of nisin *in vitro* and may be able to protect them from degradation (Salmaso *et al.* 2004). It has also been shown that microparticles are able to protect encapsulated plasmid DNA from degradation *in vivo* (Garcia del Barrio *et al.* 2003). The use of polymeric drug delivery systems such as micro or nanoparticles, and nanofibers, has been proven to be effective drug delivery systems *in vitro* and may be advantageous for the delivery of bacteriocins (Salmaso *et al.* 2004; Bilati *et al.* 2005; Garcia del Barrio *et al.* 2003; Freiberg and Zhu 2004; Siepmann *et al.* 2004; Zhang *et al.* 2008, Heunis *et al.* 2010; Ye *et al.* 2010; Heunis *et al.* 2011). There are several factors that may influence the production method used, and when working with proteins, non-degrading methods must be chosen.

This review discusses the main classes of bacteriocins produced by LAB and their possible clinical applications. Polymeric micro- or nanoparticle production and their applications will also be investigated.

Bacteriocins produced by lactic acid bacteria

The increase in number of bacteriocins discovered over the last decade is testimony of the large variety amongst these peptides. Several classification schemes have been proposed for bacteriocins produced by Gram-positive. The most widely excepted classification scheme was proposed by Klaenhammer (1993; Table 1). Recently an update of Klaenhammer's classification system has been proposed by Cotter *et al.* (2005). They suggest that there are only two bacteriocin classes produced by Gram-positive bacteria. Rea *et al.* (2011) have also suggested a rational scheme, taking into account all current classification systems (Table 2). The updated classification scheme proposed by Rea *et al.* (2011) is discussed.

Class I bacteriocins

According to previous classification systems, class I only contains heat stable post-translationally modified bacteriocins of which only lantibiotics were included (Klaenhammer 1993). In the updated schemes class I are subdivided into three subclasses which can be further subdivided into subsequent subclasses (Rea *et al.* 2011).

Class Ia

Class Ia consists of the lantibiotics and lantipeptides. These peptides contain the unusual amino acids lanthionine, and/or β-methyllantionine as well as dehydrobutyrine (Rea *et al.* 2011). In previous systems lantibiotics was classified according to structure as either type A or B (Jung 1991). Type A lantibiotics are elongated, amphiphilic, cationic peptides, illustrating activity via pore formation and subsequent dissipation of the proton motive force (Jung 1991; McAuliffe *et al.* 2001). Type B lantibiotics consists of short, globular peptides, demonstrating activity through enzymatic inhibition of cell wall biosynthesis (Jung 1991; McAuliffe *et al.* 2001). Research into the mode of action of these peptides illustrated some drawbacks to the classification system. Nisin, for example, creates pores in the membrane which makes it a type A lantibiotic (Brötz *et al.* 1998; Hasper *et al.* 2004). However, it is also capable of inhibiting peptidoglycan synthesis by binding to lipid II, which classes it as a type B lantibiotic (Brötz *et al.* 1998; Breukink *et al.* 2003). Lacticin 3147 is a two peptide lantibiotic which first targets lipid II (peptide Ltn-α) and this complex then initiates binding of Ltn-β which results in inhibition of cell wall synthesis as well as pore formation (Morgan *et al.* 2005). The mode of action of lacticin 3147 and nisin can therefore class them as both type A and type B. The update classification system divides class Ia peptides into four main subclasses according to their biosynthetic and export machinery (Table 2 and 3). Subclass I and II can be further subdivided according to amino acid sequences (Rea *et al.* 2011).

Class Ib

Class Ib or the labyrinthopeptins consists of a new class of post-translationally modified peptides. The newly identified labyrinthopeptins are distinguished by their labyrinth type structure containing a carbacyclic post-translationally modified amino acid namely; labionin (Meindl *et al.* 2010). Meindl *et al.* (2010) illustrated antiviral activity of labyrinthopeptin A2 (produced by *Actinomadura namibiensis*) as well as its ability to treat neuropathic pain in mice.

Class Ic

The small post-translationally modified cyclic peptide subtilosin A was not taken into consideration in previous classification schemes due to its unusual cysteine to α-carbon bridges (Kawulka *et al.* 2004). A new two-peptide bacteriocin thuricin CD has also been found to have cysteine to α-carbon linkages as seen in subtilosin A (Rea *et al.* 2010). Subtilosin A as well as identification of thuricin CD justifies inclusion of this emerging group in future classification systems. Rea *et al.* (2011) therefore proposed that these bacteriocins namely; ***s***ulphur to ***α-c***arbon linkage-containing an***tibiotics*** should be classed as sactibiotics (class Ic). They are further subdivided into two subclasses, single- and two-peptide bacteriocins.

Class II bacteriocins

Class II is a large heterogeneous group of bacteriocins, consisting of standard amino acid residues and heat stable bacteriocins. The traditional classification system has three classes; IIa containing the pediocin like bacteriocins, IIb made up the two-peptide bacteriocins and IIc contains the thiol activated bacteriocins (Klaenhammer 1993). Others have suggested that class IIc contain all the class II bacteriocins that do not fit into IIa or b (Nes *et al.* 1996, Eijsink *et al.* 2002). The updated scheme redefines class IIc as the cyclic bacteriocins and adds class IId which contain the non-pediocin single linear peptides (Tables 2 and 4; Cotter *et al.* 2005; Rea *et al.* 2011).

Class IIa

These bacteriocins have a narrow range of activity but are highly potent against sensitive strains. They share a conserved hydrophilic, cationic sequence at the N-terminal, referred to as the "pediocin box" containing two cysteines joined by a disulfide bond (-Y-G-N-G-V-X_1-C-X_2-K/N-X_3-X_4-C-; X_{1-4} represents polar uncharged or charged residues; Papagianni and Anastasiadou 2009). Other than this and the cleavage of the leader peptide, the peptides are unmodified. The hydrophobic/amphiphilic C-terminal is, however, not as conserved as the N-terminal (Fimland *et al.* 1996). Recent results suggest that the N- and C-terminal act independently and that the C-terminal of the bacteriocin is important for target specificity (Johnsen *et al.* 2005). The N- and C-terminal domains are separated by a flexible hinge and allows flexibility to enable insertion of the C-terminal into the hydrophobic region of the membrane (Uteng *et al.* 2003). Retention of the α-helix in the C-terminal seems to play a critical role in activity, most likely by formation of ion pores resulting in dissipation of the PMF (Chikindas *et al.* 1993; Johnsen *et al.* 2005).

Class IIb

Class IIb bacteriocins are mainly produced by LAB and range from 30-50 amino acid residues (Nissen-Meyer *et al.* 2009). They are all two-peptide bacteriocins with the genes encoding the two peptides found next to each other and the immunity gene adjacent on the same operon (Franz *et al.* 2002). The peptides are synthesized with an N- terminal leader peptide consisting of 15-30 residues (double-glycine type) and cleaved at the C-terminal side of two glycine residues upon export of the peptides outside the cell (Oppegard *et al.* 2007). The peptides are inactive on their own and need to physically interact to form an active two-peptide bacteriocin, there are however examples where one or both peptides show low individual activity (Nissen-Meyer *et al.* 1992; Anderssen *et al.* 1998; Oppegard *et al.* 2007). Mode of action of these bacteriocins has been shown to be permeabilisation of the cell membrane leading to dissipation of the PMF (Moll *et al.* 1996; Moll *et al.* 1999; Nissen-Meyer *et al.* 2009). It has been suggested that the two peptides interact through conserved GxxxG or "GxxxG like" motives present on both peptides and interrupting this interaction results in reduced activity (Rogne *et al.* 2009).

Class IIc

Class IIc bacteriocins in older schemes contained the thiol-activated bacteriocins requiring reduced cysteine residues for activity (Klaenhammer 1993). In other proposals it was suggested that all bacteriocins that do not fit into class IIa or IIb must be classed as IIc (Eijsink *et al.* 2002). In the updated system class IIc contains the cyclic antimicrobial peptides (ribosomally synthesized). Post-translational modification of these peptides results in their N- and C-terminal to be linked resulting in a circular backbone (Martinez-Bueno *et al.* 1994; van Belkum *et al.* 2011). Class IIc can further be divided into IIc (1) or IIc (2) based on their amino acid sequences (Cotter *et al.* 2005).

Class IId

Class IId enlists all unmodified, linear, non-pediocin-like one peptide bacteriocins which do not conform to the criteria set out for other class II bacteriocins (Cotter *et al.* 2005). Consequently this class contains a large number of heterogeneous antimicrobial peptides from a variety of strains and a range of ecological niches.

Bacteriolysins

The bacteriolysins are large and heat sensitive peptides with a domain-type structure with different components of the molecule functioning in translocation, receptor binding and antimicrobial activity (Simmonds *et al.* 1997). Bacteriolysins cleave the peptidoglycan protein

moiety of sensitive organisms resulting cell wall lysis (Beukes *et al.* 2000; Simmonds *et al.* 1996). There are five bacteriolysins produced by LAB, helveticin J (*Lactobacillus helveticus*; Joerger and Klaenhammer 1986), zoocin A (*Streptococcus zooepidemicus*; Simmonds *et al.* 1996), enterolysin A (*Enterococcus faecalis*; Hickey *et al.* 2003), millericin B (*Sterptococcus milleri*; Beukes *et al.* 2000) and linocin M18 (*Brevibacterium linens*; Valdes-Stauber and Scherer 1994). They share an N-terminal domain responsible for enzymatic activity that has homology to endopetidases, whereas the C-terminal is thought to contain the cell wall binding domain, as seen with the broad range of activity of enterolysin A compared to zoocin A (Hickey *et al.* 2003; Beukes *et al.* 2000; Simmonds *et al.* 1997).

Classification schemes of bacteriocins will continue to evolve, with increase knowledge of the structure of bacteriocins as well as advancements in molecular biology.

Micro or nanoparticles as polymeric protein delivery systems

Biodegradable and biocompatible polymeric particles have gained great interest as delivery systems for bioactive molecules, especially micro- and nanoparticles. Conventional delivery methods for bioactive molecules usually require daily doses to reach a level that would be considered therapeutic. With polymeric delivery systems, the release of the molecule can be delayed, requiring less frequent treatments, which may reduce patient non-compliance (Freiberg and Zhu 2004). A wide range of polymers in use are associated with bioactive agents including; poly (lactic acid) (PLA), poly (lactic-co-glycolic acid) (PLGA) and poly (ethylene oxide) (PEO) (Almeida *et al.* 1993; Salmaso *et al.* 2004; Schnieders *et al.* 2006; Yoshida *et al.* 2006). Particles prepared from these polymers proved to be effective in *in vivo* trials (Pandey *et al.* 2003; Ruhe *et al.* 2005; Schluep *et al.* 2009). Pandey *et al.* (2003) have shown that anti-tubercular drugs encapsulated in PLGA could be used to treat *Mycobacterium tuberculosis.* Loaded particles were administered every 10 days and indicated therapeutic activity throughout. Formulations like these may reduce patient non-compliance, lower production cost and increase drug delivery effectiveness. In another study, nisin was encapsulated in PLA nanoparticles (Salmaso *et al.* 2004). Loaded nanoparticles have shown prolonged antibacterial activity, indicating controlled release as well as retention of biological activity of the peptide (Salmaso *et al.* 2004). Bioactive agents such as recombinant human bone morphogenetic protein-2 (Ruhe *et al.* 2003), insulin (Builders *et al.* 2008), and platelet derived growth factor and vascular endothelial growth factor (Kang *et al.* 2008; De la Riva *et al.* 2010; Richardson *et al.* 2001) have all been encapsulated into micro or nanoparticles. The release kinetics of various antibiotics have also been investigated in

polymeric particle systems (Pandey *et al.* 2003; Yoshida *et al.* 2006; Arnold *et al.* 2007; Tsifansky *et al.* 2008)

Micro or nanoparticle fabrication

There are several methods to produce polymeric particles; the type of method will depend on the bioactive agent as well as the desired particle characteristics. Another important factor to consider is the polymer and solvent to be used for fabrication. Each bioactive agent will react differently to the polymer used as well as the solvent (Blanco and Alonso 1998; Ye *et al.* 2010). The polymer: solvent: bioactive agent solution will also affect particle characteristics and fabrication method (Griebenow and Klibanov 1996; Birnbaum *et al.* 2000; Jeon *et al.* 2000; Castellanos *et al.* 2003; Siepmann *et al.* 2004; Xu and Hanna 2006; Kang *et al.* 2008).

There are several ways to produce micro- or nanoparticles; the main methods are expanded below.

Electrospray method

Electrospraying and electrospinning follow the same basic principles in terms of electrohydrodynamic atomization (Morota *et al.* 2004; Xie and Wang 2007; Heunis *et al.* 2010). An electric field is passed through a polymer solution resulting in the formation of a meniscus (Taylor cone) at the tip of the needle. The jet emerging from the apex will then break and disperse as separate particles repelled by electrostatic forces (Xu and Hanna 2006; Xie and Wang 2007). The particles are then collected on a grounded collector. The characteristics of particles can be altered by changing parameters such as solvent and polymer (concentration, MW and composition) and potential difference through the polymer solution (Morota *et al.* 2004; Xu and Hanna 2006; Wu and Clark 2007). Examples of protein encapsulation include Bovine serum albumin into PLA and PLGA (Xu and Hanna 2006; Xie and Wang 2007). It has also been illustrated that electrospinning bacteriocin 423 results in active peptide with controlled release (Heunis *et al.* 2010; Heunis *et al.* 2011). Electrospraying for the encapsulation of proteins has not yet been used to its full potential and may prove useful in fabrication of delivery systems.

Water/oil/water double emulsion method

In the W/O/W method an aqueous protein/drug solution is dispersed into a polymer solution (e.g. PLGA dissolved in dichloromethane) resulting in the first W/O emulsion. The W/O emulsion is added to a large volume of water containing an emulsifier such as poly (vinyl alcohol), this

forms a W/O/W emulsion (Ruhe *et al.* 2003). Removing the organic phase by either solvent extraction or solvent evaporation will result in hardened particles (Cleek *et al.* 1997; Ruhe *et al.* 2003; Basarkar *et al.* 2007). Particle properties can be changed by altering the following parameters; protein concentration and type, polymer (concentration, MW and composition), solvent (type and concentration), ratio between protein and polymer and emulsification procedure (intensity and duration; Yang *et al.* 2001; Ito *et al.* 2007; Ye *et al.* 2010). Several bioactive molecules have been encapsulated using this method including; insulin, BSA as well as plasmid DNA (Cleek *et al.* 1997; Rafati *et al.* 1997; Ito *et al.* 2007; Teply *et al.* 2008,). This method uses inexpensive equipment and is relatively simple, however due to the technique protein encapsulation may not always be high.

Solid/oil/water method

This technique is used to try and prevent protein denaturisation at the solvent/water interface (Castellanos *et al.* 2003). When proteins are in a solid state it is assumed that their conformational mobility is reduced resulting in retention of bioactivity (Griebenow and Klibanov 1996; Castellanos *et al.* 2003). Solid protein or drug (crystals or powder) are added to a polymer solution to form the first emulsion (Castellanos *et al.* 2003; Siepmann *et al.* 2004). The primary emulsion is then placed in a large volume of water containing an emulsifier such as poly (vinyl alcohol). The organic phase is then removed as with W/O/W method (Siepmann *et al.* 2004). Micronization is an important parameter with this technique; it has been shown that reduction in the protein particle size influences encapsulation efficiency as well as release kinetics (Birnbaum *et al.* 2000; Takada *et al.* 2003).

Spray drying and spray freeze drying methods

In spray drying a protein solution or emulsion (S/O or W/O) is sprayed at an elevated temperature to facilitate solvent evaporation and subsequent particle atomization (Gander *et al.* 1995; Carrasquillo *et al.* 2001; Ye *et al.* 2010). This is a one-step method that has easy control over parameters that control particle characteristics (Ye *et al.* 2010). Proteins may be denatured at high temperatures required for solvent evaporation (Mumenthaler *et al.* 1994; Quaglia *et al.* 2003; Ye *et al.* 2010). Using this method particles containing insulin, BSA, tetanus toxoid and recombinant human erythropoietin have been encapsulated (Quaglia *et al.* 2003; Gander *et al.* 1995; Bittner *et al.* 1998; Johansen *et al.* 1998; Ye *et al.* 2010).

Spray freeze drying avoids high temperatures, but is much more complicated than the one step spray drying (Carrasquillo *et al.* 2001). Protein is first sprayed into liquid N_2 and lyophilized;

protein particles are then added to polymer solution to make an S/O emulsion. The S/O emulsion is then sprayed using an ultrasonic nozzle into a bath containing frozen ethanol overlaid with liquid N_2. The bath is then placed at -80°C where ethanol melts and solvent extraction begins. The particles are solidified and dried by passing through nitrogen at 2 to 8°C (Costantino *et al.* 2000). This method may exert stresses on proteins that may result in instability (Costantino *et al.* 2000; Ye *et al.* 2010)

Supercritical CO_2 particles

This technique exploits the properties of a solvent at near critical or supercritical state. A substance is at a supercritical state when its pressure and temperature is greater than its critical pressure (P_c) and temperature (T_c), respectively (Benedetti *et al.* 1997). In the supercritical state the supercritical fluid (SCF) exhibits both gas- and fluid-like properties. For pharmaceutical purposes carbon dioxide is an ideal SCF, as it has a low T_c (31.5C) and P_c (73.8 bar), which is industrially feasible and non-toxic (Benedetti *et al.* 1997). Carbon is a non-polar solvent, therefore it is not able to dissolve polar substance; in which case a different supercritical liquid can be used or a co-solvent can be added to increase the solubility properties of CO_2 (Benedetti *et al.* 1997). Two basic techniques are used in the production of particles using $scCO_2$; e.g. rapid expansion of supercritical solutions (RESS) and supercritical antisolvent process (SAS).

When using RESS, a non-volatile solute is dissolved in the SCF (supercritical fluid). The solution is then extruded at high velocity through a capillary/nozzle, as the pressure rapidly decreases solvent-free particles precipitate (solute nucleation; Benedetti *et al.* 1997). The disadvantage of RESS is the low solubility of various polar pharmaceutical compounds (including polymers) and the large amount of CO_2 needed (Sekhon 2010). A co-solvent can be added to the CO_2, but this however takes away RESS's solvent free advantage (Benedetti *et al.* 1997; Mishima *et al.* 2000).

SAS requires an organic solvent, in addition to the SCF and the solid solute (Benedetti *et al.* 1997). The solute is dissolved in the organic solvent which is then added to the SCF which is miscible with the solvent but not the solute, resulting in the precipitation of the solute (Benedetti *et al.* 1997). Two methods are used for the precipitation of the solute; gas anti-solvent (GAS) precipitation and precipitation with compressed anti-solvents (PCA). In the GAS method high pressure CO_2 is added to the solvent: solute solution resulting in a volumetric expansion of the liquid phase and the precipitation of the solute (Elvassore *et al.* 2001; Fusaro *et al.* 2005). In the PCA method the solvent: solute solution is sprayed into a SCF, resulting the rapid two-way mass

transfer (SCF diffusing into solute and solute diffusing into SCF) results in supersaturation of the solute and its precipitation (Jarmer *et al.* 2003; Fusaro *et al.* 2005).

A wide variety of pharmaceutical compounds have been used in both GAS and PCA, including nisin (using PCA; Elvassore *et al.* 2001; Salmaso *et al.* 2004; Lee *et al.* 2008).

Future prospects for bacteriocins and micro or nanoparticle delivery systems

The discovery of antimicrobials plays a crucial role with the increase in antibiotic resistant microorganisms. The role new antimicrobial peptides, such as bacteriocins, may play in treatment of resistant pathogens may be invaluable. The discovery of new antibiotics over the past few decades does not look promising and mostly relies on the development of synthetic antibiotics (Fischbach and Walsh 2009). The identification of novel bacteriocins that may be active against resistant pathogens is however on the increase (Chen and Hoover 2003). Research into their discovery, testing (safety) and characterization is therefore essential. Sensitivity of bacteriocins to degradation *in vivo* is a drawback for their use in the treatment/prevention of infection (De Kwaadsteniet *et al.* 2009; Brand *et al.* 2010; De Kwaadsteniet *et al.* 2010). Future research has to focus on selecting for more robust broad spectrum bacteriocins that are capable of remaining active *in vivo.* Identifying the major factors involved in their *in vivo* degradation may also provide insight into how to select bacteriocins for *in vivo* applications.

Polymeric delivery systems may provide the solution to degradation of bacteriocins *in vivo*. The development of more effective and efficient ways to treat disease is essential if the medical industry to evolve. The use of micro and nanoparticles to provide controlled release and protection of bioactive agents may present possible improvements in treatment (Pandey *et al.* 2003; Salmaso *et al.* 2004; Ye *et al.* 2010). With the increase of drug effectiveness using polymeric particles parameters such as cost, patient compliance and effective treatment all benefit.

Research into the combination of polymeric drug delivery systems, such as particles and bacteriocins is ongoing. An important factor is to make sure that these combinations actually work in an *in vivo* situation. *In vivo* experiments are therefore essential to lay the ground work for the eventual use of bacteriocins and polymer systems in the treatment of infections. Although bacteriocins are able to kill sensitive organisms it is important to realize that resistance to these peptides are possible. It has been shown that organisms obtaining resistance to bacteriocins indicate increase resistance to antibiotics. The combination of antibiotics and bacteriocins must also be investigated; these combinations may lead to more effective antimicrobials.

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Table 1: Original classification scheme of Klaenhammer 1993 (from Rea *et al*. 2011)

Class	*Description*	*Distinctive features*
Class I	Post-transcriptionally modified bacteriocins	Contain unusual amino acids lanthionine, and/or β-methyllantionine and dehydrobutyrine
Class II	Unmodified peptides	Small (<10 kDa) heat stable membrane active peptides
Class III	Unmodified proteins	Large (>30 kDa) heat liable proteins
Class IV	Complex proteins	Contains lipid or carbohydrate moieties

Table 2: Updated classification scheme for Gram-positive bacteriocins and bacteriocin-like peptides and proteins (adapted from Rea *et al.* 2010)

Class	*Description*	*Subdivision*
Class I	**Modified peptides**	
	a. Lantibiotics and lantipeptides	Subclass I and II based on modification of pre-peptides with LanB, LanC (I) and LanM (II). Can be further divided into 12 subclasses based on aa sequences.
		Subclass III and IV based on modifications with RamC-like (III) LanL (IV) proteins.
	b. Labyrinthopeptins	
	c. Sactibiotics	Single- and two-peptide bacteriocins
Class II	**Non-modified peptides**	
	a. Pediocin-like bacteriocins	Four subclasses I-IV
	b. Two-peptide bacteriocins	Two subclasses; A and B
	c. Circular bacteriocins	Two subclasses; 1 and 2
	d. Linear-non pediocin-like single bacteriocins	
Bacteriolysins	**Non-bacteriocin lytic proteins**	

Table 3: Class I subclasses and lantibiotic groups in each class (adapted from Piper *et al.* 2009 and Rea *et al.* 2011)

Class and Subclass	*Lantibiotic group or peptide*
Class Ia	
Subclass I	Nisin, planosporicin, epidermin, Pep5 and streptin.
Subclass II	Lacticin, mersacidin, LtnA2, cytolysin, lactosin S, cinnamycin and sublancin.
Subclass III	RamS, SapT and AmfS.
Subclass IV	Venezuelin.
Class Ib (Labyrinthopeptins)	Labyrinthopeptins A1 and A2.
Class Ic (Sactibiotics)	Subtilosin A (single-peptide) and Thuricin CD (two-peptide).

Table 4: Class II subclasses and main bacteriocins in each class (adapted from Martin-Visscher *et al.* 2009 and Nissen-Meyer *et al.* 2009)

Class and Subclass	*Main bacteriocins in subclass*
Class IIa	
Subclass I	Coagulin, divergicin (M35 and V41), enterocin A, leococin C, listeriocin 743 A, munticin (- and KS), pediocin PA-1, pisciocin CS 526, piscicolin 126 and sakacin (P and 5X).
Subclass II	Lactococcin MMFII, leucoccin A, mesentericin Y105, plantaricin (423 and C19), sakacin G.
Subclass III	Curvicin A, Carnobacteriocin BM1 and enterocin P
Subclass IV	Bacteriocin 31, bacteriocin RC714, bacteriocin T8, penocin A, enterocin SE-K4 and carnobacteriocin B2.
Class IIb (Two-peptide bacteriocins)	
Subclass A	ABP-118, brochocin C, lacticin F, lactocin 705, mutacin IV, plantaricin (E/F, J/K, NC8 and S), Salvaricin P and thermophilin 13.
Subclass B	Lactococcin (G and Q) and entrocin 1071.
Class IIc (Circular bacteriocins)	
Subclass 1	Carnocyclin A, circularin A, enterocin AS48, uberolysin and lactocyclicin Q.
Subclass 2	Butyrivibriocin AR10, gassericin A, reutericin 6 and acidocin B[1].
Class IId (Linear-non pediocin-like single bacteriocins)	Lactococcin (A, B and 972), Enterocin (B, EJ97, L50A, L50B, Q and RJ-11), Carnobacteriocin A, Bac32, Aureocin (A70 and A53), Lacticin (Q and Z), BHT-B, Acidocin (A, 1B and CH5), Bacteriocin OR-7, Divergicin 750, Weissellicin 110, Cripacin A, Lacticin RM, Micrococcin GO5, Lsb (A and B) Mesenterocin 52B, Mesentericin B105, Dextranicin 24, Leucocin B-TA33a, Bovicin 255.

[1] Circular structure not yet determined but has 98% sequence identity to gassericin A and reutericin 6.

Chapter 3

Release of *Enterococcus mundtii* bacteriocin ST4SA from self-setting brushite bone cement*

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Release of *Enterococcus mundtii* bacteriocin ST4SA from self-setting brushite bone cement

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Abstract

Maxillofacial and craniofacial surgery is on the increase, which exposes more patients at risk of acquiring microbial infections. The use of antibiotic loaded calcium phosphate bone cements have been shown to reduce the incidence of infection. A marked increase in antibiotic resistant pathogens, including multidrug-resistant pathogens, has been reported. This has led to the investigation of various compounds as alternatives to conventional treatments. In this paper we report on the incorporation and release of a broad-spectrum class II antimicrobial peptide, bacteriocin ST4SA produced by *Enterococcus mundtii*, into a calcium orthophosphate-based bone cement (CPC). Our results suggest class II bacteriocins may be incorporated into self-setting bone cements to produce implants with antimicrobial activity over extended periods of time.

Keywords

Bacteriocin, Brushite Bone Cement, Antimicrobial Activity

Introduction

With the increase in life expectancy, orthopedic implant surgery is on the rise, which in turn puts more patients at risk of acquiring microbial infections [17, 26]. In maxillofacial and craniofacial surgery, antibiotics are usually administered prophylactically to prevent post-operative infections. However, the increase in the number of antibiotic resistant bacteria, especially methicillin-resistant *Staphylococcus aureus* (MRSA), has narrowed the choice of antibiotics down to only a few [13, 14, 26, 34]. Furthermore, antibiotics administered intravenously or orally are not always effective [13, 14]. The main reason for this is the restriction in blood flow in areas surrounding the implant, and hence less contact between the antibiotic and site of infection [14, 37]. In severe cases of infection, surgical intervention is often the only alternative to a cure [37, 38].

A number of papers have been published on the incorporation of antibiotics and therapeutic agents in bone cement [2, 6, 15, 18, 20, 32, 33, 36]. Thornes and co-workers [34] observed a decrease in *Streptococcus epidermis* infection with gentamicin incorporated into bone cement (41% infection rate compared to 73% in the absence of gentamicin). However, the number of *S. epidermis* cells that developed resistance to gentamicin increased from 19% to 78% [34]. In other studies antibiotic-loaded bone cements used as a prophylactic significantly reduced the incidence of prosthetic joint associated infection [13, 19].

Bacteriocins with a broad or narrow spectrum of activity, and mechanisms of action different from conventional antibiotics, may serve as an alternative in the treatment of antibiotic resistant pathogens. Some of these peptides have shown activity against MRSA, vancomycin resistant enterococci as well as other clinical isolates [7, 11, 22, 23, 28]. Most of the bacteriocins are small, ribosomally synthesized, cationic and hydrophobic/amphiphilic peptides with 20 to 60 amino acid residues. They are grouped into two main classes [9, 10, 21]. Class I bacteriocins are defined as lantibiotics and are divided into subgroups A and B based on structure and mode of action [10]. Class II bacteriocins are heat-stable, non-lanthionine-containing peptides and are separated into four subgroups [4]. Pediocin-like bacteriocins [1, 35] are grouped into class IIa, bacteriocins that require two peptide for activity are grouped in class IIb, cyclic peptides in class IIc and single non-pediocin like peptides in class IId [10]. Class III contains the bacteriolysins [10].

Bacteriocin ST4SA, produced by *Enterococcus mundtii* ST4SA, belongs to the class IIa bacteriocins, and is active against various Gram-positive bacteria, including *S. pneumonia* and *S.*

aureus, as well as the Gram-negative bacterium *Pseudomonas aeruginosa* [22]. This paper describes the incorporation of bacteriocin ST4SA in calcium orthophosphate-based cement (CPC) and reports on the release rate and antimicrobial activity of the peptide *in vitro*. We have chosen CPC due to its excellent osteoconductive properties and stability during *in situ* molding [29]. Furthermore, CPCs are usually resorbed by osteoclasts and do not need to be removed, thus reducing the risk of infection caused by secondary operations [8, 27].

Materials and Methods

The β-tri calcium phosphate (βTCP, 95%) and monocalcium phosphate monohydrate (MCPM, 85%) was acquired from Sigma-Aldrich (Sigma-Aldrich, Germany). Tri-sodium citrate dihydrate was obtained from Saarchem (Saarchem, Gauteng, South Africa), and ammonium sulfate (99.5%) and Listeria Enrichment Broth (LEB) from Merck (Merck, Darmstadt, Germany). Dialysis membranes (1 kD, Spectra/Por® 6) were from Spectrumlabs (Spectrum Inc., CA, USA). The BCA protein assay kit was from Thermo Scientific (Pierce Biotechnology, Rockford, IL, USA). All other growth media used were from Biolab (Biolab Diagnostics, Midrand, South Africa).

Preparation of Bacteriocin ST4SA

E. mundtii ST4SA was cultured in 1 L MRS broth for 24 h at 30°C. Cells were harvested at 8000 *g* for 10 min at 4°C. The pH of the resulting supernatant was adjusted to 6.5-7.0 and then incubated at 80°C for 10 min to inactivate proteolytic enzymes. Proteins were precipitated from the cell-free supernatant with 80% saturated ammonium sulfate [25]. The precipitate was collected by centrifugation (10000*g* for 1 h at 4°C) and the pellet re-suspended in 10 mL sterile distilled water. The concentrated bacteriocin was dialyzed against 4 L sterile distilled water using a 1 kDa cut-off dialysis membrane (Spectrumlabs). The dialyzed product was concentrated by freeze-drying and stored at -20°C. Antimicrobial activity was determined by using the agar-spot method [22]. Activity was expressed in arbitrary units per mL (AU/mL). One AU is defined as the reciprocal of the highest serial two-fold dilution showing a clear zone of growth inhibition. *Listeria monocytogenes* EGD-e, grown in LEB supplemented with 7.5 µg/mL chloramphenicol, and incubated at 37°C on a shaker, was used as model sensitive strain.

Preparation of Bone Cement

Cement samples were prepared according to the method described by Barralet and co-workers [3]. β-TCP and MCPM were ground to a fine powder with a pestle and mortar and mixed in

equimolar amounts. The powder (250 mg) was mixed with 75.76 µL 500 mM tri-sodium citrate (P/L ratio of 3.3 g/mL), which served as retardant. Samples containing bacteriocin ST4SA were prepared by first adding the peptide to the dry cement powder (5%, w/w) before mixing with 500 mM tri-sodium citrate. Mixing was performed on a glass slab for 30 seconds to form a homogeneous paste. The paste was moulded into insulin syringes, from which the tips have been cut off, and dried at 37°C for 2 h. The set cement cylinders were approx. 8 mm long and 4 mm in diameter.

Characterization of Bone Cement

The crystal structure of the bone cement was studied by using a Zeiss EVO MA15VP scanning electron microscope (SEM). The cement cylinders were fractured, placed on an adhesive stub and coated with gold before SEM analysis. Powder X-ray diffraction (XRD) patterns of the set cements were recorded on a PANalytical X'Pert PRO MPD (Multi-Purpose Diffractomator). Data were collected from $2\theta = 10° - 60°$ with a step size of 0.02° and a normalized count time of 1 s/step using Cu Ka_{α} radiation. Phase composition was checked by means of ICDD (international centre for diffraction data) reference patterns for β-TCP (00-009-0348), MCPM (01-070-0359), brushite (00-009-0077 & 01-072-0713) and hydroxyapatite (00-001-1008).

Release of Bacteriocin ST4SA from Bone Cement

Bone cement cylinders, made from 250 mg β-TCP/MCPM powder mixed with 12.5 mg bacteriocin ST4SA preparation, were incubated in 5 mL PBS buffer (pH 7.4) at 37°C and slowly stirred. At selected time intervals the entire volume of buffer was collected and centrifuged at 8000 *g* for 1 min to remove non-dissolved particles. Protein concentration (mg/mL) in the particle-free buffer was determined by using the colorimetric BCA protein assay method at 562 nm, to determine release of bacteriocin ST4SA. Readings were recorded from standard curves prepared with bovine serum albumin. Buffer extracted was replaced with fresh buffer and the bone cement cylinders incubated until the next reading. Readings were recorded over 120 h. Release of protein from the bone cement was expressed as percentage of the original protein content present in the sample.

In a separate experiment, the bone cement cylinders were removed from the PBS buffer (pH 7.4) and inserted into 10 mL soft agar (1.0%, w/v), seeded with 1 mL *L. monocytogenes* EGD-e (1×10^7 cfu/mL). The plates were incubated at 37°C for 24 h and bacteriocin ST4SA activity recorded by formation of a clear zone of growth inhibition around the cement cylinders.

Antimicrobial Activity of Peptide ST4SA Released from Bone Cement

To determine the rate at which bacteriocin ST4SA is released from the bone cement, a delayed-agar diffusion method was used [5]. Bone cement (250 mg) containing 12.5 mg peptide ST4SA, equivalent to 102400 AU/mL, were incubated in 20 mL 1% LEB soft agar seeded with 1 mL *L. monocytogenes* EGD-e (1 x 10^7 cfu/mL). After 24 h of incubation at 37°C, the diameter (cm) of growth inhibition zones was recorded. The cement cylinders were then aseptically removed and placed into fresh soft agar seeded with *L. monocytogenes* EGD-e. The experiment was repeated until no zones of growth inhibition zones could be detected.

Statistical Analysis

Data were collected in Microsoft Excel 2007 and data points, indicated with standard deviations, represent an average of three repeats.

Results

Cement Characteristics

Addition of bacteriocin ST4SA to the β-TCP/MCPM powder produced a slightly more viscous cement paste compared to samples without the peptide. SEM of cement cylinders with and without bacteriocin ST4SA revealed similar textures, i.e. a dense surface with characteristic plates and blocks (Fig. 1A and B). XRD patterns corresponded well with that recorded for ICDD patterns of brushite (00-009-0077 & 01-072-0713), although some peaks for β-TCP (00-009-0348) and MCPM (01-070-0359) could be seen in samples with and without bacteriocin ST4SA (Fig. 2). No peaks characteristic of hydroxyapatite were observed in any of the samples.

Release of bacteriocin ST4SA from the bone cement

Most of bacteriocin ST4SA (approx. 80%) was released from the bone cement during the first 12 h (Fig. 3). This was followed by a 4% increase in release over the next 108 h. After 120 h of submersion in PBS buffer, bacteriocin ST4SA-loaded bone cement was still active, i.e. repressed the growth of *L. monocytogenes* (not shown).

Bacteriocin ST4SA incorporated into the bone cement inhibited the growth of *L. monocytogenes* EGD-e for up to 13 consecutive days, as shown by clear zones of growth inhibition (Fig. 4A). Zone sizes ranged from 3.5 cm in diameter, reported at day 2, to approx. 1.7 cm in diameter on day 13 (Fig. 4B).

Discussion

Incorporation of bacteriocin ST4SA into β-TCP/MCPM did not lead to drastic structural changes (Fig. 1), suggesting that crystal formation and setting of the cement was not altered. XRD results indicated that the main phase of the set cement is brushite and addition of bacteriocin ST4SA did not cause any significant changes (Fig. 2). Peaks for β-TCP and MCPM could be seen in control samples and bacteriocin ST4SA-loaded samples, possibly due to the presence of non-reacting starting material [6]. Similar results have been reported with the incorporation of other therapeutic agents in bone cement [24, 33].

Release of the active agent from bone cement depends on several factors, such as cement-drug interaction, porosity of the cement and solubility of the drug [16, 20, 33]. Release of most therapeutic agents from bone cement is characterized by a sudden burst, followed by slow release over several hours [6, 15, 20, 33, 36]. Rapid release of bacteriocin ST4SA (Fig. 3) suggests a high initial diffusion from the bone cement, which is supported by the small size (3.5 kDa) of the peptide. Bacteriocin ST4SA, on the other hand, was not completely released from the bone cement, as evident by the prolonged antimicrobial activity recorded (Fig. 4B) as well as the release study (Fig. 3). The release profile of bacteriocin ST4SA out of the cement was slightly higher than that reported for other therapeutic agents in unmodified cement [6, 15, 20, 33, 36]. Solubility also plays a major role in the release rate. Hofmann and co-workers [20] reported a higher burst release rate for vancomycin (80% in 24 h) as opposed to the less soluble ciprofloxacin (60% in 24 h). Concluded from results obtained in our study, bacteriocin ST4SA was released from bone cement for at least 120 h, suggesting that a certain percentage of the peptide takes longer to dissolve. It may also be that the cationic peptide adheres to the bone cement by electrostatic forces. However, XRD and SEM results did not indicate chemical or structural changes (Figs 2 and 3). Another possibility may be that the peptide is only released upon cement dissolution/resorption [20, 33]. *L. monocytogenes* EGD-e was used as a model pathogen in this study. In future *in vitro* and *in vivo* experiments *Streptococcus epidermidis* and *S. aureus* will be included.

Release of bacteriocin ST4SA from the bone cement may be enhanced by incorporating the peptide into micro- or nanoparticles. The concept has been shown in several other studies [12, 29-32]. The porosity of bone cement is easily changed by increasing the powder:liquid ratio, which in turn will enhance the release of a therapeutic agent [20].

Conclusions

Bacteriocin ST4SA proved stable and active for at least 13 days in β-TCP/MCPM cement. The possibility of incorporating bacteriocins into bone cement and developing implants with inherent antimicrobial activity seems possible. The challenge would be to select antimicrobial peptides with broad-spectrum activity that would not elicit a host immune response and with a mode of action that would prevent the emergence of microbial resistance. The possibility of combining antibiotics with a suitable bacteriocin may also reduce the chances of microbial resistance. Encapsulation of antimicrobial peptides with micro- and nanoparticles *in vitro* as well as *in vivo* trials with mice are currently being investigated.

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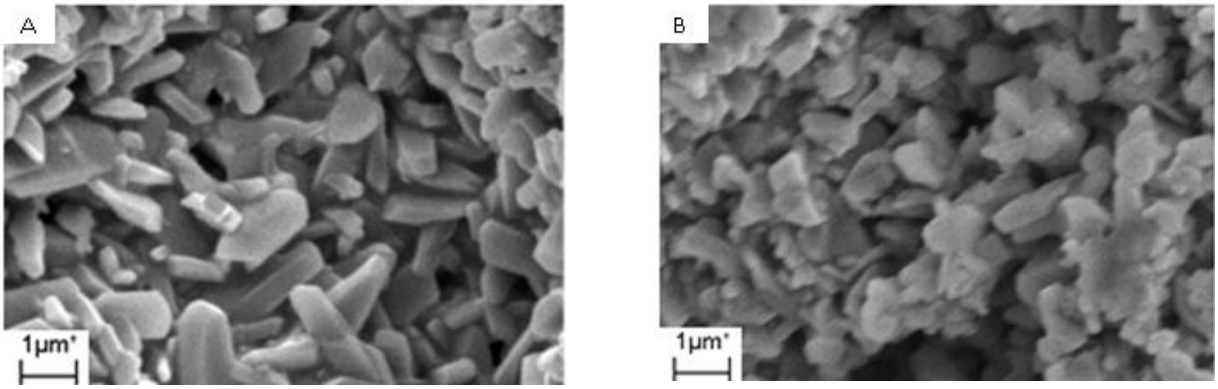

Fig. 1 SEM images (5.00K) of fractured cement samples without (A) and with (B) bacteriocin ST4SA (5%, w/w)

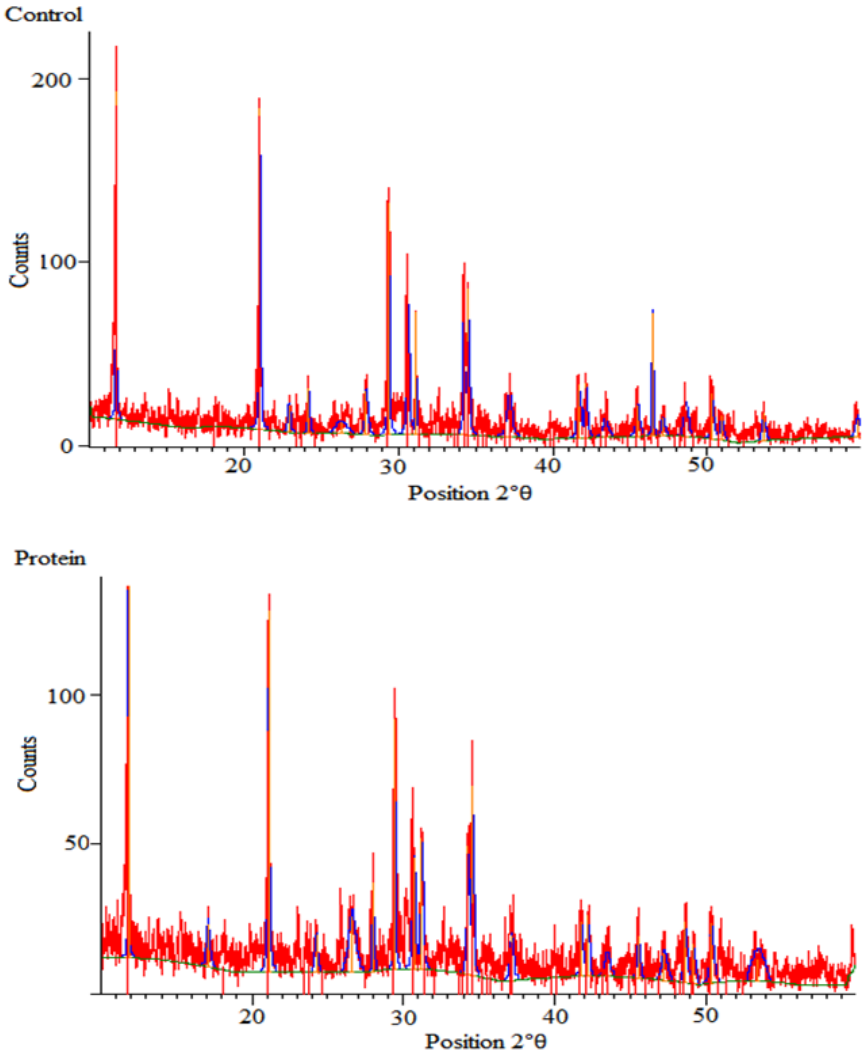

Fig. 2 XRD patterns of unloaded (control) cement samples and bacteriocin ST4SA-loaded samples (protein). Red lines indicate main brushite peaks (ICDD 00-009-0077)

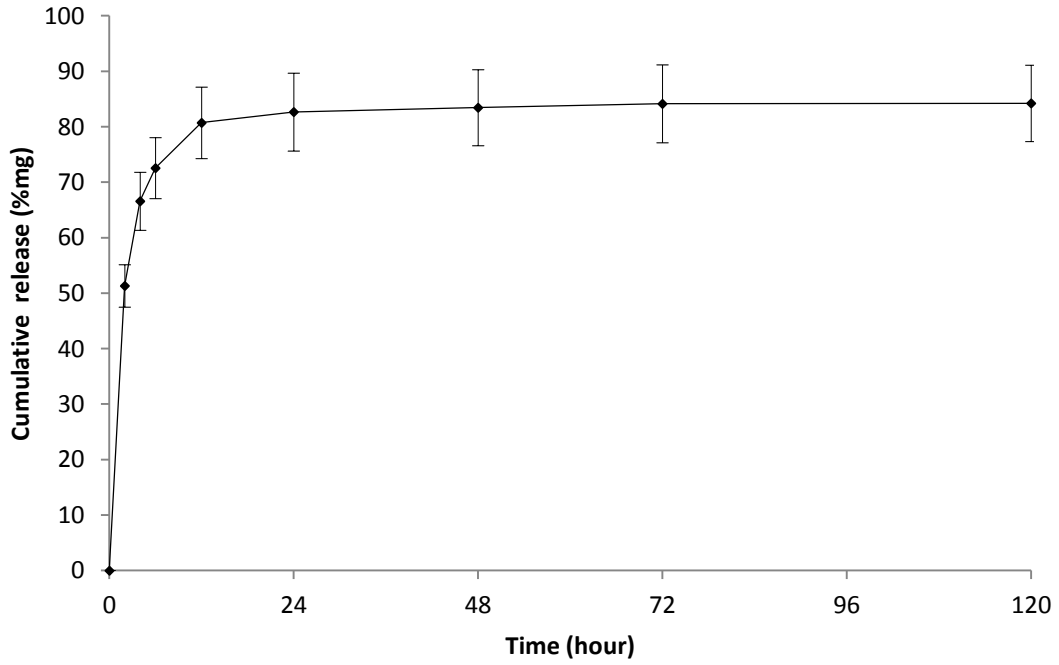

Fig. 3 Cumulative release of bacteriocin ST4SA (5%, w/w) from brushite cement, expressed as a percentage of the original protein content

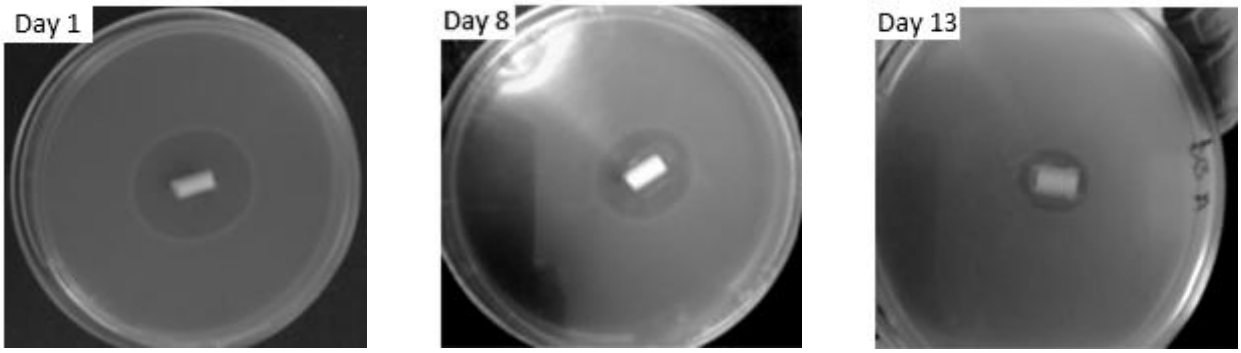

Fig. 4A Antimicrobial activity of bacteriocin ST4SA incorporated into brushite cement. *L. monocytogenes* EGD-e served as sensitive strain. Images of antimicrobial activity are as recorded on days 1, 8 and 13

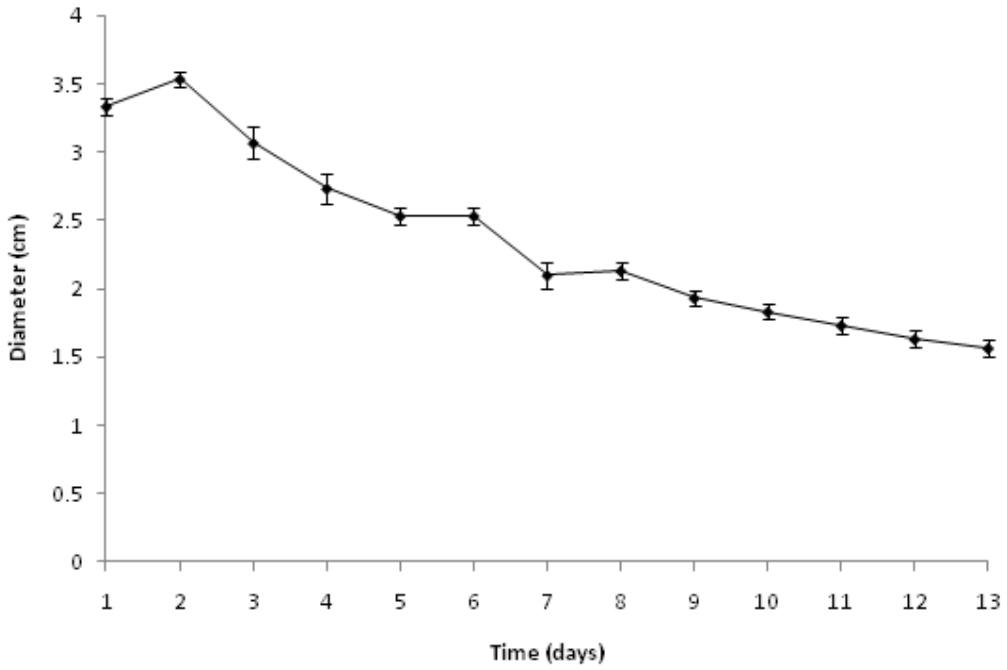

Fig. 4B Release of peptide ST4SA (5%, w/w) from brushite cement over 13 days. *L. monocytogenes* EGD-e served as sensitive strain

Chapter 4

Incorporation of bacteriocin associated Poly (D,L-lactide-*co*-glycolide) particles into self setting brushite bone cements

*This chapter is written accourding to the style of Journal of Applied Microbiology.

Incorporation of bacteriocin-associated poly (D,L-lactide-*co*-glycolide) particles into self setting brushite bone cements

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Abstract

Aims: To determine if peptide ST4SA-poly (D, L lactice-*co*-glycolide) (PLGA) microparticles could be incorporated into self-setting brushite bone cement and if peptide ST4SA will be released in its active form.

Methods and Results: Peptide ST4SA was suspended in an N, N-Dimethylformamide PLGA solution and electrosprayed to produce ST4SA-associated PLGA microparticles (SPM). Brushite cement was prepared by mixing equimolar concentrations of β-tricalcium phosphate and monocalcium phosphate monohydrate. SPM was added to brushite cement, to a concentration of 10% (w/w), and moulded into cylinders. Release of peptide ST4SA from the cement and microparticels was determined by using BCA protein assay. Based on SEM analysis SPM had an average size of 1048·4 nm and did not significantly affect crystal entanglement when added to the cement. According to XRD analysis the addition of SPM to the cement did not interfere with the setting reaction. After the first 12 h, 36·5% and 23·2% of the total bacteriocin was released from particles and brushite-SPM composites, respectively. This was, followed by a 1·6% and a 7·9% release over the next 228 h (for particles and brushite-SPM composites, respectively). Activity of the particles and cement was tested against a sensitive strain embedded in soft agar. Particle and brushite-SPM composites released active bacteriocin and inhibited the growth of *L. monocytogenes* EGD-e.

Conclusions: Electrospraying was successfully used to generate ST4SA-associated PLGA submicron particles able to release active bacteriocin, when incorporated into brushite bone cement.

Significance and Impact of the Study: ST4SA-associated PLGA particles prolong the release of ST4SA when incorporated into brushite bone cement and may provide protection to the peptide.

Keywords

Peptide ST4SA, brushite bone cement, *in vitro* activity, poly (D, L lactice-*co*-glycolide), microparticles

Introduction

Infections associated with orthopedic surgery are difficult to treat and result in increase cost and morbidity (Cosgrove and Carmeli 2003, Frommelt and Kühn 2005, Jiranek *et al.* 2006, Zimmerli 2006). Antibiotic-loaded bone cements have been used with some success as a prophylactic in various orthopedic procedures (van de Belt *et al.* 2001). The main cement type used in orthopedics is polymethylmethacrylate (PMMA) based, which is a non-resorbable polymer (Van de Belt *et al.* 2001). Calcium phosphate cements (CPC) have several advantages over the traditional PMMA cements, of which bioresorbability and biocompatibility are the most important (Theiss *et al.* 2005). CPC unfortunately suffer from low mechanical strength as appose to PMMA cements and are only used in low load bearing areas (Schmitz *et al.* 1999). CPC have, however, been used clinically with some success and have been included in several *in vitro* and *in vivo* studies (Miyamoto *et al.* 1995, Ohura *et al.* 1996, Kurashina *et al.* 1997, Otsuka *et al.* 1997, Petruzzelli and Stankiewicz 2002, Kasperk *et al.* 2005, Ruhe *et al.* 2005, Schnieders *et al.* 2006, Ginebra *et al.* 2006, Alkhraisat *et al.* 2010, Fullana *et al.* 2010, Neovius and Engstrand 2010). The release of therapeutic drugs from CPC is dependent on several factors, including drug cement interaction, solubility of the drug and cement porosity. Changing these factors may result in a change in release kinetics of the agent (Young *et al.* 2008, Hofmann *et al.* 2009, Fullana *et al.* 2010). Release is usually coupled by a high initial burst release, followed by partial or complete release of the agent (Hofmann *et al.* 2009). Controlling release rates can be achieved by incorporation of the therapeutic agent into polymeric micro-or nanoparticles. Release of therapeutic agents from polymeric scaffolds such as fibers and particles have been investigated (Birnbaum *et al.* 2000, Jeon *et al.* 2000, Elvassore *et al.* 2001, Pandey *et al.* 2003, Salmaso *et al.* 2004, Xu and Hanna 2006, Builders *et al.* 2008, Teply *et al.* 2008, Heunis *et al.* 2010; Heunis *et al.* 2011). The release rate depends on several polymer characteristics. Hydrophobic polymers, e.g. poly (lactic acid) (PLA) or poly (ε-caprolactone) polymer provides a slow release and, in some cases, only degrades over several months/years (Iooss *et al.* 2001, Salmaso *et al.* 2004). In contrast to this, a hydrophilic polymer such as poly (ethylene oxide), releases the therapeutic agent at a rapid rate (Kenawy *et al.* 2002). Co-polymers such as poly (lactide-*co*-glycolide) (PLGA) have a slower release rate and provide a more gradual release (Klose *et al.*2008). Incorporation of polymeric particles has shown to be promising in delaying release when incorporated into CPC (Ruhe *et al.* 2003, Schnieders *et al.* 2006). Several therapeutic agents have been incorporated into particles and then into cement, including gentamicin and recombinant human bone morphogenetic protein-2 (Ruhe *et al.* 2003, Schnieders *et al.* 2006)

Pathogens acquiring resistance to antibiotics is a major problem in the treatment of infections (Thornes *et al.* 2002, Pulido *et al.* 2008). Thornes *et al.* (2002) illustrated that incorporation of antibiotics into bone cement may also lead to increase incidence of antibiotic resistance (Thornes *et al.* 2002). Alternatives to antibiotics are therefore needed to reduce the emergence of antibiotic resistant pathogens (Joerger 2003).

Bacteriocins produced by lactic acid bacteria are possible alternatives to antibiotics. These are small, ribosomally synthesized cationic, hydrophobic or amphiphilic peptides composed of 20-60 amino acid residues and are divided into three classes (Class I, Class II and bacteriolysins; Rea *et al.* 2011). Bacteriocins show antimicrobial activity against closely related species and in several cases against antibiotic resistant microorganisms such as MRSA (methicillin resistant *Staphylococcus aureus*) and VRE (vancomycin resistant enterococci) (Van Reenen *et al.* 2003, Kruszewska *et al.* 2004, De Kwaadsteniet *et al.* 2010, Piper *et al.* 2010).

The main drawback of using bacteriocins is their instability *in vivo* (De Kwaadsteniet *et al.* 2009, Brand *et al.* 2010, De Kwaadsteniet *et al.* 2010). Instability may be overcome by encapsulating the bacteriocins with biocompatible polymers. Nisin has been successfully incorporated into particles and showed prolonged release and activity (Salmaso *et al.* 2004).

The current study aims to investigate the *in vitro* properties of bacteriocin-associated PLGA microparticles (SPM) incorporated into brushite bone cement. Peptide ST4SA produced by *Enterococcus mundtii* ST4SA was used as model peptide.

Materials and Methods

Materials

Poly (D,L-lactide-*co*-glycolide; PLGA) lactide: glycolide 65:35 and mol wt 40 000-75 000 was purchased from Sigma (Sigma Aldrich, Germany). N, N-Dimethylformamide, DMF (99% min) was purchased from Saarchem (Saarchem, Gauteng) and ammonium Sulfate (99·5%) was acquired from Merck (Merck, Germany). The β-tri calcium phosphate (βTCP, 95%) and monocalcium phosphate monohydrate (MCPM, 85%) was acquired from Sigma-Aldrich (Sigma-Aldrich, Germany). Tri-sodium citrate dihydrate was obtained from Saarchem (Saarchem, Gauteng, South Africa), and ammonium sulfate (99·5%) and Listeria Enrichment Broth (LEB) from Merck (Merck, Darmstadt, Germany). Dialysis membranes (1 kD, Spectra/Por® 6) were from Spectrumlabs (Spectrum Inc., CA, USA). The BCA protein assay kit was from Thermo

Scientific (Pierce Biotechnology, Rockford, IL, USA). All growth media used were from Biolab (Biolab Diagnostics, Midrand, South Africa).

Preparation of peptide ST4SA

E. mundtii ST4SA was cultured in 1 liter MRS broth for 24 h at 30°C. Cells were harvested at 8000 ***g*** for 10 min at 4°C. The pH of the resulting supernatant was adjusted to 6·5-7·0 and then incubated at 80°C for 10 min to inactivate proteolytic enzymes. Proteins were precipitated from the cell-free supernatant with 80% saturated ammonium sulfate (Maniatis *et al.* 1982). The precipitate was collected by centrifugation (10000 ***g*** for 1 h at 4°C) and the pellet re-suspended in 10 ml sterile distilled water. The concentrated bacteriocin was dialyzed against 4 liters sterile distilled water using a 1 kDa cut-off dialysis membrane. The dialyzed product was concentrated by freeze-drying and stored at -20°C. Antimicrobial activity was determined by using the agar-spot method (Van Staden *et al.* 2011). Activity was expressed in arbitrary units per ml (AU ml^{-1}). One AU is defined as the reciprocal of the highest serial two-fold dilution showing a clear zone of growth inhibition. *Listeria monocytogenes* EGD-e, grown in LEB supplemented with 7·5 µg ml^{-1} chloramphenicol, and incubated at 37°C on a shaker, was used as sensitive strain.

Preparation of microparticles

Bacteriocin loaded microparticles were produced using an electrospraying method. PLGA (15% w/v) was dissolved in DMF containing peptide ST4SA (15 mg ml^{-1}). No bacteriocin was added for un-associated particles (UP). A setup using a sterile tapered glass tube and high voltage supply was used for the production of SPM and UP (Fig. 1). The cathode was placed in the glass tube containing the polymer solution and anode was connected to the collector. A constant potential difference of 12 kV was applied to polymer solution and -5 kV to the collector (1.133 kV/cm). The glass tube was kept at an angle of 0 to 5°. The relative humidity was kept below 30% and the temperature at 25°C. Particles were collected and used in further experiments.

The bacteriocin concentration in 25 mg SPM was determined by dissolving the particles in DMF. The protein concentration (mg ml^{-1}) was determined by using the colorimetric BCA protein assay method at 562 nm and compared to a bovine serum albumin standard curve.

Preparation of bone cement composites

Cement samples were prepared according to the method described by Barralet *et al.* (2004). β-TCP and MCPM were mixed in equimolar amounts with a pestle and mortar. The powder (250

mg) was mixed with 75·76 µL 500 mM tri-sodium citrate (P/L ratio of 3·3 g ml^{-1}), which served as retardant. Particles were added to the powder component of the cement (10% w/w) before mixing with 500 mM tri-sodium citrate. Mixing was performed on a glass slab for 30 s to form a homogeneous paste. The paste was moulded into sterile insulin syringes, from which the tips have been cut off, and left to dry until hard tablets formed. The set cement cylinders were approx. 8 mm long and 4 mm in diameter.

Characterization of bone cement composites and particles

The crystal structure of the bone cement and particle morphology was studied by using scanning electron microscopy (SEM; Zeiss EVO MA15VP, Zeiss, Oberkochen, Germany). The cement cylinders were fractured and particles were sprayed onto foil, placed on an adhesive stub and coated with gold, before SEM analysis. Images were used to determine size distribution of particles using SEM Image studio software (version 10·1).

Powder X-ray diffraction (XRD) patterns of the set cement cylinders were recorded on a multi-purpose diffractometer (PANalytical X'Pert PRO, PANanyltical, Almelo, The Netherlands). Data were collected from $2\theta = 10° - 60°$ with a step size of 0·02° and a normalized count time of 1 s/step using Cu Ka_{α} radiation. Phase composition was checked by means of ICDD (international centre for diffraction data) reference patterns for β-TCP (00-009-0348), MCPM (01-070-0359), brushite (00-009-0077 & 01-072-0713) and hydroxyapatite (00-001-1008). XRD was performed on samples containing 10% w/w SPM or unloaded cement samples.

Release studies

All release studies were carried out in PBS buffer (pH 7·4) at 37°C and agitation; 25 mg SPM were added to 1 ml buffer and incubated for several hours. Particles were spun down at 8000 ***g*** for 1 min to avoid accidental removal. Removed buffer was replaced with fresh buffer and incubated again. Cement samples were prepared as previously described and incubated in 5·0 ml PBS buffer for release studies, un-dissolved particles were harvested at 8000 ***g*** for 1 min. Protein concentration (mg ml^{-1}) in the particle-free buffer was determined by using the colorimetric BCA protein assay method at 562 nm and the concentration determined by using a bovine serum albumin standard curve.

Activity of particles and cement composites

Particles were scattered over an LEB agar (1·0% w/w) plate seeded with *L. monocytogenes* EGD-e and incubated at 37°C 24 h. Brushite-SPM or -UP composites were placed into LEB agar (1·0% w/w) plate seeded with *L. monocytogenes* EGD-e and incubated at 37°C 24 h. Observation of clear zones around samples was indicative of antimicrobial activity.

Calculations and analysis of data

Data were collected in Microsoft Excel 2007. Data points indicated with standard deviations; represent an average of three repeats. The rate of bacteriocin release is presented as the 2-point moving average. Student's t test was used to compare release curves.

Results

The average bacteriocin concentration in 25 mg SPM was 3·73 mg ml^{-1} (±0·63). Electrosprayed UP had sizes ranging from 1014 nm to 1992 nm (Fig. 2), with an average of 1101·51 nm (±179·1). Particles sprayed with bacteriocin had sizes ranging from 330 nm to 2520 nm (Fig. 3), with an average size of 1048·4 nm (±322). Incorporation of the SPM into the cement did not result in significant structural changes (Fig. 4 A and B). Particles could, however, be visualised imbedded in the cement (Fig. 4B). XRD patterns of control cement and brushite-SPM composites compared well with each other (Fig. 5). The diffraction patterns also corresponded well to that of brushite (ICDD 00-009-0077 & 01-072-0713). Bacteriocin was released from SPM over a period of 240 h (Fig. 6). After the first 12 h, 36·5% (± 1·43) of the total bacteriocin associated with the particles was released, followed by a 1·6% (±0·18) release over the next 228 h. Brushite-SPM composites had a significant reduction in burst release of the bacteriocin in the first 12 h ($p<0·001$; Fig. 6) compared with SPM not incorporated. Within the first 12h 23·2% (±1·21) was released from the brushite-SPM composites, followed by a 7·9% (±1·8) release up to 240 h. Particle and brushite-SPM composites released active bacteriocin and inhibited the growth of *L. monocytogenes* EGD-e (Fig. 7).

Discussion

Electrospraying was used to produce particles in the submicron range; SPM had a wider size distribution than UP. The wider range in size distributions seen in SPM may be due to variations in conductivity and viscosity of the bacteriocin polymer solution (Morota *et al.* 2004, Xu and Hanna 2006). Addition of SPM to cement did not have a significant effect on the setting reaction or entanglement of crystals, as shown with XRD and SEM analysis. Similar results have been obtained with the addition of PLGA and other microparticles to CPC (Schnieders *et al.* 2006,

Fullana *et al.* 2010). Low concentrations of bacteriocin were released from PLGA micro particles (i.e. 38·1% ±1·5). Salmaso *et al.* (2004) also reported a low release for nisin A from PLA particles. However, they found that the nisin A released from particles inhibited the growth of a sensitive strain for up to 40 days in broth (Salmaso *et al.* 2004). Bacteriocins can be released at small concentrations over longer periods of time thus they are very potent and can be active at picomolar concentrations (Morgan *et al.* 2005, Nissen-Meyer *et al.* 2009). Release of bacteriocin from particles was with an initial burst in the first 12 h. After addition of SPM to the cement a more gradual initial release was noted, followed by a steady release of bacteriocin. Schnieders *et al.* (2006) reported a similar result with the addition of gentamicin-loaded PLGA particles into CPC. Addition of the particles to the cement significantly reduced burst release (Schnieders *et al.* 2006). This can be attributed to a combination of embedding of SPM in the cement matrix, polymer degradation (depended on buffer contact) and interaction between the bacteriocin and cement. The properties of PLGA may also contribute to the slow release profiles. In PLGA lactic acid and glycolic acid are chemically bound together as a result ester bonds need to be hydrolysed for PLGA to be degraded (Nijenhuis *et al.* 1996; Wu and Wang 2001). Lactic acid is more hydrophobic than glycolic acid therefore lactide rich PLGA degrades more slowly resulting in delayed release kinetics (Nijenhuis *et al.* 1996; Wu and Wang 2001). All of these factors influence release kinetics of the bacteriocin from the particles and cement (Schnieders *et al.* 2006). The release of unloaded peptide ST4SA is associated with a high burst release with more than 80% of peptide ST4SA released (van Staden *et al.* 2011). Reducing the release of therapeutic agents may be advantageous for prolonged activity and protection from degrading substances (i.e. proteases). Bacteriocin released from the particles and the brushite-SPM composites inhibited the growth of *L. monocytogenes* EGD-e, indicating that biological activity was retained. Heunis *et al.* (2010) reported a decrease in activity of plantaricin 423 after electrospinning into fibers. However Xie and Wang (2007) found that after electrospraying BSA retained its secondary structure and was not degraded after prolonged release, suggesting that the technique does not significantly influence protein structure (Xie and Wang 2007). They also reported a high retention of lysozyme bioactivity of 92% (Xie and Wang 2007). The effect of electrospraying on the activity of peptide ST4SA needs to be determined.

In conclusion, peptide ST4SA was electrosprayed in association with PLGA to form submicron particles. The particles released active bacteriocin at a delayed rate and incorporation of the SPM into brushite bone cement resulted in a decrease in initial burst release. The results reported here suggest that it is possible to associate bacteriocins with a polymer to form submicron PLGA particles using electrospraying and produce brushite-SPM composites.

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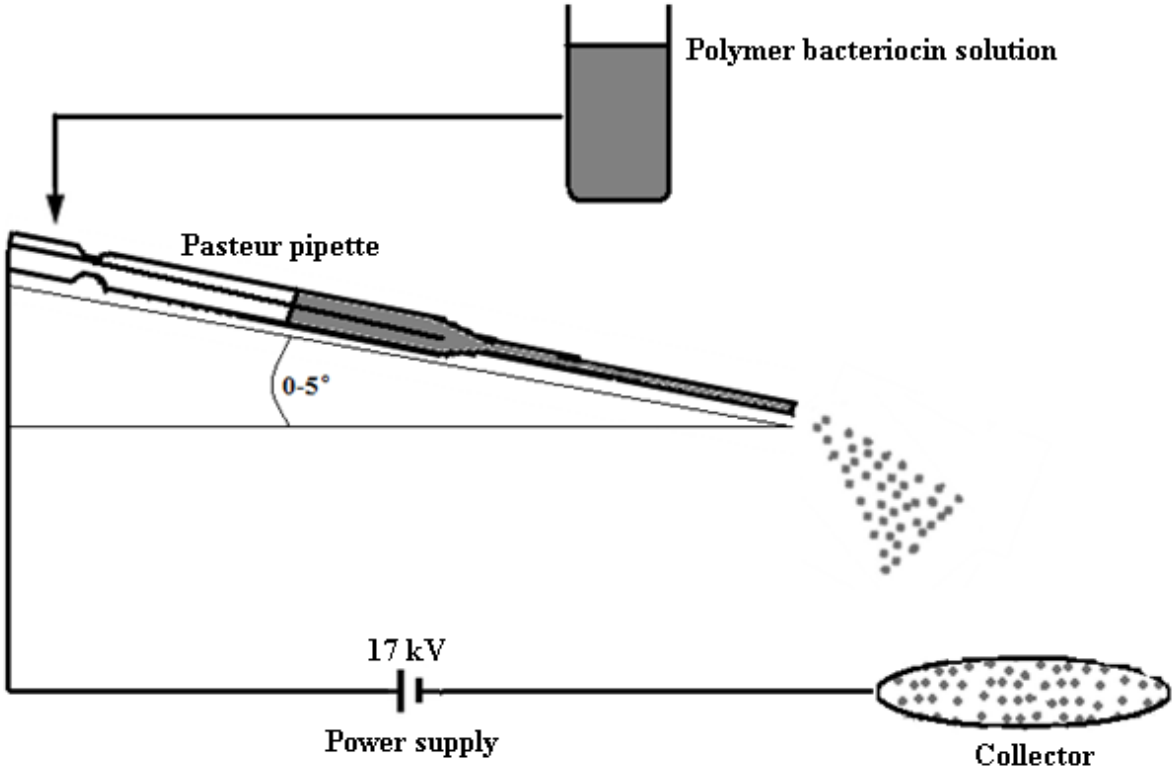


Figure 1 Diagram of the electrospraying setup used.

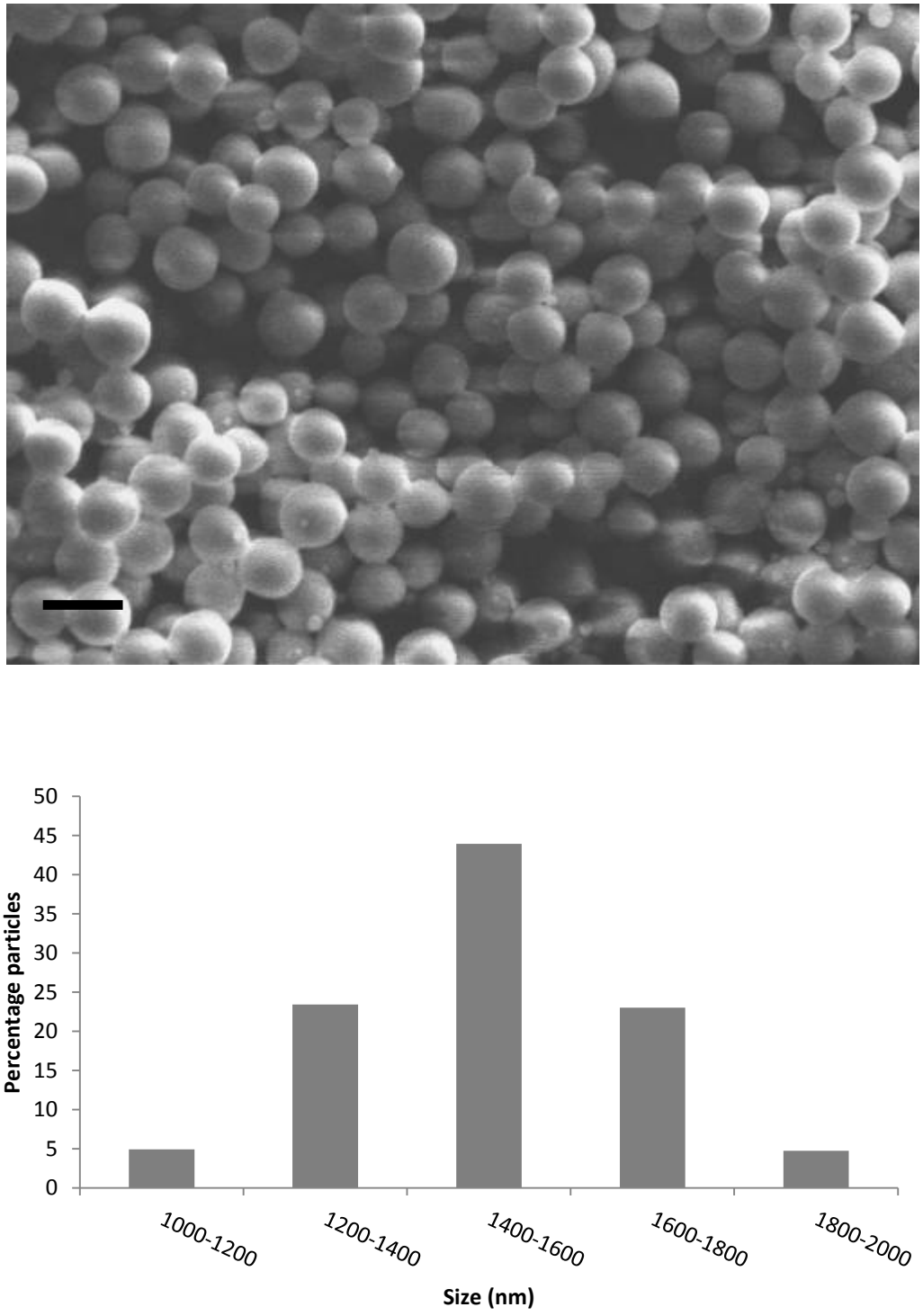
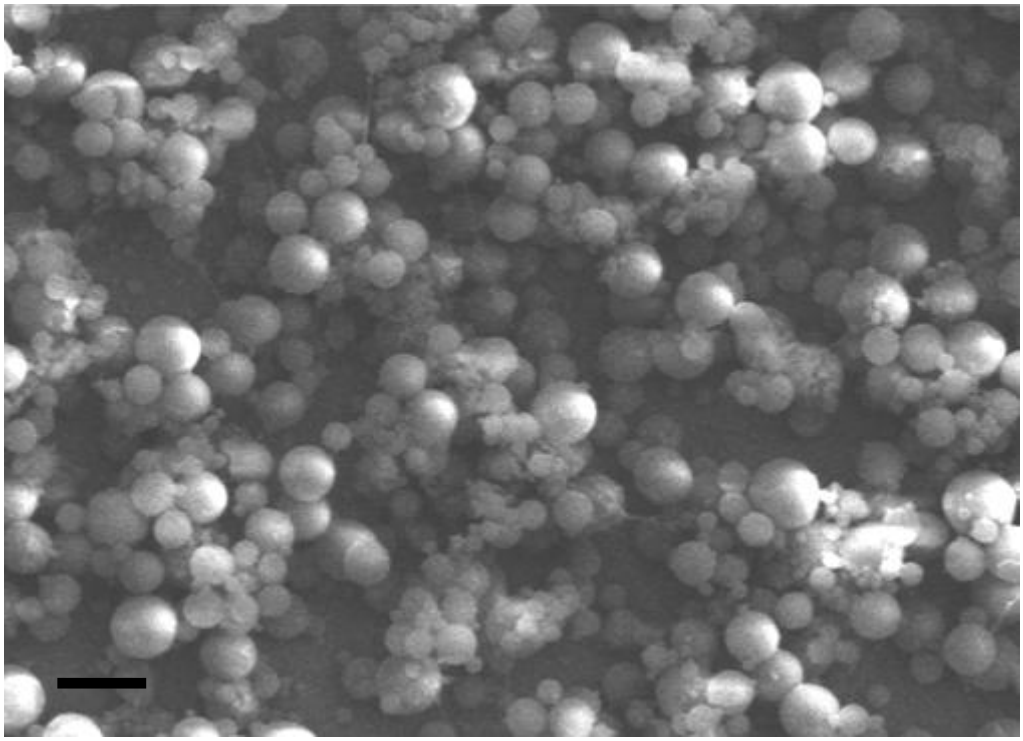


Figure 2 Scanning electron microscope (SEM) images of UP The scale bar represents 2 μm (image captured at 5000 times magnification). Graph represents size distribution of UP according to analysis with SEM Image studio.



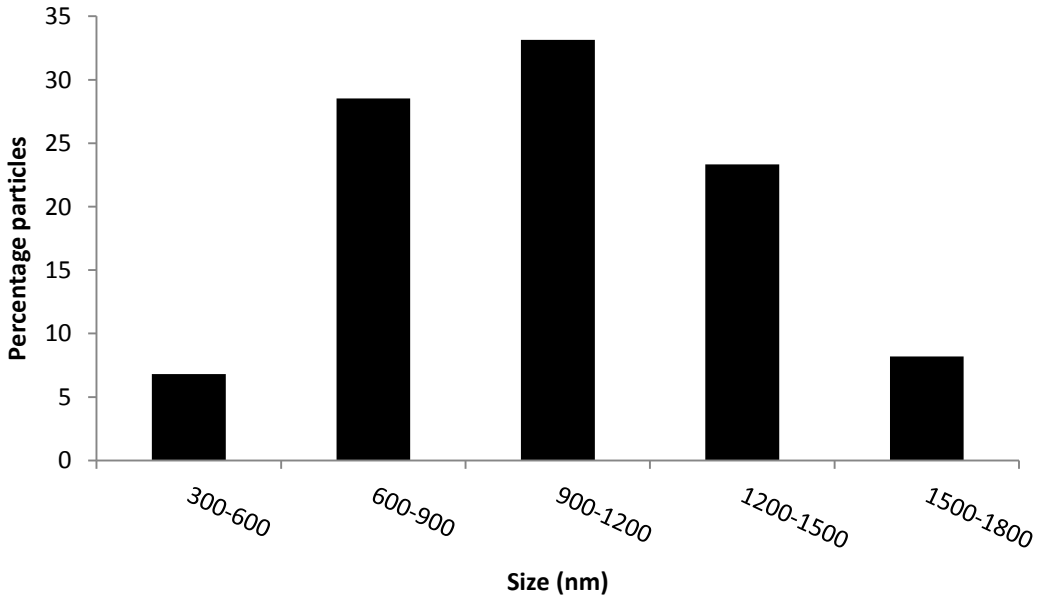


Figure 3 SEM images of SPM. The scale bar represents 2 μm (image captured at 5000 times magnification). Graph represents size distribution of SPM according to analysis with SEM Image studio.

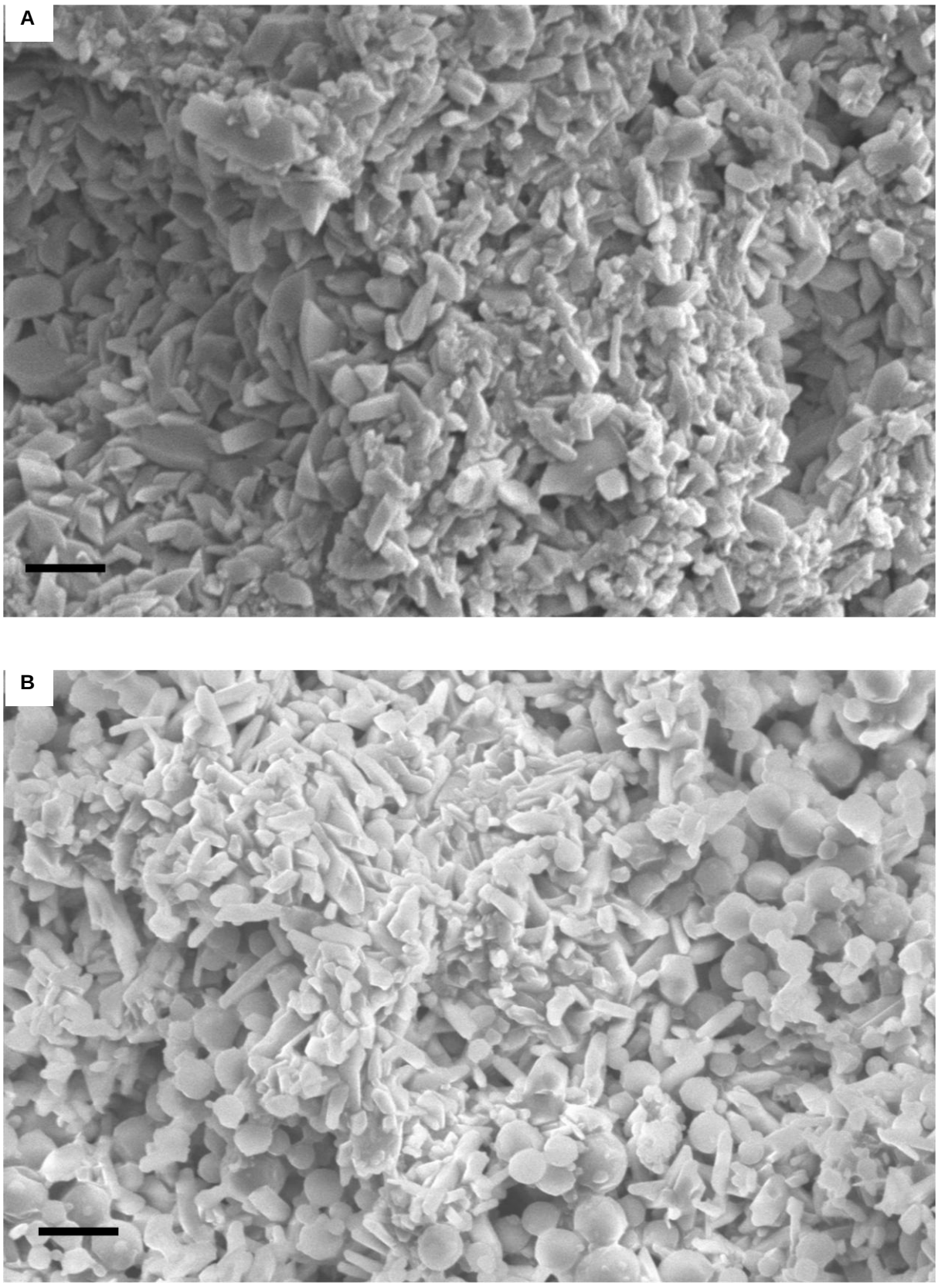


Figure 4 SEM images of brushite cement samples without SPM (**A**) and cement samples containing 10% w/w, SPM (**B**). The scale bar represents 2 μm (image captured at 5000 times magnification).

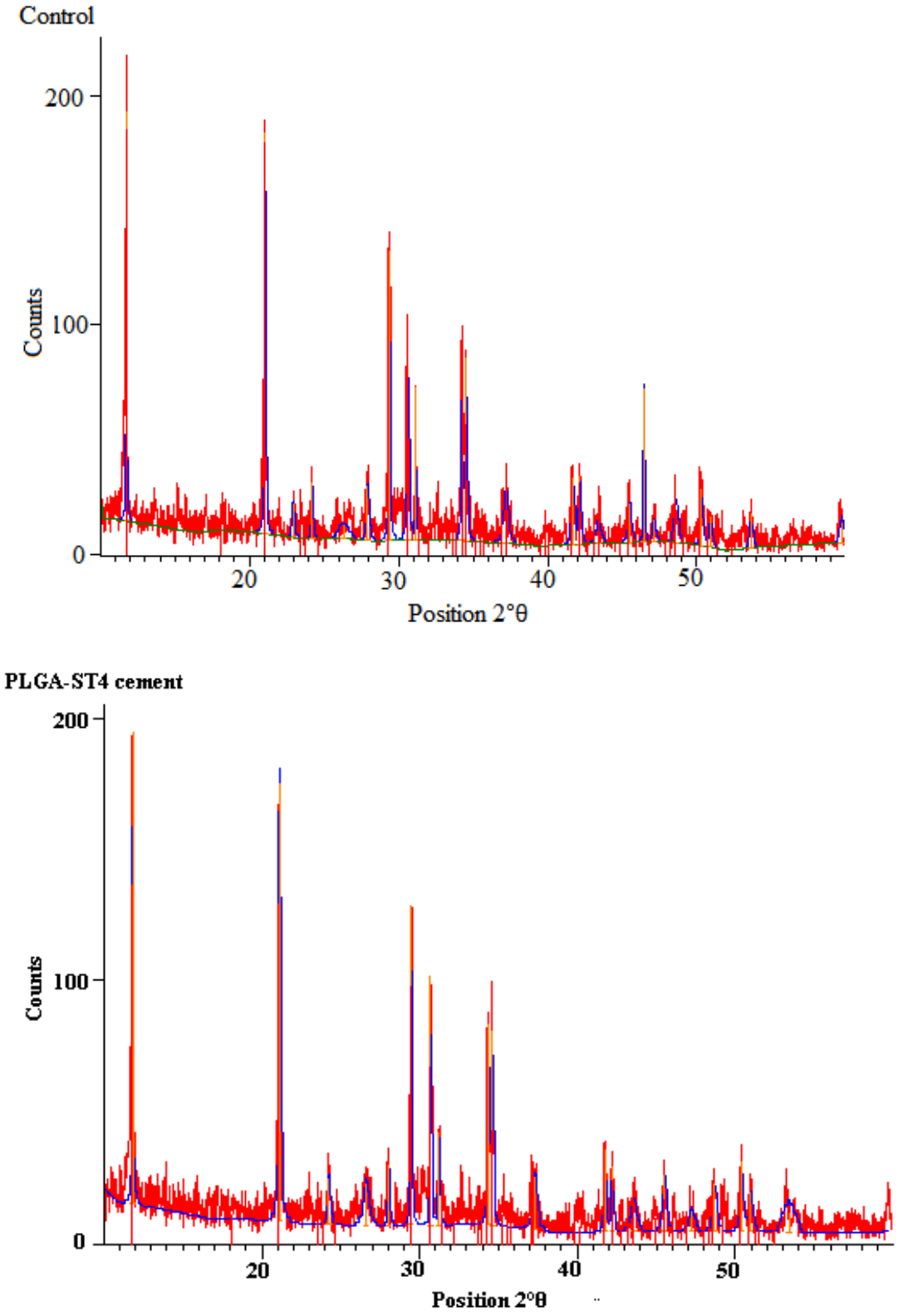


Figure 5 XRD patterns of unloaded (control) cement samples and brushite-SPM composites (PLGA-ST4 cement). The red lines indicates the main brushite peaks (ICDD 00-009-0077).

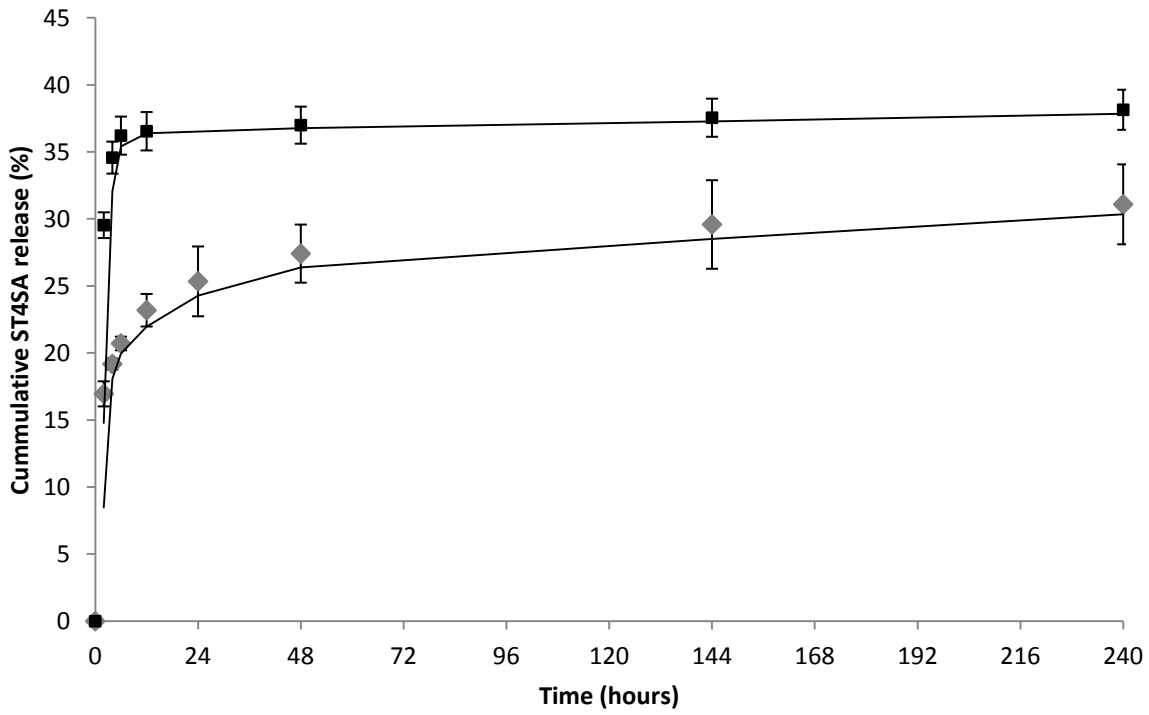


Figure 6 Cumulative release of peptide ST4SA from SPM (■) and brushite-SPM composites (◆), expressed as percentage of original protein content.

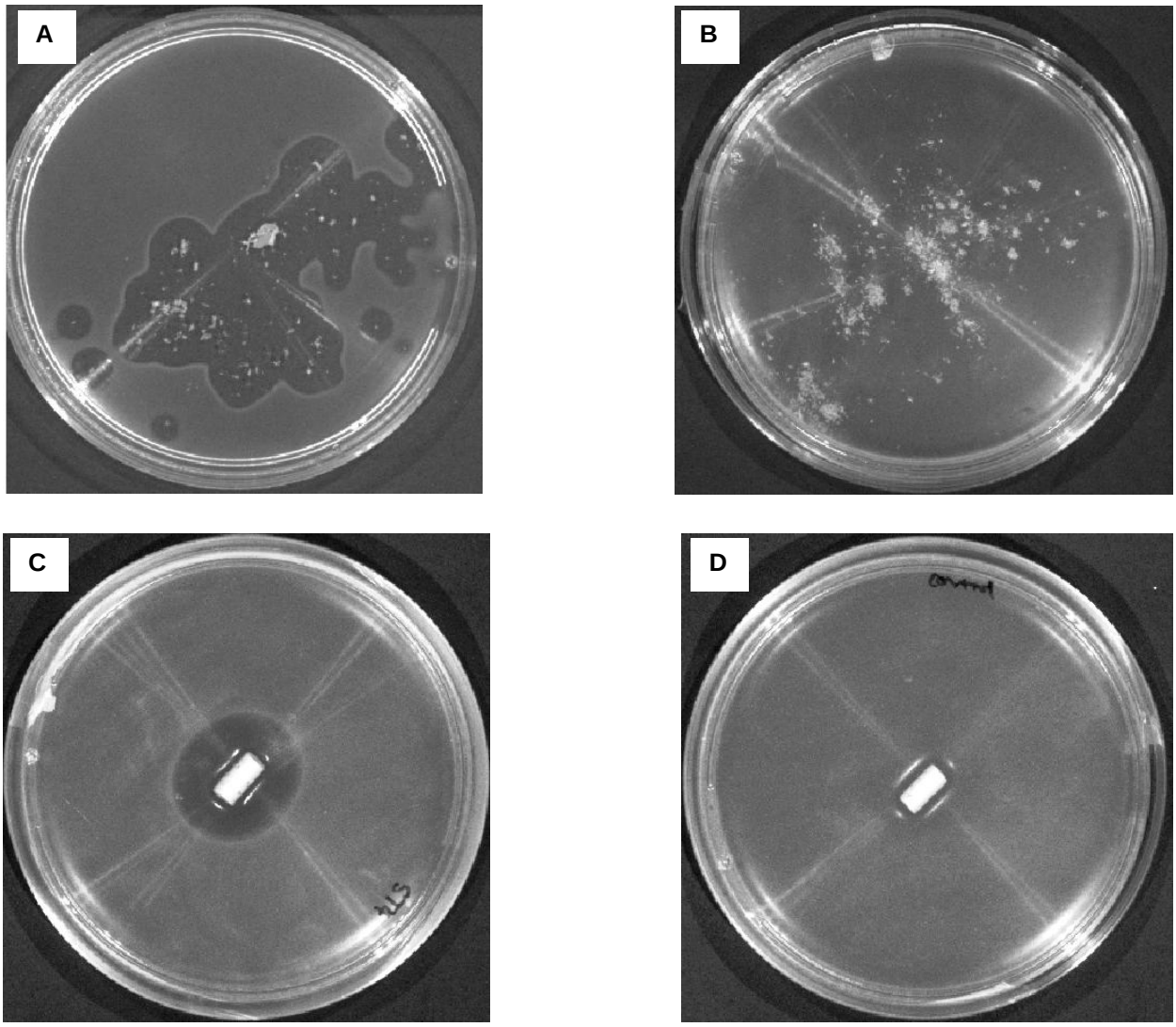


Figure 7 Soft agar (1% w/w) plates seeded with *Listeria monocytogenes* EGD-e, scattered SPM (**A**) and UP (**B**). Imbeded with brushite-SPM composites (**C**) and brushite-UP composites (**D**).

Chapter 5

Nisin F-loaded brushite bone cement prevented the growth of *Staphylococcus aureus in vivo*[*]

[*]This chapter has been provisionally accepted for publication in Journal of Applied Microbiology.

Nisin F-loaded brushite bone cement prevented the growth of *Staphylococcus aureus in vivo*

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Abbreviated running headline: Inhibition of *S. aureus* by nisin F in bone cement

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Abstract

Aims: To determine if nisin F-loaded self-setting brushite cement could control the growth of *Staphylococcus aureus in vivo*.

Methods and Results: Brushite cement was prepared by mixing equimolar concentrations of β-tricalcium phosphate and monocalcium phosphate monohydrate. Nisin F was added at 5·0%, 2·5% and 1·0% (w/w) and the cement moulded into cylinders. *In vitro* antibacterial activity was determined using a delayed agar diffusion assay. Release of nisin F from the cement was determined using BCA protein assays. Based on scanning electron microscopy and X-ray diffraction analysis, nisin F did not cause significant changes in cement structure or chemistry. Cement containing 5·0% (w/w) nisin F yielded the most promising *in vitro* results. Nisin F-loaded cement was implanted into a subcutaneous pocket on the back of mice and then infected with *S. aureus* Xen 36. Infection was monitored for 7 days, using an *in vivo* imaging system. Nisin F prevented *S. aureus* infection for 7 days and no viable cells were isolated from the implants.

Conclusions: Nisin F-loaded brushite cement successfully prevented *in vivo* growth of *S. aureus*.

Significance and Impact of the Study: Nisin F incorporated into bone cement may be used to control *S. aureus* infection *in vivo*.

Keywords

Nisin F, brushite, bone cement, *in vivo* activity, *S. aureus* growth inhibition

Introduction

Bacterial infection is one of the major complications in orthopedic surgery and may lead to permanent damage of tissue or bone (Krishnan *et al*. 1993; Fialkov *et al.* 2001; Cosgrove and Carmeli 2003). Antibiotics orally or intravenously administered are often ineffective, due to poor penetration of infected sites (Frommelt and Kühn 2005; Zimmerli 2006). Furthermore, biofilms that form on implants may also prevent the migration of immune cells to infected areas (Stewart and Costerton 2001). Antibiotic-loaded bone cement may be a solution to the control of orthopedic related bacterial infections (Jiranek *et al.* 2006).

Polymethylmethacrylate (PMMA) antibiotic-loaded bone cements have been used in orthopedic surgery (Van de Belt *et al*. 2001). Antibiotic-loaded bone cement can lower the rate of infection by 50% in primary hip arthoplasty and with 40% in revision hip arthoplasty of previously infected implants (Parvizi *et al*. 2008). PMMA bone cements are however not bioresorbable. Calcium phosphate bone cement (CPC) has excellent bioactivity, bioresorbability and biocompatibility properties and is considered more practical than PMMA by some researchers (Ruhe *et al.* 2005; Ginebra *et al.* 2006b; Schnieders *et al.* 2006; Zhang *et al.* 2008; Alkhraisat *et al.* 2009, Hofmann *et al.* 2009; Alkhraisat *et al.* 2010; Fullana *et al.* 2010). CPC can easily be molded to defects as well as be injected, and sets *in situ* (Ginebra *et al.* 2006a). In addition to these properties, CPC sets at low temperatures, which allows the incorporation of heat sensitive drugs or proteins (Bohner 2000). Antibiotic-loaded CPC proved effective in the control of *in vivo* infections (Hamanishi *et al.* 1996; Otsuka *et al.* 1997; Stallmann *et al.* 2004; Joosten *et al.* 2005; Ginebra *et al.* 2006b). However, CPC has a low mechanical strength and can only be used in non- or low load-bearing areas (Ginebra *et al.* 2006a).

The frequent use of antibiotic-loaded bone cement may increase the chances of developing antibiotic-resistant strains (Thornes *et al.* 2002). Bacteriocins may offer an alternative to conventional antibiotics; these are small cationic or amphiphilic antimicrobial peptides that inhibit the growth of closely related species (Cotter *et al.* 2005). A few bacteriocins, including nisin, have been described with activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) (Piper *et al.* 2010). Nisin binds to lipid-II on bacterial cell walls and inserts into the cell membrane to form pores, resulting in the efflux of intracellular material and dissipation of the proton motive force (Hsu *et al.* 2004; Bauer and Dicks 2005).

In the current study we determine the antibacterial activity of nisin F incorporated into brushite bone cement. *In vivo* studies were conducted on mice that have been infected with a bioluminescent strain of *S. aureus*. Changes in infection were recorded by monitoring changes in bioluminescence.

Materials and methods

Monocalcium phosphate monohydrate (MCPM, 85%) and β-tri calcium phosphate (βTCP, 95%) were purchased from Sigma-Aldrich (Sigma-Aldrich, Steinheim, Germany). Tri-sodium citrate dihydrate was purchased from Saarchem (Saarchem, Gauteng, South Africa), and ammonium sulfate (99·5%) from Merck (Merck, Darmstadt, Germany). Dialysis membranes (1 kD, Spectra/Por® 6) were purchased from Spectrumlabs (Spectrum Inc., CA, USA). *Bac*klight™ LIVE/DEAD bacterial viability kits (L7012) were from Invitrogen (Invitrogen, Leiden, The Neterlands). All growth media used were from Biolab (Biolab Diagnostics, Midrand, South Africa).

Preparation of nisin F

Lactococcus lactis subsp. *lactis* F10 (De Kwaadsteniet *et al.* 2008) was cultured in 1 litre De Man Rogosa and Sharpe (MRS) broth for 24 h at 30°C. Cells were harvested at 8000 ***g*** for 15 min at 4°C. The pH of the cell-free supernatant was adjusted to 6·5 to 7·0 and then treated at 80°C for 10 min to inactivate proteolytic enzymes. Proteins were precipitated from the cell-free supernatant with 80% saturated ammonium sulphate (Maniatis *et al.* 1982). The precipitate was collected by centrifugation at 10000 ***g*** for 1 h at 4°C and the pellet re-suspended in 10 ml sterile distilled water. The suspension containing the lantibiotic was dialyzed against 4 litres sterile distilled water using a 1 kDa cut-off dialysis membrane. The dialyzed product was concentrated by freeze-drying, resulting in semi-purified nisin F and stored at -80°C. Antimicrobial activity was determined by using the agar-spot method (Knoetze *et al.* 2008). Activity was expressed in arbitrary units per ml (AU ml^{-1}). One AU is defined as the reciprocal of the highest serial two-fold dilution showing a clear zone of growth inhibition. *S. aureus* Xen 36, grown in Brain Heart Infusion (BHI) broth or BHI soft agar (1·0%, w/v, agar), supplemented with 200 µg ml^{-1} kanamycin, was used as sensitive strain. Incubation was at 37°C for 24 h.

Preparation of bone cement containing nisin F

Bone cement samples were prepared according to the method described by Barralet *et al.* (2004). Equimolar amounts of βTCP and MCPM were mixed using a pestle and mortar. Freeze-dried

nisin F was added to 250 mg dry cement powder to yield final concentrations of 5·0%, 2·5% and 1·0% (w/w), respectively. The same quantity of freeze-dried nisin F was suspended in sterile distilled water and activity determined by using the agar-spot method as described before. Tri-sodium citrate (75·76 µl of 500 mM; P/L ratio of 3·3 g ml^{-1}) was added to the cement powder to slow down setting. Mixing to a homogeneous paste was done on a glass slab for 30 s. The paste was moulded into sterile disposable insulin syringes, of which the tips have been cut off, and left to set. The set bone cement cylinders were approx. 8 mm long and 4 mm in diameter.

Structural analysis of bone cement

Powder X-ray diffraction (XRD) patterns of the set bone cement cylinders were recorded on a multi-purpose diffractometer (PANalytical X'Pert PRO, PANanyltical, Almelo, The Netherlands). Data were collected from 2θ = 10° to 60° with a step size of 0·02° and a normalized count time of 1 s $step^{-1}$ using Cu Ka_{α} radiation. Phase composition was checked by means of ICDD (international centre for diffraction data) reference patterns for β-TCP (00-009-0348), MCPM (01-070-0359), brushite (00-009-0077 and 01-072-0713) and hydroxyapatite (00-001-1008). The crystal structure of the bone cement was studied using a scanning electron microscope (SEM; Zeiss EVO MA15VP, Zeiss, Oberkochen, Germany). The bone cement cylinders were fractured and placed on an adhesive stub and sputter-gold coated before analysis. Images were taken at 5000 times magnification at 7.00 kV and a working distance of 10 mm.

***In vitro* antimicrobial activity and release of nisin F from bone cement**

The activity of nisin F incorporated into bone cement was studied using a delayed agar-diffusion method (Bayston and Milner 1982). Bone cement cylinders containing the three different concentrations of nisin F were each covered with 20 ml BHI soft agar (1·0%, w/v, agar) that has been seeded with 1·0 ml *S. aureus* Xen 36 (1·0 x 10^7 CFU ml^{-1}) and then incubated at 37°C for 24h. Antimicrobial activity was recorded by measuring the diameter of the growth inhibition zone through the centre of the bone cement cylinder. The cylinders were then aseptically removed, placed into a fresh plate of soft agar seeded with *S. aureus* Xen 36 (1·0 x 10^7 CFU ml^{-1}) and incubated at 37°C for 24h. Transfer of the cylinders from one plate to the other was repeated until no growth inhibition was observed.

In another experiment, washed cells of *S. aureus* Xen 36 (1·0 x 10^8 CFU ml^{-1}) were stained with the *Bac*klight™ LIVE/DEAD fluorescent probes SYTO 9 (emits green when excited with 472 nm) and propidium iodide (emits red when excited with 572 nm), according to the

manufacturer's instructions. *S. aureus* Xen 36 (1·0 x 10^7 100 μl^{-1}) was added to 20 mg bone cement, supplemented with 5·0% (w/w), 2·5% (w/w) and 1·0% (w/w) nisin F, respectively, and incubated for 60 min at 37°C. Bone cement without nisin F served as control. At 5·0, 30 and 60 min, samples were exposed to 472 nm and 572 nm, using a Xenon-Arc burner (Olympus Biosystems GmbH, Germany). Fluorescence was recorded using an inverted fluorescence microscope fitted with an Olympus Cell^R system attached to an IX-81 (Olympus Biosystems GmbH, Hamburg, Germany). Images were taken with a F-view-II cooled CCD camera (Soft Imaging Systems, Hamburg, Germany). Emission was collected using a UBG triple- bandpass emission filter cube. For the *z*-stack image frame acquisition, an Olympus Plan Apo N 60x/1·4 Oil objective and the Cell^R imaging software were used. Images were processed, background-subtracted, and expressed as maximum intensity projection.

The rate at which nisin F diffused from the bone cement cylinders was estimated from the rate at which proteins were released. Bone cement cylinders containing 5·0% (w/w) nisin F were incubated in 5·0 ml Phosphate Buffered Saline (PBS) buffer (pH 7·4) at 37°C and slowly stirred. At selected time intervals the entire volume of buffer was collected and centrifuged at 8000 ***g*** for 1·0 min to remove non-dissolved particles. The protein concentration (mg ml^{-1}) in the supernatant was determined by using the colorimetric BCA protein assay method at 562 nm. Readings were recorded from standard curves prepared with bovine serum albumin. Buffer extracted was replaced with fresh buffer and the cylinders incubated until the next reading. Readings were recorded over 120 h. Release of protein from the bone cement cylinders was expressed as percentage of the protein content originally present in the sample. In another experiment, samples were incubated in PBS buffer (pH 7.4) for 24 h after which antimicrobial activity of released nisin F, in the PBS buffer, was tested against *S. aureus* Xen 36 seeded in BHI soft agar.

Hemolytic activity of nisin F bone cement

The hemolytic activity of nisin F-loaded bone cement was determined spectrophotometrically using a hemoglobin release assay. Sheep erythrocytes were collected by centrifugation at 800 ***g*** for 10 min and washed three times with PBS (pH 7·0; 10 min at 800 ***g***). Diluted (10x in PBS) red blood cells (RBC; 200 µl) were added to 20 mg bone cement samples with 5·0% (w/w) nisin

F and without nisin F. Samples were incubated at 37°C for 1·0 h. Aliquots (100 µl) were removed from samples and centrifuged at 4000 ***g*** for 10 min. Supernatants (50 µl) were collected and read spectrophotometrically at 541 nm to determine hemoglobin release. Zero and 100% hemolysis was determined using PBS (pH 7·0) and 0·1% Triton X100, respectively.

Insertion of bone cement cylinders into mice

Experiments on animals have been approved by the Ethics Committee of the University of Stellenbosch (ethics reference number: 11NM_DIC01). Male mice (BALB/c), weighing between 20 and 30 g, were fed a standard rodent diet and kept under controlled environmental conditions (12 h light-dark cycles, 20-22˚C).

Twenty-four mice (six per group) were anaesthetized via inhalation with 2·0% (v/v) isoflurane (Isofor, Safe Saline pharmaceuticals, Florida, South Africa). The back of each mouse was shaved and disinfected with Betadine (10%, v/v). A mid-scapular incision, approx. 1·0 cm in length, was made and the bone cement cylinder inserted into the subcutaneous pocket. Bone cement implanted in mice of groups 1 and 3 did not contain nisin F, whereas the implants used in groups 2 and 4 contained 5·0% (w/w) nisin F. The subcutaneous pockets of mice in groups 3 and 4 were infected with 100 µl *S. aureus* Xen 36 (1·0 x 10^3 CFU 100 μl^{-1}), whereas 100 μl sterile saline was injected in the subcutaneous pockets of the control mice (groups 1 and 2). Incisions were closed using CliniSut 1·5 (4/0) sutures (Akacia health, Port Elizabeth, South Africa) and dabbed with 10% (v/v) Betadine. Pain was controlled by injecting the mice with bupronorphine (0·03 mg kg^{-1} bodyweight; Temgesic, Schering-Plough (Pty) Ltd., Woodmead, South Africa) directly after surgery and 24 h later.

Evaluation of *in vivo* infection

S. aureus infections were recorded using the Xenogen *in vivo* bioluminescent imaging system (Xenogen IVIS®, Caliper Life Sciences, Hopkinton, MA, USA). Images were recorded directly after closing of the subcutaneous pocket and every 24 h thereafter for 7 days. Readings are expressed as average radiance (photons cm^{-2} $second^{-1}$ $steridian^{-1}$) within a circular region of interest (1·0 x 10^3 pixels) and calculated by using Living Image® software (Bernthal *et al.* 2010; Caliper Life Sciences). After 7 days of observation the animals were killed with an overdose of pentabarbitione sodium (Euthapent, Kyron Laboratories (Pty) Ltd., Benrose, South Africa) and the bone cement cylinders removed. Swabs were taken from the subcutaneous pockets and streaked onto BHI agar supplemented with 200 μg ml^{-1} kanamycin. The bone cement implants

were vigorously vortexed in 1·0 ml sterile saline for 5·0 min. The suspensions were serially diluted and plated out onto the same growth medium. All plates were incubated at 37°C for 24 h. Blood were collected one day before surgery and on days 0, 4 and 5 form selected mice in group 1 and 2 (n=6) for preliminary analysis of total and differential leukocyte counts.

Statistical analysis

All experiments were conducted in triplicate, unless otherwise stated. Standard deviation was calculated using Student's t-test (two-tailed) with $p < 0{\cdot}05$, $p < 0{\cdot}01$ and $p < 0{\cdot}001$ considered as significant.

Results

Activity of nisin F

Freeze-dried nisin F suspended in sterile distilled water at 5·0%, 2·5% and 1·0% (w/v) yielded an activity of 6,400, 3,200 and 1,280 AU ml^{-1}, respectively. Thus, the activity of nisin F in the bone cement cylinders (250 mg) corresponded to 6,400 AU, 3,200 AU and 1,280 AU.

Structural analysis of bone cement

The XRD patterns showed minor peaks for βTCP and MCPM in the bone cement cylinders, with and without nisin F (Fig. 1 A and B). The patterns for samples with and without nisin F corresponded well with the ICCD pattern of brushite and did not indicate significant changes with the addition of nisin F. SEM images of fractured bone cement cylinders, with and without nisin F (5·0% w/w) showed densely packed plates and blocks of brushite as well as clusters of smaller particles (Fig. 1 A and B). This indicates that the addition of nisin F did not interfere with crystal entanglement and therefore crystal structure.

***In vitro* antimicrobial activity and release of nisin F from bone cement**

All nisin F-loaded bone cement samples (6,400 AU, 3,200 AU or 1,280 AU) released active nisin F. Samples containing 6,400 AU and 3,200 AU nisin F clearly inhibited the growth of *S. aureus* Xen 36 on agar plates for at least 72 h (Fig. 2). There was no statistical difference in the activity between bone cement samples containing 6,400 AU and 3,200 AU nisin F ($p > 0{\cdot}05$), except after 24 h ($p < 0{\cdot}05$). Samples incorporated with 1,280 AU nisin F inhibited the growth of *S. aureus* Xen 36 for only 48 h. There was an increase in inhibition zone size in samples

containing nisin F 6,400 AU from 24 h to 48 h, however this was not statistically significant ($p > 0{\cdot}05$).

A few membrane compromised bacteria were observed in bone cement without nisin F. However, in the presence of all nisin F-loaded bone cement formulations the majority of bacteria showed signs of membrane damage (stained red) after 30 min (Fig. 3). Most of the nisin F samples (containing 6,400 AU) was released from bone cement into PBS buffer (pH 7·4) during the first 12 h (Fig. 4; 71·7 % ±0·81). This was followed by a steady release of 4·98 % (±0·81) over the next 108 h. Release studies were only done on samples containing 6,400 AU. PBS buffer (pH 7.4), containing released nisin F from samples incubated for 24 h, retained activity against *S. aureus* Xen 36 seeded in BHI soft agar.

Hemolytic activity of nisin F bone cement

Nisin F-loaded samples showed minimal hemolysis, with 9·28 % (±2·08) relative hemolysis as compared to 0·1 % v/v Triton X100 after 1·0 h incubation at 37°C.

***In vivo* activity of nisin F incorporated into bone cement**

No bioluminescent cells of *S. aureus* Xen 36 were recorded in mice of groups 1 and 2 which were the uninfected control groups (Fig. 5). Mice implanted with nisin F-loaded (5·0% w/w) bone cement do not indicate any bioluminescent signals (group 4; Fig. 5) Bioluminescent cells were, however, recorded in mice of group 3 (bone cement implant without nisin F and infected with *S. aureus* Xen 36), from day one throughout the 7 day trial (Fig. 5). Implants collected from group 3 had high bacterial cell numbers associated with implants ($2{\cdot}3 \times 10^7$ CFU ml^{-1}) and *S. aureus* were isolated from the swabs. No viable cells were isolated from bone cement implants and wound swabs collected from mice in groups 1, 2 and 4. Preliminary blood analysis of mice in group 1 and 2 indicate that total and differential leukocyte counts did not undergo any significant changes in either total or subpopulation counts (not shown).

Discussion

Incorporation of nisin F into the bone cement did not interfere with the setting reaction or crystal entanglement of the cement. The incorporation of bacteriocin ST4SA into the same cement reported similar results (Van Staden *et al.* 2011). The residual levels of βTCP and MCPM are possibly due to un-reacted starting material and correlates with results reported for other therapeutic agents incorporated into bone cement (Bohner *et al.* 1997, Le Nihouannen *et al.*

2008, Tamimi *et al.* 2008). Nisin F was released in its active form from brushite bone cement and inhibited *S. aureus* growth, similar to results for bone cement loaded with bacteriocin ST4SA. Brushite bone cement sets at about 37°C and pH < 6·0 which provides a more favorable environment for the bacteriocin.

The steady decline in antimicrobial activity recorded over 72 h suggests that nisin F slowly diffused from the bone cement. At lower levels (1,280 AU per cylinder), the residual concentration of nisin F was too low to inhibit the growth of *S. aureus* Xen 36 for longer than 48 h. Release of antimicrobial agents from bone cement is influenced by a number of factors, e.g. solubility, interaction with cement particles and porosity of the bone cement (Ginebra *et al.* 2006a, Hofmann *et al.* 2009, Tamimi *et al.* 2009). Release of nisin F from bone cement is most likely dose dependent, as observed when higher concentrations were incorporated into bone cement and tested against *S. aureus* Xen 36. Bacteriocins are very potent at low concentrations and are able to dissipate the proton motive force of cell membranes within a few minutes (Winkowski *et al.* 1994). The fluorescent microscopy results illustrate the antimicrobial potency of nisin F, in killing the bacterial cells upon contact. Similar to results reported for other therapeutic agents, a high burst release of nisin F was observed in the first 12 h. (Bohner *et al.* 1997; Tamimi *et al.* 2008; Young *et al.* 2008; Hofmann *et al.* 2009; Fullana *et al.* 2010) and bacteriocin ST4SA (Van Staden *et al.* 2011).

Three concentrations of nisin F were evaluated *in vitro* to determine the optimal concentration for use in *in vivo* studies. From the *in vitro* results and preliminary *in vivo* trials (data not shown) it was seen that samples containing 6,400 AU nisin F most effectively inhibited *S. aureus*. Bioluminescent imaging has been used in a number of antimicrobial studies (Brand *et al.* 2010, De Kwaadsteniet *et al.* 2010) and proved effective in studying antimicrobial implants in mice (Bernthal *et al.* 2010). Nisin F incorporated (6,400 AU) in bone cement controlled *S. aureus* infection for 7 days. Infection in group 3 (infected control) could only be visualized with bioluminescence from day 1 onwards. This is most likely due to a combination of insufficient bacterial numbers present at day 0 and absorption of bioluminescent signal by animal tissue (Doyle *et al.* 2004). Brand *et al.* (2010) has shown that nisin F, intraperitoneally injected into mice, could control the growth of *S. aureus*, although for only 15 min, based on changes recorded in bioluminescence. The inability to suppress *S. aureus* over a longer period has been attributed to the rapid degradation of nisin F by proteolytic enzymes (Brand *et al.* 2010), or possibly non-specific binding of the peptide.

In another study, nisin F inhibited the growth of *S. aureus* in the respiratory tract of immune-compromised rats when administered through the nasal cavity (De Kwaadsteniet *et al.* 2009). Attempts to treat subcutaneous *S. aureus* infections with nisin F, on the other hand, were unsuccessful (De Kwaadsteniet *et al.* 2010). The reason for this has also been attributed to possible degradation of the peptide (De Kwaadsteniet *et al.* 2010). However, intravenous Teflon® catheters, which have been coated with nisin, implanted into the jugular veins of sheep have shown antimicrobial activity for more than 5 h (Bower *et al.* 2002).

Incorporation of nisin F into bone cement may facilitate controlled release of the peptide, and most probably delays the rate at which the peptide is degraded. Nisin used as a prophylactic seems to more effective than treatment of bacterial infections. This can be seen here as well as in several other studies (Ghiselli *et al.* 2004, De Kwaadsteniet *et al.* 2009, Brand *et al.* 2010, De Kwaadsteniet *et al.* 2010).

Hemolysis release assays can be used to study preliminary cytotoxicity of therapeutic agents (Maher and McClean 2006). Nisin has showed low toxicity against RBC which makes it a possible candidate for use in systemic infections (Maher and McClean 2006). Bower *et al.* (2002) also reported no reaction to implanted materials coated with nisin when implanted *in vivo*. Preliminary analysis of blood collected substantiates the hemolysis assay results with no significant changes observed in either total or subpopulation leukocyte counts of uninfected groups.

The prophylactic use of antimicrobial-loaded bone cement may result in the prevention of infection. However increase antibiotic-resistance has been reported *in vivo* with the use of antibiotic-loaded bone cement (Thornes *et al.* 2002). Nisin F may be an effective alternative to antibiotics. Of all nisin variants, nisin F showed the most promising results *in vitro* against MRSA and VRE (Piper *et al.* 2010). As with any antimicrobial the risk of the target organism acquiring resistance is still a possibility (Neu 1992, Crandall and Montville 1998, Walsh 2003, Kramer et al. 2004). Due to different mechanisms it may be beneficial to combine antibiotics and bacteriocins or two different peptides to reduce the risk of developing resistance (Brumfitt et al. 2002, Ghiselli *et al.* 2004, Naghmouchi *et al.* 2010).

The current study evaluated the effectiveness of nisin F-loaded brushite bone cement *in vitro* and *in vivo.* We have shown that *S. aureus* infection could be controlled for 7 days. Furthermore, nisin F implants that were removed did not show signs of contamination, compared with untreated implants. The results suggest the possible use of nisin F in bone cement implants.

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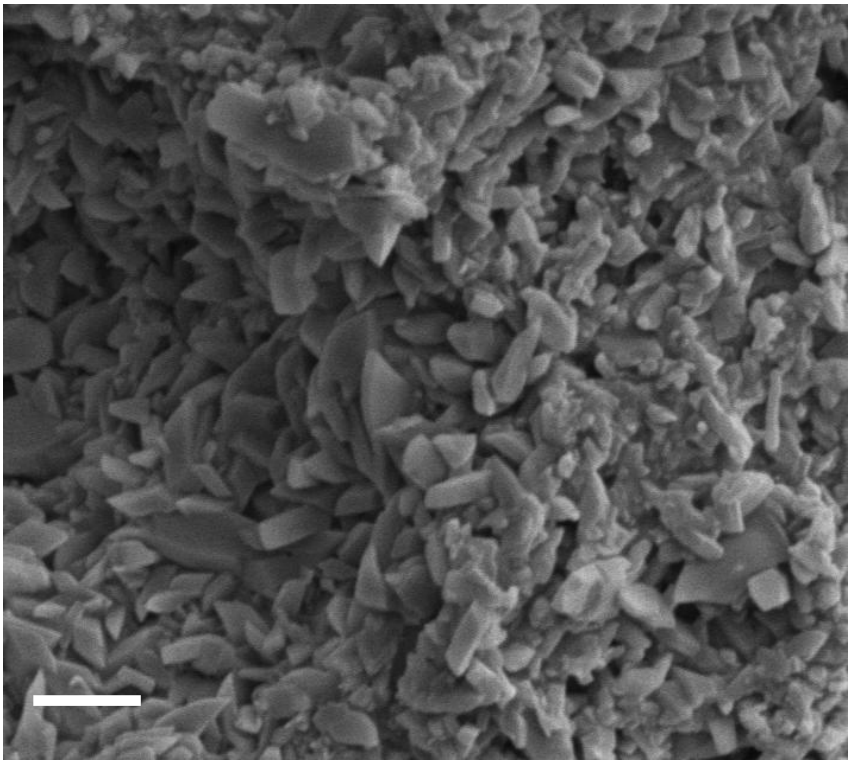
Figure 1A



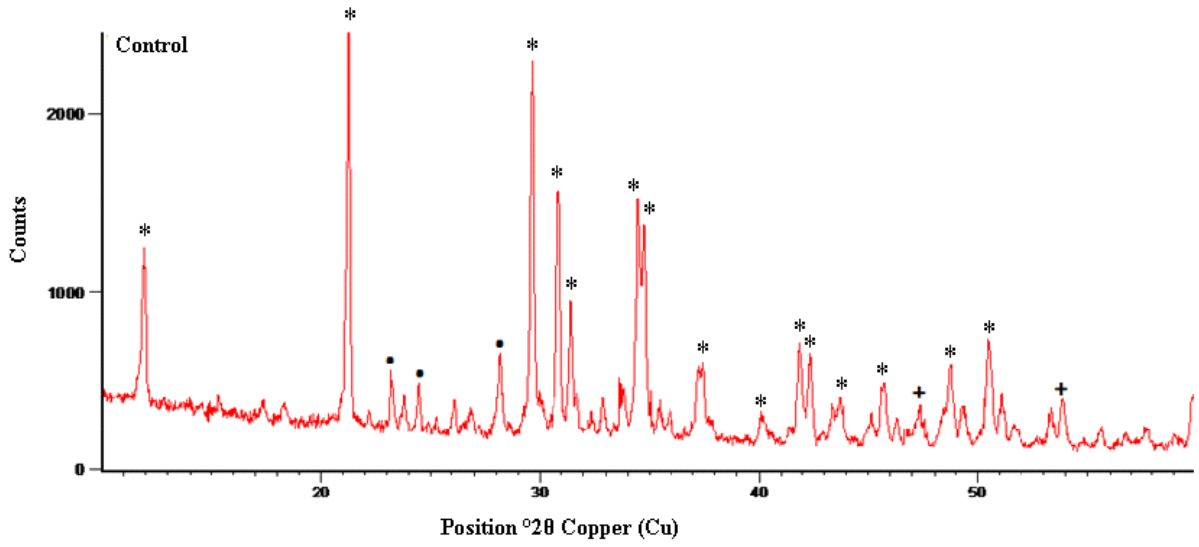


Figure 1B

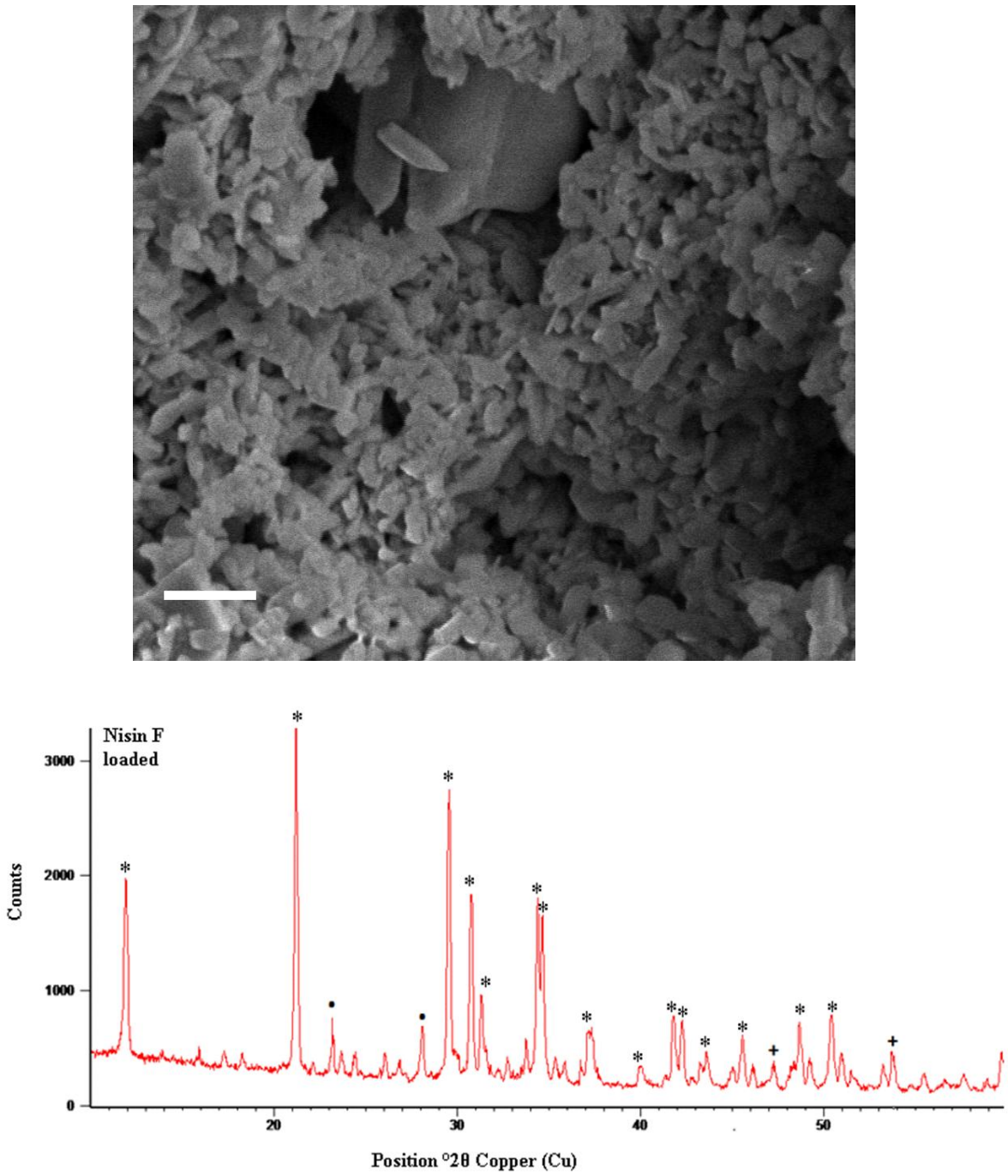


Figure 1 Scanning electron microscopy (SEM) images and X-ray diffraction (XRD) patterns of (A) control brushite bone cement samples (without nisin F) and (B) nisin F-loaded (5·0%, w/w) brushite bone cement samples. SEM images were captured at 5000 times magnification. The scale bar indicates 2 μm. Main brushite peaks (*) and peaks observed for β-TCP (•) and MCPM (+) are indicated in the XRD graphs.

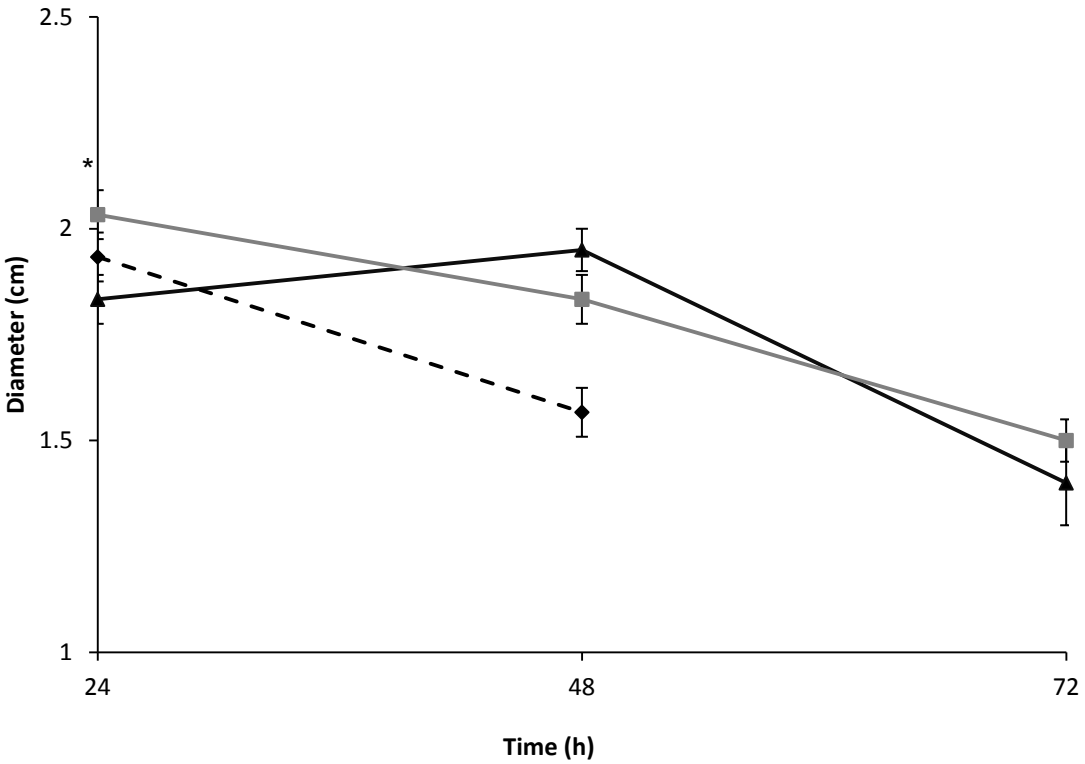


Figure 2 Release of nisin F from brushite bone cement over three days. *S. aureus* Xen 36 served as sensitive strain. Nisin F 6,400 AU (▲); Nisin F 3,200 AU (■); Nisin 1,280 AU (◆). * $p < 0{\cdot}05$; nisin F 6,400 AU vs. 3,200 AU (Student's t-test).

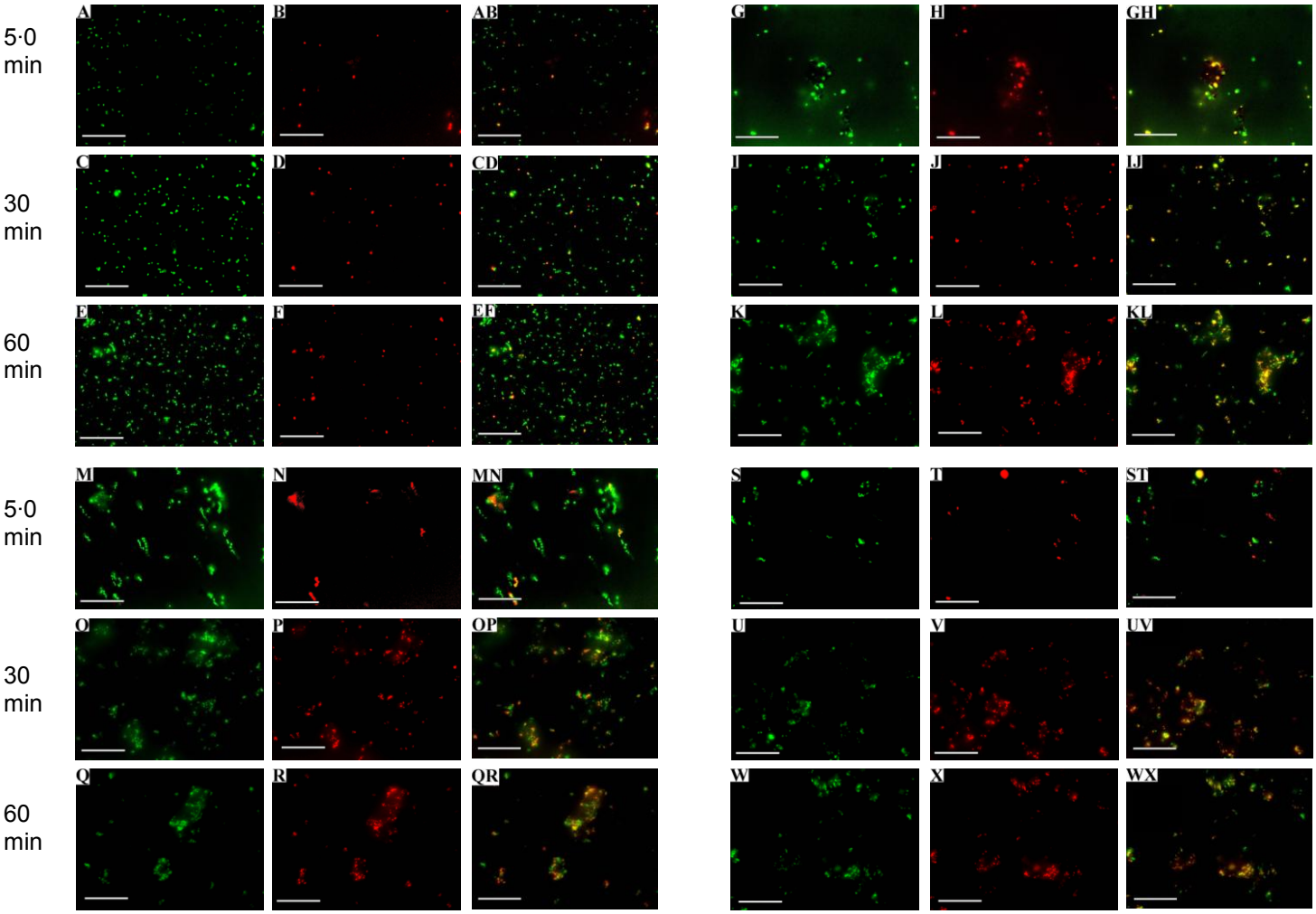


Figure 3 Bacterial cells were dyed and added to bone cement samples containing different bacteriocin concentrations; 6,400 AU (G-L), 3,200 AU (M-R) and 1,280 AU (S-X). Green cells indicate all bacteria in the sample and red cells indicate membrane compromised bacterial cells. Images were taken at 5·0 min, 30 min and 60 min. Images A-F are control bone cement samples containing no bacteriocin. Images AB-WX indicate overlaid images (green and red fluorescence), membrane compromised cells are seen as yellow. Scale bar represents 0·02 mm.

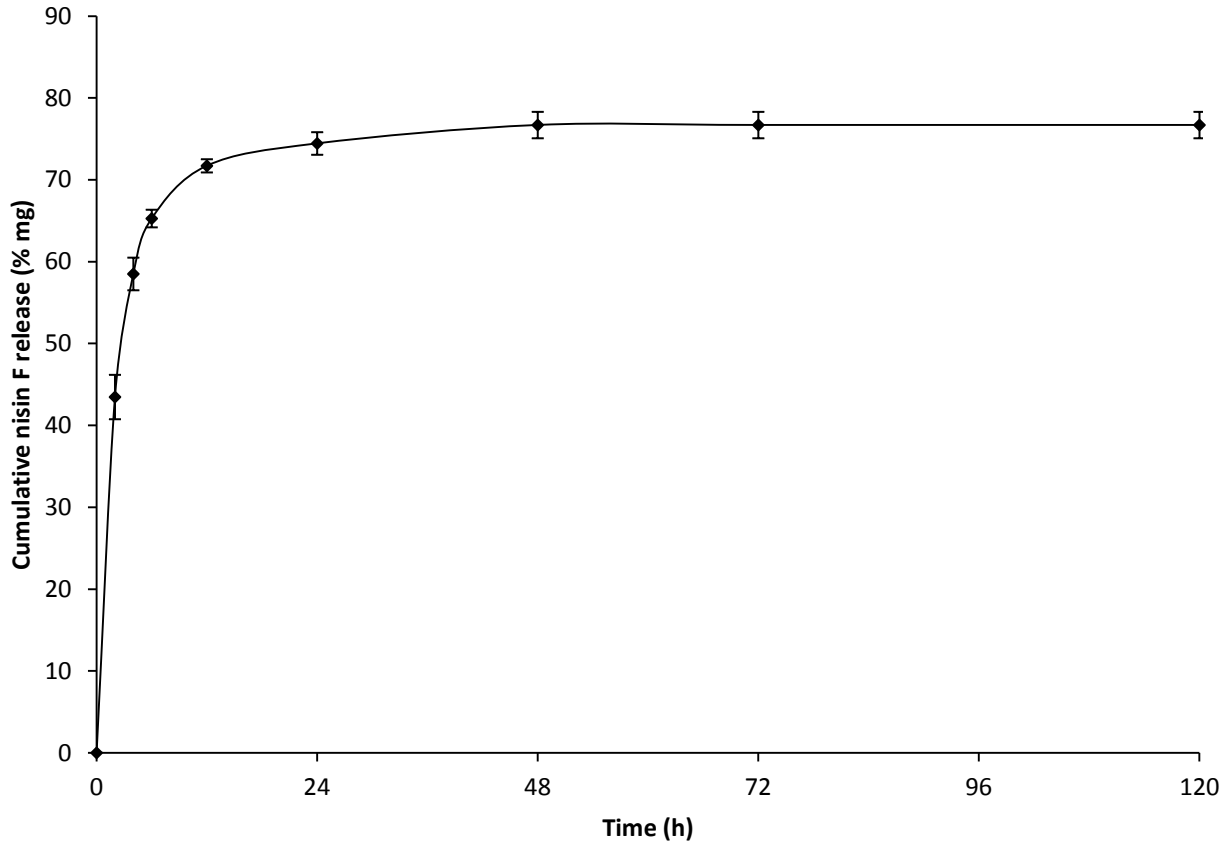


Figure 4 Cumulative release of nisin F (5·0%, w/w) from brushite cement, expressed as a percentage of the original protein content.

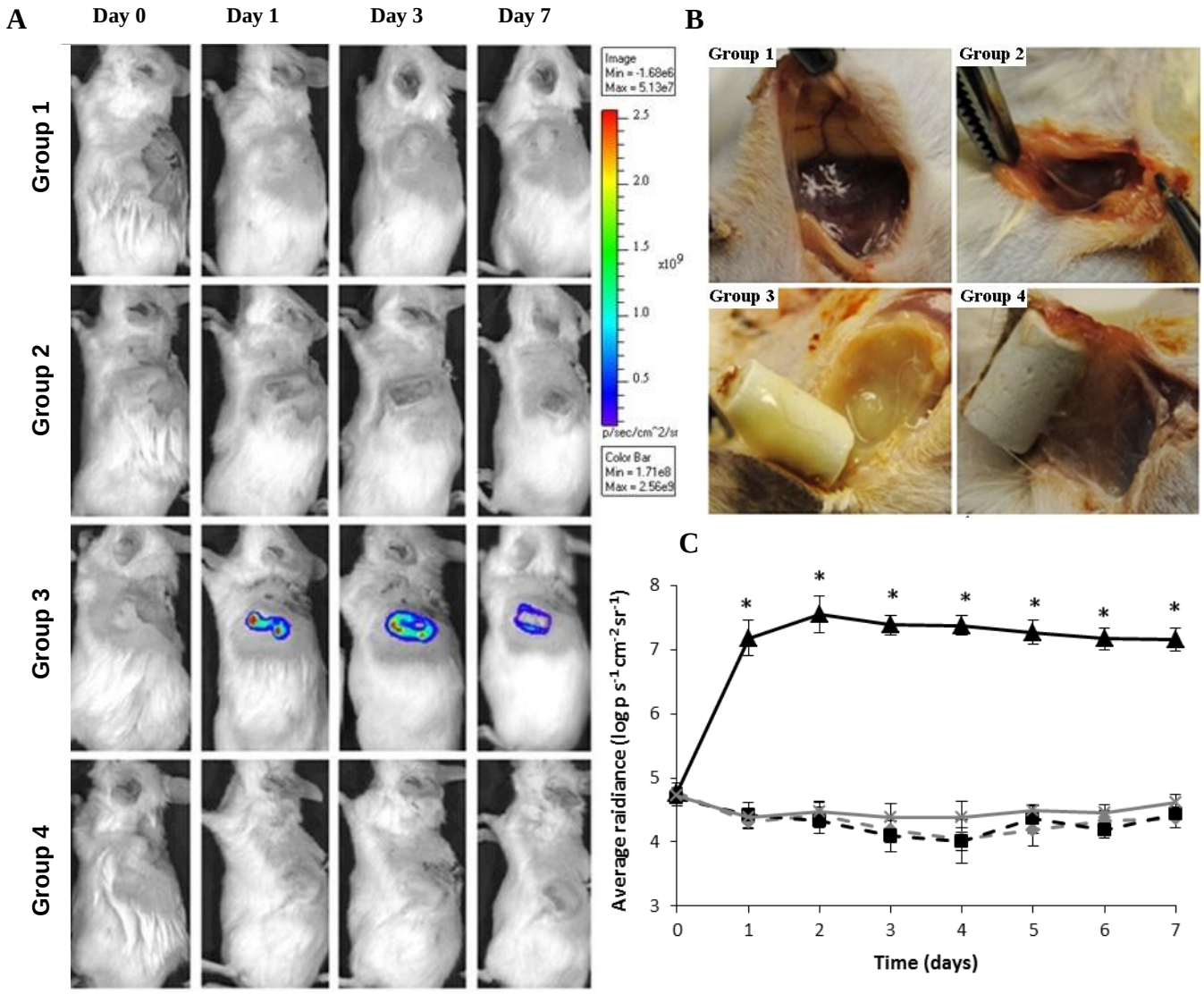


Figure 5 Bioluminescent images are represented on a colour scale overlaid on top of a greyscale image. A: Group 1 (untreated and uninfected), group 2 (treated and uninfected), group 3 (untreated and infected), group 4 (treated and infected). B: Representative images of implantation sites after a seven day trial. C: Graph represents average radiance of *S. aureus* Xen 36. Group 1 (◆), group 2 (■), group 3 (▲) and group 4 (✕). * $p < 0{\cdot}001$ group 3 vs. group 4 (Student's t-test).

Chapter 6

General Discussion and Conclusions

*This chapter is written accourding to the style of Journal of Applied Microbiology

General Discussion and Conclusions

Characterization of brushite composites

Incorporation of peptide ST4SA, ST4SA-PLGA particles, or nisin F into cement did not result in substantial changes in the cement setting reaction or crystal structure. Incorporation of other therapeutic agents have also shown little effect on setting reaction or crystal entanglement (Bohner *et al.* 1997; Schnieders *et al.* 2006; Young *et al.* 2008; Fullan a *et al.* 2010), rendering the results of this study not that surprising. The type and concentration of the drug plays an important role, for example addition of high gentamicin results in longer setting times whereas high chlorhexidine concentrations results in faster setting times (Bohner *et al.* 1997; Young *et al.* 2008). The effects bacteriocins may have on cement setting and structural characteristics need to be studied. In addition to these tests, mechanical tests should also be performed on the bacteriocin-cement composites.

Release of bacteriocins from brushite composites

Several therapeutic agents have been incorporated into calcium phosphate cements (CPC), including several antibiotics, growth factors and proteins (Otsuka *et al.* 1994; Hamanishi *et al.* 1996; Bohner *et al.* 1997; Otsuka *et al.* 1997; Young *et al.* 2008; De la Riva *et al.* 2009). The release of these substances from a cement matrix is dependent on several factors, e.g. drug solubility, drug cement interaction and porosity of the cement (Bohner *et al.* 1997; Young *et al.* 2008; Hofmann *et al.* 2009). However, the general trend is a high burst release within the first 24 h, followed by a steady release, resulting in partial or complete release of the incorporated substance. Incorporation of peptide ST4SA or nisin F resulted in a high burst release, followed by a steady release. The release trend was, however, slightly more rapid than reported for other drugs. Release kinetics can be changed by altering porosity of the cement or by incorporating polymeric particles associated with the desired substance (Schnieders *et al.* 2006; Hofmann *et al.* 2009; Fullana *et al.* 2010).

Several researchers have reported a decrease in burst release when incorporating therapeutic agents into polymeric particles (Schnieders *et al.* 2006; Basarkar *et al.* 2007; Fullana *et al.* 2010; Santander-Ortega *et al.* 2010). Schnieders *et al.* (2006) reported a significant difference; not only in burst release, but total release when gentamicin-PLGA microspheres were incorporated into cement. A similar result was seen in the current study when incorporating peptide ST4SA-PLGA-associated particles into brushite cement. The burst release was significantly reduced, compared to microparticles and normal peptide ST4SA cement. Because bacteriocins are active at low concentrations against sensitive bacteria, they can be released at low concentrations

(Morgan *et al.* 2005; Nissen-Meyer *et al.* 2009). This was seen in a study where nisin was incorporated into poly (L, lactic acid) particles and released over a period of 40 days, still in an active form able to inhibit the target organism. Less than 35% of the nisin was released over the 40 day trail, illustrating the antimicrobial potency of the peptide (Salmaso *et al.* 2004). From these results, it seems as if the release of bacteriocins at low concentrations, above the minimum inhibitory concentration, is sufficient to control bacterial growth. Protection of peptide ST4SA in PLGA has not been studied. This may provide valuable information on the use of microparticles to protect bacteriocins from harsh environments. The effect of electrospraying on the activity of peptide ST4SA must also be investigated.

Prevention of *S. aureus* infection with nisin F brushite composites

The possible use of bacteriocins as an alternative antimicrobial therapy (AAT) relies on extensive research; not only on *in vtiro* characterization and *in vivo* properties of the peptides, but also on the applications. *In vitro* research into the possible application of bacteriocins as an AAT has been investigated by some researchers (Joerger 2003; Gillor *et al.* 2005; Knoetze *et al.* 2008; Piper *et al.* 2010). However, it is important to test and confirm the *in vitro* results *in vivo.* Certain characteristics in complex *in vivo* situations cannot be simulated *in vitro.* Research has focused on the *in vivo* efficacy of several bacteriocins (Ingham *et al.* 2003; Ghiselli *et al.* 2004; Damasko *et al.* 2005; De Kwaadsteniet *et al.* 2009; Brand *et al.* 2010; De Kwaadsteniet *et al.* 2010). For this reason, nisin F brushite composites were tested for their *in vivo* efficacy. Most antibiotic-loaded bone cements are used on a prophylactic basis and are not used to treat infections, except in certain implant revision cases (Hanssen 2004; Jiranek *et al.* 2006; Parvizi *et al.* 2008). Therefore, the model used in this study was based on nisin F brushite cement as a prophylactic for the prevention of *S. aureus* infection. The bacterial concentration used to infect the subcutaneous pockets, created for implant insertion, was high enough to form an established infection that lasted for seven days (Bernthal *et al.* 2010). The nisin F-loaded cement samples prevented the growth of *S. aureus* over the seven day period. The lantibiotic most likely killed the bacteria within the first few minutes of contact, resulting in insufficient viable cells being available to sustain the infection. In other *in vivo* studies with bacteriocins it has been reported that the bacteriocins are only able to inhibit bacterial infection for a short time period (Ingham *et* al. 2003; Brand *et al*. 2010). However, in some cases bacteriocins were more effective in preventing infection, especially when associated with medical devices (Bower *et al.* 2002; Ghiselli *et al.* 2004). When a lower concentration of nisin F-loaded cement was used (i.e. 2·5%), infection could not be controlled, suggesting that the inhibition is dose dependent (Appendix A2). Similar findings have been reported by Otsuka *et al.* (1997) and Bernthal *et al.* (2010). If

purified nisin F is used, a lower concentration may be necessary to obtain the same result. Further research must also be performed on the stability of nisin F *in vivo*.

Conclusion

The *in vitro* results of the current study showed that bacteriocins incorporated into brushite cement did not significantly alter the characteristics of the matix and that the peptides were released in an active form. The incorporation of ST4SA-associated PLGA particles resulted in a delayed release profile with a decreased burst release. Finally it was shown that nisin F-loaded brushite cement controlled *S. aureus* infection in mice. Bacteriocins may thus be a possible alternative to antibiotics incorporated into bone cement. Further research is needed to support the findings obtained in this study.

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Addendum A1

Incorporation of bacteriocin associated PEO: PDLLA blend nanoparticles into self setting brushite bone cements*

*The addendum is supplementary to chapter 4: Incorporation of bacteriocin associated Poly (D, L-lactide-*co*-glycolide) particles into self setting brushite bone cements.

Incorporation of bacteriocin associated PEO: PDLLA blend nanoparticles into self setting brushite bone cements

Aims: The aim of the study was to investigate the incorporation of peptide ST4SA associated Poly (ethylene oxide); PEO: poly (D, L-lactide); PDLLA blend nanoparticles (produced by electrospraying) into brushite cement.

Methods and Results: Peptide ST4SA was isolated from *Enterococcus mundtii* ST4SA and concentrated using ammonium sulfate precipitation, dialysis and freeze-drying. *Listeria monocytogenes* EGDe was used as sensitive strain.

Bacteriocin-associated particles (BP) were produced using an electrospraying method. Three 2·0% (wt/vol) blends of PEO: PDLLA were prepared, namely 70:30, 50:50 and 30:70. The polymer blends were dissolved in DMF containing bacteriocin. A setup using a sterile tapered glass tube and high voltage supply was used for the production of particles. A constant potential difference of 15 kV was applied to polymer solution and -5 kV to the collector (1·33 kV/cm).

Brushite cement was prepared by mixing equimolar concentrations of β-tricalcium phosphate and monocalcium phosphate monohydrate. Different BP blends were added at 10% (w/w) and ST4SA at 5·0% w/w to brushite cement and moulded into cylinders. Release of BP and brushite-BP composites were determined by using BCA protein assays (Fig. 3 and 6). BP were characterized using SEM analysis (Fig. 1 and 2), cement composites were characterized using XRD and SEM analysis (Fig. 4 and 5),

Electrosprayed BP had sizes ranging from 161·443 nm to 800 nm with an average of 783·74 nm (±62·4) for the PEO 50: PDLLA 50 blends. Sizes ranging from 173·5 nm to 1973·4 nm with an average of 499·7 nm (±173·5) for the PEO 70: PDLLA 30 blends. Sizes ranging from 222·2 nm to 2234·2 nm with an average of 562·3 nm (±273·7) for the PEO 30: PDLLA 70 blends. Most of the bacteriocin was release within the first 4 h for all blends. The PEO 50: PDLLA 50 BP-cement composite differed statistically (p<0·05) with the control group at 2 and 4 hours. Cement composite containing the PEO 30: PDLLA 70 blend differed statistically (p<0·05) with the ST4SA-loaded group at 2, 4 and 6 h. Bacteriocin used for BP production had a protein concentration of 10mg mL^{-1} < [protein] < 20mg mL^{-1} and antimicrobial activity of 102400 AU mL^{-1}. Particle associated bacteriocin retained activity upon release from the particles and BP-composites (Fig. 7).

Conclusions: Peptide ST4SA was successfully associated with PEO: PDLLA blend particles and was released in its active form, from BP and brushite-BP composites. Incorporation of PEO 50: PDLLA 50 and PEO 30: PDLLA 70 BP to brushite cement resulted in statistically different release profiles at certain time points.

Significance: The current study showed that it is possible to associate a class II bacteriocin with PEO: PDLLA blends using an electrospraying method. In addition to this the particles were also incorporated into brushite cement.

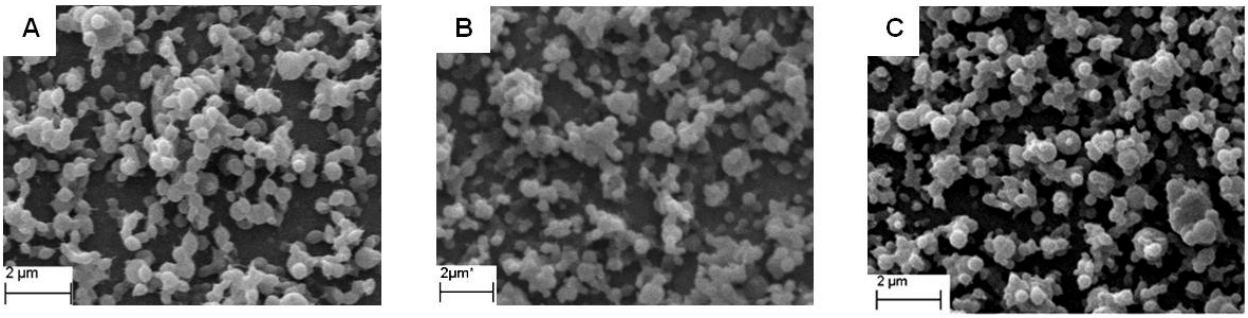

Figure 1 SEM images of blend BP, Scale bar represents 2 μm, image captured at 5000 times magnification. Images represent blend BP PEO 70: PDLLA 30 (**A**), PEO 50: PDLLA 50 (**B**) and PEO 30: PDLLA 70 (**C**).

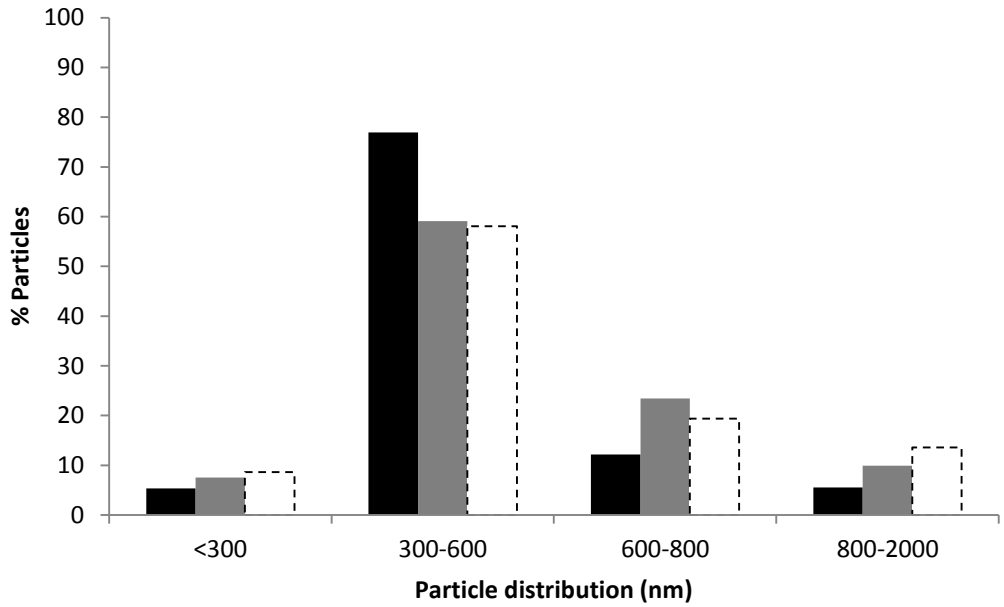

Figure 2 Graph represents size distribution of blend BP according to analysis of SEM images with SEM Image studio. Bars represent blend BP PEO 70: PDLLA 30 (■), PEO 50: PDLLA 50 (■) and PEO 30: PDLLA 70 (▭).

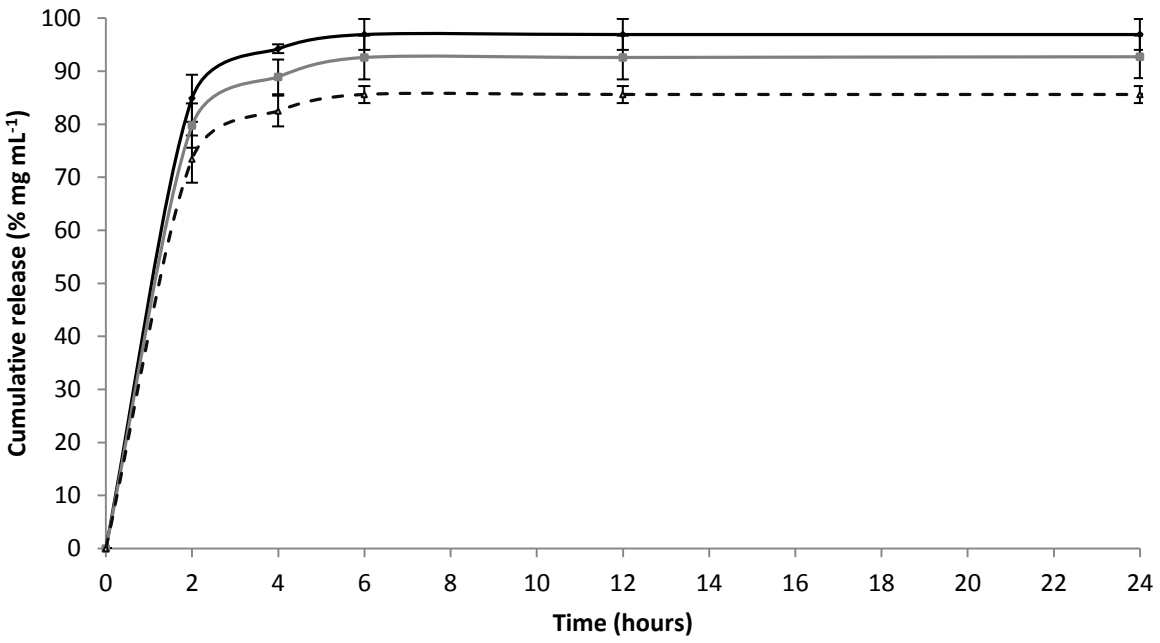

Figure 3 Cumulative release of peptide ST4SA from PEO 70: PDLLA 30 (—•—), PEO 50: PDLLA 50 (—▪—) and PEO 30: PDLLA 70 (- - -▲- - -) blend BP.

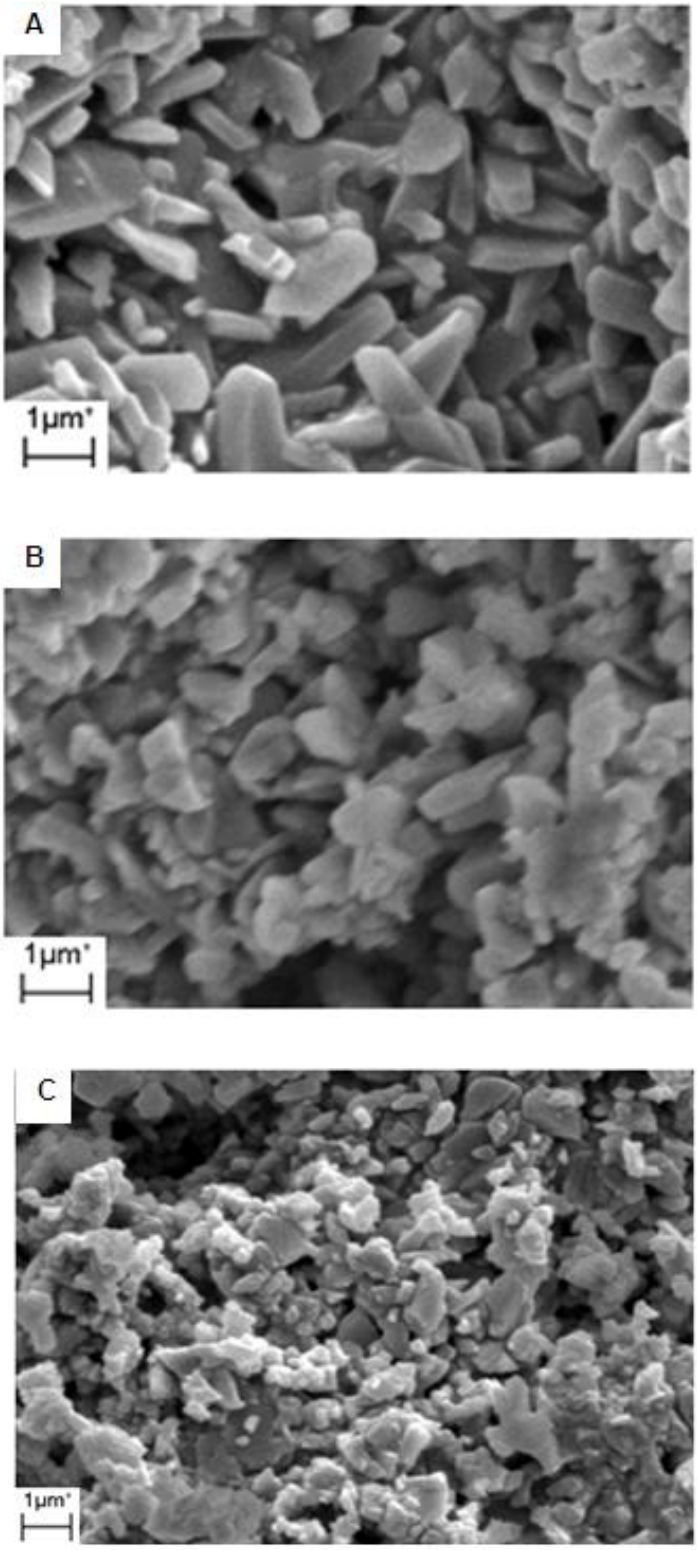

Figure 4 SEM images of brushite cement samples without BP (**A**), ST4SA-loaded (5·0% w/w; **B**) and cement samples containing 10% w/w PEO 50: PDLLA 50 blend BP (**C**). Scale bar represents 1 μm, image captured at 5000 times magnification.

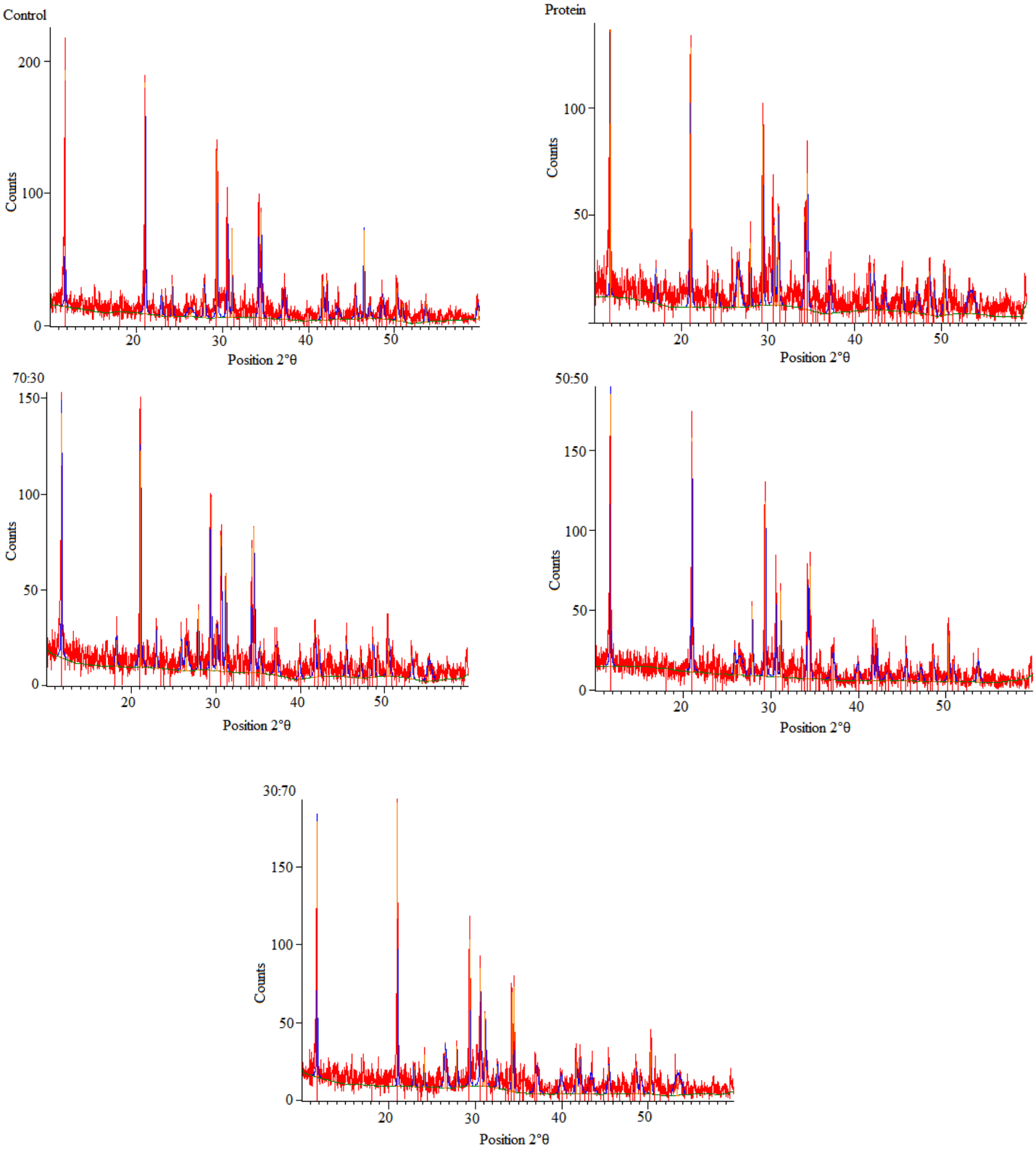

Figure 5 XRD patterns of unloaded (control) composites, ST4SA composites (protein), PEO 70: PDLLA 30 (70:30), PEO 50: PDLLA 50 (50:50) and PEO 30: PDLLA 70 (30:70) blend BP composites. Red lines indicate main brushite peaks (ICDD 00-009-0077)

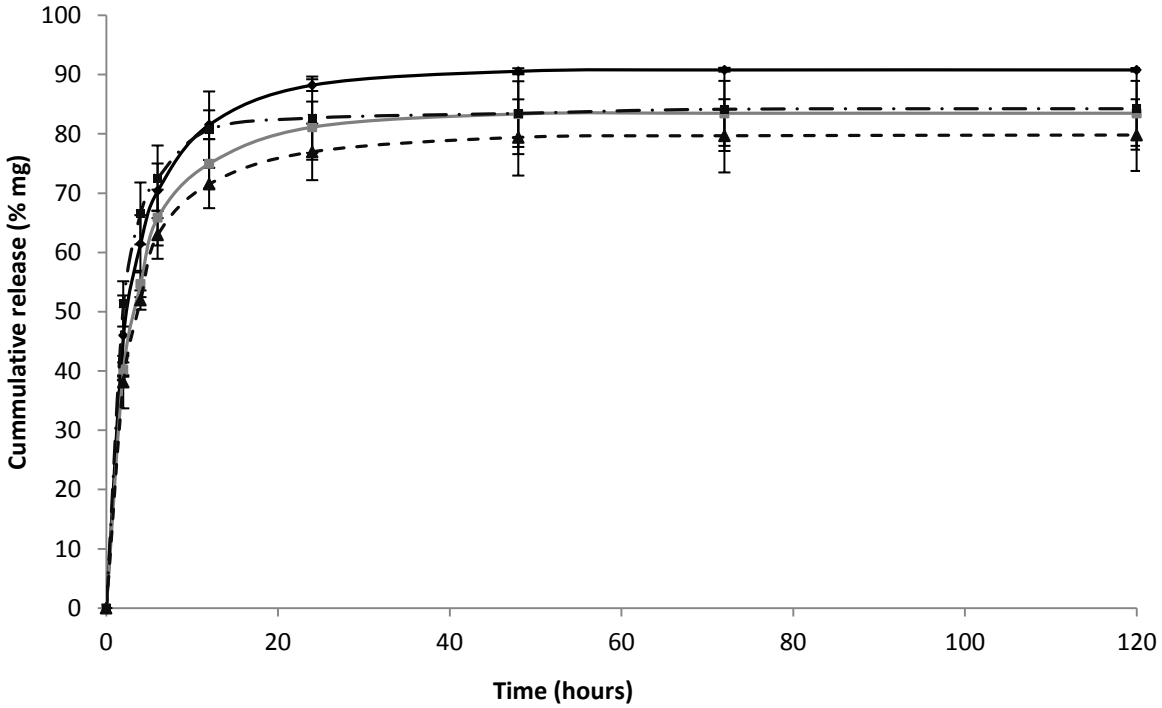

Figure 6 Cumulative release of peptide ST4SA from ST4SA composites ( ) PEO 70: PDLLA 30 ( ), PEO 50: PDLLA 50 ( ) and PEO 30: PDLLA 70 ( ) blend BP composites, expressed as percentage of original protein content.

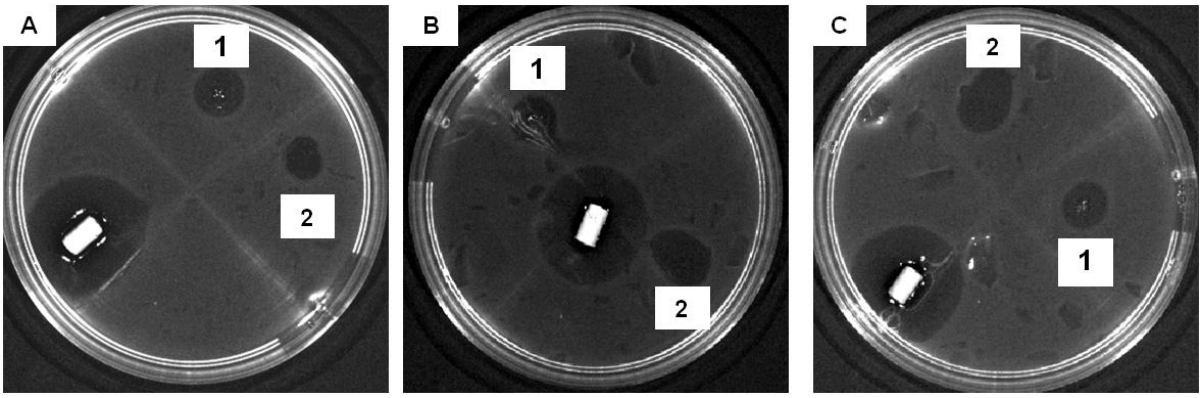

Figure 7 Antimicrobial activity of BP and BP blend composites on soft agar seeded with 1% *L. monocytogenes* EGD-e. Images of PEO 70: PDLLA 30 (**A**), PEO 30: PDLLA 70 (**B**) and PEO 50: PDLLA 50 (**C**) blends are shown. Zones numbered 1 indicate particles placed on plate and 2 positive ST4SA control.

Addendum A2

In vivo evaluation of brushite cement loaded with 2·5% and 5·0% nisin F as a method to prevent the growth of *Staphylococcus aureus**

*The addendum is supplementary to the in vivo section of chapter 5: Nisin F-loaded brushite bone cement prevented the growth of *Staphylococcus aureus in vivo.*

In vivo evaluation of brushite cement loaded with 2·5% and 5·0% nisin F as a method to prevent the growth of _Staphylococcus aureus_

Aims: The aim of the study was to determine the optimal concentration of nisin F needed to prevent infection with *S. aureus* Xen 36.

Methods and Results: Brushite cement was prepared by mixing equimolar concentrations of β-tricalcium phosphate and monocalcium phosphate monohydrate. Nisin F was added at 5·0% and 2·5% (w/w), respectively, to brushite cement and moulded into cylinders. Nisin F-loaded or unloaded cement was implanted into a subcutaneous pocket on the back of mice (n=9) and then infected with a bioluminescent *S. aureus* strain (Xen 36). Infection was monitored for 6 days, using an *in vivo* imaging system (IVIS; Fig. 1A and B). After the 6 day trial implants were removed and vortexed in 1 ml sterile saline. Samples were diluted and plated out onto BHI agar containing 200 µg ml^{-1} kanamycin (Fig. 2 A and B). Mice implanted with samples containing no nisin F showed bioluminescence throughout the 6 day trial period. Viable cells were also isolated from removed implants. No bioluminescent signals could be detected for infected mice implanted with 5·0% and 2·5% w/w nisin F containing samples. Nisin F containing 5·0% w/w prevented *S. aureus* infection for 6 days and no viable cells were isolated from the implants. Viable bacteria were isolated from implants containing 2·5% w/w nisin F.

Conclusions: Nisin F-loaded (5·0% w/w) brushite cement successfully prevented *in vivo* growth of *S. aureus*. However, results suggest that samples containing 2·5% w/w nisin F was able to suppress bioluminescence but unable to completely eradicate bacteria.

Significance and Impact of the Study: A correlation must be found between bioluminescence and bacterial load (from traditional plating techniques) when examining different therapeutic concentrations of a drug. This will help determine the lower limit of bioluminescence that can be detected from the bacteria used. It is also important to confirm the absence of infection after *in vivo* bioluminescent trails using traditional plating techniques. This was done in chapter 5 as well as in the current study and was found to be useful in determining the optimal concentration for total prevention and not just suppression of bacterial infection. However these are preliminary results and further investigation is needed to determine optimal concentration.

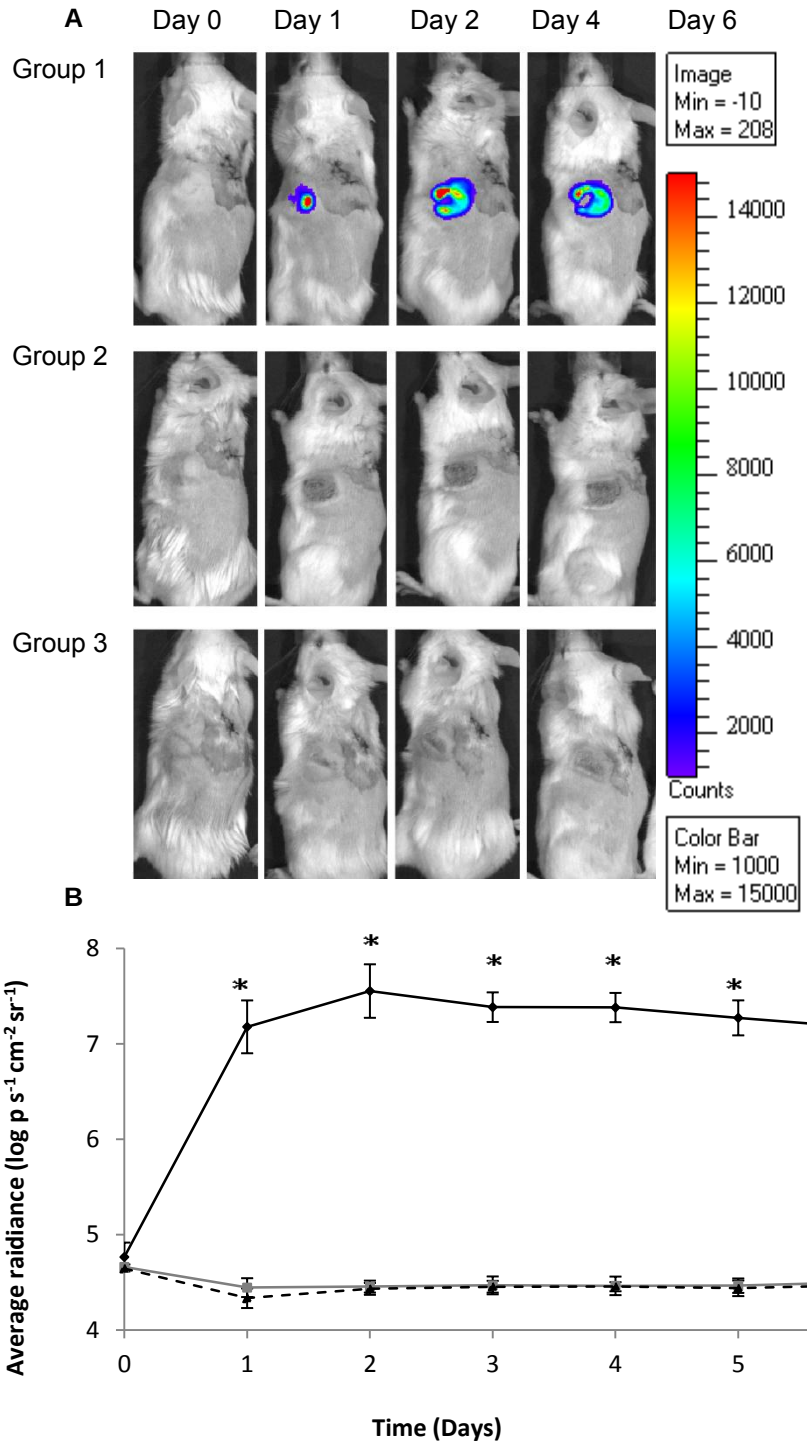

Figure 1 Bioluminescent images are represented on a colour scale overlaid on top of a greyscale image. A: Group 1 (untreated and infected), group 2 (treated with 2·5% nisin loaded cement and infected) and group 3 (treated with 5·0% nisin loaded cement and infected). Graph represents average radiance of *S. aureus* Xen 36. Group 1 (—◆—), group 2 (—■—) and group 3 (- -▲- -). * $p < 0{\cdot}001$ group 3 vs. group 4 (Student's t-test).

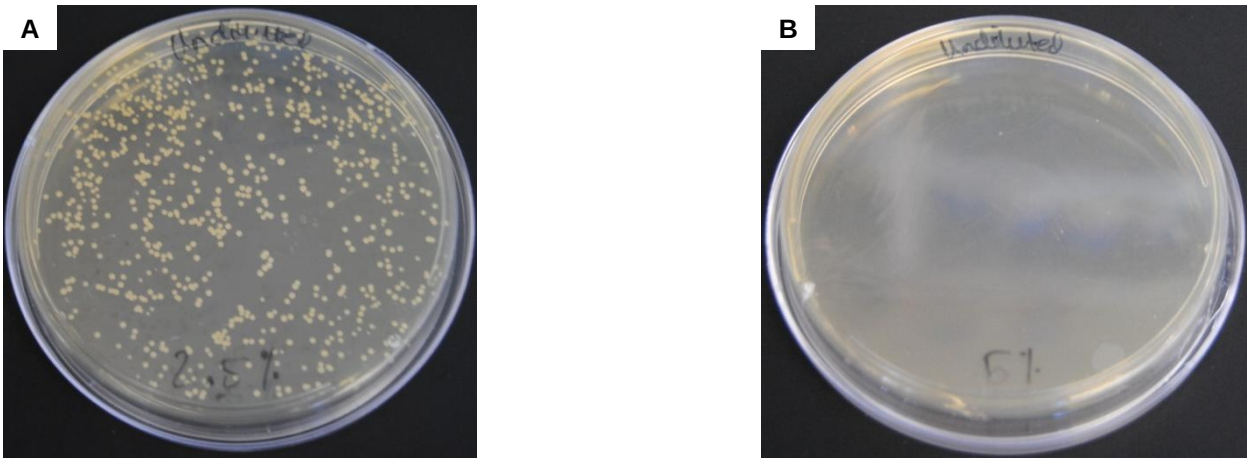

Figure 2 Undiluted suspensions from removed implants plated out onto BHI (200 µg mL^{-1} kanamycin). Images indicate samples plated out from cement loaded with 2·5% (**A**) and 5·0% (**B**) nisin F, respectively.