Delivery of Gentamicin from Resorbable Polymeric Carriers as Anti-Infective Strategy for Implant-Associated Osteomyelitis

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DELIVERY OF GENTAMICIN FROM RESORBABLE POLYMERIC CARRIERS AS ANTI-INFECTIVE STRATEGY FOR IMPLANT-ASSOCIATED OSTEOMYELITIS

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Chapter 1

General Introduction

General Introduction

The number of patients requiring fracture fixation after trauma is increasing yearly [1, 2]. In the US alone, 440 thousand fractures were reduced with internal fracture fixation implants in 2010 [3]. The insertion of orthopaedic- and trauma implants creates a localized deficiency in phagocytosis of bacteria in the vicinity of the implant. Therefore, the susceptibility to bacterial colonization of the implant substantially increases [4, 5].

The fixation of closed fractures results in infection rates of 0.5 % to 2 %. However, the infection rate might exceed 30% in patients with severe open fractures [1, 6]. On average 5% of the internal fracture fixation devices get colonized by bacteria, resulting in implant infection [2]. The socio-economic effects of implant infection are tremendous. The treatment of chronic osteomyelitis is difficult and time-consuming. A standard protocol for chronic osteomyelitis and infected non-unions may involve: identification of the bacterial species and antibiotic susceptibility testing, removal of fracture fixation devices, debridement of the infected bone and soft tissue and systemic delivery of antibiotics for a 4-6 weeks period [7]. The average cost for removing infected internal fracture fixation devices and subsequent treatment of these infections were estimated to be $ 15,000 on average per case around the year 2000 in the United States [8]. A large survey performed amongst hospitals located in New York City revealed similar figures. A total of 13,550 cases of *S. aureus* infection with a total cost of $ 435.5 million for treatment were reported, on average $ 32,100 per case [9].

 As such, the prophylactic delivery of antibiotics plays a crucial role in the prevention of infections in trauma surgery. However, systemic delivery of antibiotics is often insufficient to protect the implant site from bacterial colonization as the bony structures and local vascularity might be disturbed [10]. Furthermore, the systemic delivery of certain antibiotics is associated with toxic side effects [11, 12].

Local delivery of antibiotics by antibiotic loaded biomaterials (ALBs) can overcome the aforementioned problems, by on-site delivery of high concentrations of antibiotics. One of these ALBs that is most commonly used, either as carrier in the shape of polymeric bead chains, blocks or putty, is non-degradable

poly(methylmethacrylate) (PMMA) [13]. A follow-up surgery is required to remove the material after its useful lifetime, involving the risk for new intraoperative infection. Other commercially available ALBs are antibiotic loaded collagen fleece [14] and antibiotic-loaded poly(lactic acid) (PLA) as a coating on fracture fixation implants. Although both biomaterials are degradable, they do not take the nature of trauma wounds into account as these materials have confined dimensions and may have limited distribution in complex wounds. Hyaluronic acid (HA) hydrogels are biocompatible and degradable and used across many biomedical applications, notably as vehicle for drug delivery systems [15]. HA hydrogels with lower critical solution temperature (LCST) behaviour can be obtained by grafting thermosensitive (co)polymers to one of the functional groups on the HA backbone. Although many polymers exhibit thermo-responsiveness, some have gained particular interest because their LCSTs are just below physiological temperature [16]. These comprise poly(*N*-isopropylacrylamide) (pN) and its copolymers, poly(*N,N*-diethylacrylamide), block copolymers of poly(ethylene oxide) and poly(propylene oxide) (PEO-PPO) and block copolymers of poly(ethylene oxide) and poly(lactic-co-glycolic acid) (PEO-PLGA) [16, 17]. Importantly, conjugation of pN onto a polymer chain yields thermo-responsive polymers with sharp phase transition [18].

Scope of the studies

In this thesis several investigations are performed. Firstly, the potential of hyaluronic acid and several HA derivatives to complex gentamicin sulfate, a commonly used antibiotic in ALBs, is investigated. Additionally, HA derivatives are prepared with 2 different polymers with LCST behaviour. The thermo-responsive behaviour of PEO/PPO grafted HA is compared with pN functionalized HA. Secondly, thermo-responsive hydrogels loaded with gentamicin sulfate are prepared from pN grafted HA (HApN) and characterized *in vitro*. Their potential to prevent infection *in vivo* is studied in a rabbit humerus model including fracture, implant and high bacterial load. Their influence on bone healing upon clearing an infection is investigated. Finally, the hydrophobic modification of gentamicin and encapsulation in hydrophobic matrices

and particles are explored to obtain gentamicin loaded ALBs more suited for infection treatment purposes.

Outline of the thesis

In **Chapter 2** an introduction to antibiotic loaded biomaterials (ALBs) is provided. Protocols for systemic antibiotic infection prophylaxis and current commercial available ALBs are described. Finally, new developments in this field are reviewed, including the incorporation of antimicrobial agents other than antibiotics into ALBs, development of improved polymeric carriers for the passive delivery of antibiotics and the description of responsive polymeric carriers for the active delivery of antibiotics.

In **Chapter 3** the complexation of positively charged gentamicin with negatively charged hyaluronic acid (HA) into polyelectrolyte complexes (PECs) is investigated. Subsequently, several HA derivatives are reported that have been synthesized and characterized with respect to their thermo-responsiveness and their complexation into PECs with- and release of gentamicin.

Chapter 4 describes the preparation of a thermo-responsive hyaluronic acid poly(*N*-isopropylacrylamide) hydrogel and its loading with gentamicin sulfate. The rheological properties and *in vitro* release from the gentamicin-loaded HApN hydrogel is studied. The efficacy of prophylactic antibiotic delivery from these hydrogels is investigated in a contaminated osteotomy model with plating osteosynthesis in the rabbit humerus.

In **Chapter 5** the effect of the thermo-responsive hydrogel on fracture healing is assessed. Firstly, the effect of HApN hydrogels on fracture healing is assessed and compared to normal healing in the presence of a fracture fixation implant. Finally, clearance of a high bacterial load and subsequent fracture healing in the presence of gentamicin-loaded HApN hydrogels is investigated.

Chapter 6 describes the preparation of a hydrophobic salt of gentamicin by hydrophobic ion pairing. The potency of the hydrophobic salt of gentamicin to kill bacterial species relevant to traumatology and orthopaedics, and its toxicity towards mammalian cells is investigated. Finally, both the hydrophobic and hydrophilic gentamicin salts are encapsulated within poly(trimethylene carbonate) matrices.

In **Chapter 7** the preparation of polymeric microspheres loaded with gentamicin is described by means of the double emulsion solvent evaporation technique and the preparation of monodisperse microspheres loaded with either the hydrophobic or hydrophilic salt of gentamicin by membrane emulsification is described.

Chapter 8 focuses on the future perspectives of local antimicrobials delivery

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Chapter 2

Antimicrobial delivery systems for local infection prophylaxis in orthopedic- and trauma surgery

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Abstract

Infectious complications occur in a minor but significant portion of the patients undergoing joint replacement surgery or fracture fixation, particularly those with severe open fractures, those undergoing revision arthroplasty or those at elevated risk because of poor health status. Once established, infections are difficult to eradicate, especially in the case of bacterial biofilm formation on implanted hardware. Local antibiotic carriers offer the prospect of controlled delivery of antibiotics directly in target tissues and implant, without inducing toxicity in non-target organs. Polymeric carriers have been developed to optimize the release and targeting of antibiotics. Passive polymeric carriers release antibiotics by diffusion and/or upon degradation, while active polymeric carriers release their antibiotics upon stimuli provided by bacterial pathogens. Additionally, some polymeric carriers gelate in-situ in response to physiological stimuli to form a depot for antibiotic release. As antibiotic resistance has become a major issue, also other anti-infectives such as silver and antimicrobial peptides have been incorporated in research. Currently, several antibiotic loaded biomaterials for local infection prophylaxis are available for use in the clinic. Here we review their advantages and limitations and provide an overview of new materials emerging that may overcome these limitations.

Introduction

Developments in the field of trauma and orthopedic biomaterials have improved the life of millions of patients undergoing surgery. However, infectious complications can delay successful healing. Infection after orthopedic or trauma surgery occurs when bacteria enter the surgical site and cause pathological conditions or diseases [1]. Surgical site infections (SSI) are infections that encompass the surgical wound and all other tissues involved in the operation [2]. Clinical studies have identified several risk factors for the development of SSI, which may be classified into patient-related factors and operation-related factors (Table 2.1) [2, 3].

One of the most significant risk factors for the development of infection is the presence of an implant. The reasons why implanted medical devices are at such high risk for developing infection has been a topic of research for decades. In 1957, Elek and Conen showed that the presence of a single silk suture could reduce the number

of bacteria required to cause an infection in human subjects by 10,000 fold [4]. Zimmerli *et al.* showed similar data in a guinea pig model, whereby the minimal infectious dose of *Staphylococcus aureus* (*S. aureus*) was 100,000 fold lower when a device was implanted subcutaneously compared to the situation in which no implant was inserted [5]. This increased susceptibility to infection appears to be partly due to a localized deficiency in phagocytosis of bacteria in the vicinity of an implant, and to the growth of bacteria in a biofilm on the surface of the implanted device [6, 7]. Published infection rates of about 5 % have been reported for internal fracture fixation devices (FFDs) [8]. Increased infection rates have been reported within certain high-risk groups: for example, the infection rate in patients with open fractures may exceed 30% [9], in comparison with 0.5-2% for equivalent closed fractures [9]. Similarly, the infection rate for revision of failed prosthetic joints may be up to 40% [9], whereas the rate for primary joint replacement is approximately 1-4% [3, 10-12].

A guideline document outlining best clinical practice for the prevention of SSI has been published by the U.S. department of health and human services [13]. Several key-actions for surgical personnel are described which are based upon well-designed studies that have proven to reduce the risk of SSI significantly. Most importantly, preoperative intravenous administration of antimicrobial agents that are effective against the most common species causing SSI should be provided. The timing of the administration should be such that the minimal bactericidal concentration of the antimicrobial agent for these species should be reached and that it stays above this level at least until a few hours after the incision is closed. Finally, surgical personnel should adhere to the principles of asepsis when placing intravascular devices, spinal or epidural anesthesia catheters or when providing intravenous drugs [13].

Table 2.1: Risk factors for surgical site infection [2, 14]

Risk factors for the development of SSI	
Patient factors	Extremes of age
	Poor nutritional state
	Obesity (>20% of ideal body weight)
	Diabetes mellitus
	Smoking
	Infections at sites other than the surgical site
	Inflammatory arthritis
	Malignancy
	Bacterial colonization (e.g. nares colonization)
	Immunosuppression (steroids, other immunosuppressive drug use, cytotoxic drugs or previous antibiotics)
	Preoperative hospitalization
	Prolonged postoperative hospital stay (nosocomial infection)
Operation factors	Too short surgical scrub (shorter than 2 minutes)
	Poor skin antisepsis
	Preoperative shaving
	Type of agent used for preoperative skin preparation
	Emergency procedure
	Length of operation
	Antimicrobial prophylaxis
	Operating theather ventilation
	Inadequate instrument sterilization
	Traumatic or unfamiliar surgical technique (hematoma, devitalized tissue, dead space, electro cautery etc.)
	Foreign material in surgical site (orthopedic implant, fracture fixation devices)
	Surgical drains
	Surgical technique (hemostasis, poor closure, tissue trauma)
	Postoperative hyperthermia

Most infections associated with orthopedic implants are caused by opportunistic pathogens and bacteria that are regularly found within the microflora of the human skin [15]. The initial colonization of the wound tissues may occur preoperatively, in the case of open wounds, or perioperatively, i.e. after incision but before complete healing of the surgical wound [8]. Considering the large number of bacterial species

present on the human skin, only relatively small percentages have been implicated in SSI infections involving orthopedic hardware. The staphylococci account for the majority of cases of device related infections for most classes of implants. *S. aureus* and various coagulase negative staphylococci such as *S. epidermidis* account for more than 50% of infection cases related to FFDs and for up to 65% of prosthetic joint infections (PJIs) [8, 16-19].

The increasing prevalence of multiple antibiotic resistant bacteria such as methicillin resistant *S. aureus* (MRSA), both in the community and in the hospital setting, has been attributed to the misuse of antibiotic agents in the medical and agricultural sector [20, 21]. This has an additional downstream impact upon the treatment of infections associated with implanted orthopedic hardware. Even when medically required, antibiotic use can have secondary consequences. For example, colonization of the hip joint with antibiotic- resistant bacteria may reach up to 88% upon hip revision surgery in which antibiotics had been used in bone cement at a previous operation [22].

Infection of the bone and bone marrow, osteomyelitis, can be divided into different types according to the origin of the condition: 1) osteomyelitis from hematogenous spread of infection, 2) osteomyelitis secondary to a continguous focus of infection, 3) osteomyelitis associated with vascular insufficiency [23] and 4) osteomyelitis from iatrogenic inoculation during surgery or trauma.

If symptoms appear early and are correctly diagnosed, treatment of acute osteomyelitis is usually successful [24]. If the diagnosis is delayed, or infection of the bone progresses without successful treatment, a significantly more challenging complication arises. Chronic osteomyelitis may involve bacterial biofilm formation on the implant and devitalized bone fragments, which become sequestra [25]. The bacterial biofilm is a central factor in implant related osteomyelitis. A biofilm consists of adherent bacteria within a polymeric matrix made out of exopolysaccharides surrounded by interstitial voids in which nutrients circulate between cells [26]. These polysaccharides are required for intercellular adhesion so that bacteria can accumulate in an adherent muli-layered biofilm [27]. Besides these polysaccharides, the extracellular polymeric substances in which bacteria live, are composed out of proteins, nucleic acids (extracellular DNA), lipopolysaccharides, glycolipids and lipids

[28, 29]. Biofilms readily form on surfaces such as prostheses and implants [30] as well as on the surface of dead bone and living tissue [31, 32]. The presence of bacteria in a biofilm drastically reduces their susceptibility to antimicrobial drugs and host defense cells. Reduced susceptibility seems not to be caused by limited antibiotic penetration, but the reduced growth rate of bacteria in a biofilm makes them less susceptible to growth-dependent antimicrobial killing [17, 26, 33]. Sub-populations may also differentiate into a phenotypically resistant state and bacteria might express biofilm-specific antimicrobial resistance genes [34, 35].

The treatment of chronic osteomyelitis is difficult, time-consuming and expensive. A standard treatment protocol for chronic osteomyelitis and infected non-unions may involve: identification of the bacterial species and antibiotic susceptibility testing, removal of orthopedic or fracture fixation devices, debridement of infected bone and soft tissue and systemic delivery of antibiotics for a 4 to 6 week period. Antibiotic-loaded spacers may be placed in the area where infected bone is removed. Irrigation with abundant antiseptic after debridement of infected bone may be an adjunct to this standard procedure [36]. Furthermore, a viable and stable soft-tissue environment has to be created and reconstruction, alignment and stabilization of the skeleton has to be performed [25].

The average costs for removing infected internal fixation devices and subsequent treatment of these infections were estimated to be $ 15,000 on average per case around the year 2000 in the United States [37]. A similar figure was obtained from a large survey from hospitals located in New York City in 1995, where 13,550 cases of *S. aureus* infection were reported with a total cost of $ 435.5 million for treatment; on average $ 32,100 per case [38]. Treatment of SSI caused by *S. aureus* cost on average $ 21,800 and treatment of osteomyelitis $ 35,000 [38]. The morbidity and socioeconomic costs of implant related osteomyelitis emphasize the need for effective prophylactic measures to prevent infection in orthopedic- and trauma surgery.

Systemic antibiotic prophylaxis is mandated for certain surgical procedures, though not for all [39]. The requirement for antibiotic prophylaxis is largely based on the risk assessment of the surgeon, regular practice in the hospital in question, and

numerous guidelines published on the topic. Antimicrobial prophylaxis is indicated for operations considered to have contamination, as these are associated with a high rate of infection [40]. When an implant is inserted during surgery, it is standard practice that perioperative antibiotic prophylaxis is provided, since the presence of a foreign material at the surgical site is an important risk factor for the development of SSI [41].

For arthroplasty, usually a single dose or 24h administration of a first or second generation cephalosporin is provided [14, 42]. A similar regime is indicated for the fixation of closed fractures [14]. For the fixation of open fractures in trauma surgery, the number of antibiotic doses and the duration of administration are related to the severity of the open fracture and the status of the wound, with greater antibiotic protection required for cases with greater soft tissue damage and greater exposure of bone and higher degree of contamination [43] (Table 2.2). When there is potential fecal contamination of the wound, either piperacillin/tazobactam, or a carbapenem or a third generation cephalosporin plus metronidazole should be provided [14].

For elective surgery like an arthroplasty, the optimal effect is obtained when sufficient antibiotic tissue levels are achieved at the time of incision. The incidence of SSI during these surgeries increases to 3.8% when prophylaxis is given too early (>2h before surgery) or to 3.3% when prophylaxis is given too late (>3h after surgery) in comparison with 0.6% when prophylaxis is given just before surgical incision is made [44]. In the case of severe trauma with open fractures after an accident, one would like to give antibiotic prophylaxis as soon as possible, since the possibility of bacterial colonization of the wound increases over time. In surgical practice, however, the antibiotics are administered upon arrival in hospital.

One of the problems related to systemic delivery of antibiotics is that insufficient concentrations are reached at vascular compromised locations, such as a fracture site or other compromised tissues. In fractures, the bony structure may be affected and the local vascularity may be disturbed [45] making it impossible to achieve appropriate local antibiotic concentrations via systemic delivery via the bloodstream. Increasing the dose of systemically delivered antibiotics is not a suitable approach since high concentrations of antibiotics over an extended period might cause

systemic toxicity problems; e.g. like ototoxicity for aminoglycosides [46] and nephrotoxicity for aminoglycosides [46-48], glycopeptides [48, 49], polymyxins [48-50], quinolones, rifampicin and sulfonamides [48].

Table 1.2: *Guidelines for antibiotic prophylaxis according to AO principles [14]*

Open fracture classification		Fracture description	Likely bacterial pathogen	Antibiotic course (IV)	Duration following wound closure
I.		Skin wound more than 1 cm, Clean, Simple fracture pattern	Gram positive cocci	1st or 2nd generation cephalosporin	24 h
II.		Skin wound less than 1 cm, Soft tissue damage not extensive, No flaps or avulsions, Simple fracture pattern	Gram positive cocci	1st or 2nd generation cephalosporin	24 h
III.	A B C	High-energy injury involving extensive soft tissue damage, Or multifragmentary fracture, segmental fractures, or bone loss irrespective of the size of skin wound, Or severe crush injuries, Or vascular injury requiring repair, Or severe contamination including farmyard injuries	Gram positive cocci + Gram negative rod	Amoxicillin / clavulanic acid or ampicillin/ sulbactam or 3rd generation cephalosporin	120 h

Local antibiotic infection prophylaxis

Local delivery of antibiotics maximises target tissue concentration, and minimizes systemic toxicity risks. Any drug delivery device intended for prophylactic use should have a broad-spectrum antibiotic incorporated in order to prevent both a wide of Gram-positive (e.g. *Staphylococcus aureus* and *S. epidermidis*) and Gram-negative (e.g. *Pseudomona aeruginosa* and *Escherichia coli*) bacteria from colonizing the surgical site.

Aminoglycosides are broad-spectrum antibiotics covering the species most frequently encountered in trauma surgery such as *S. aureus* and *S. epidermidis* [51]. Aminoglycosides have two mechanisms of action. First of all, aminoglycosides inhibit protein synthesis in bacteria. Sensitive bacteria accumulate aminoglycosides on the 30s subunit of ribosomes associated with the cell membrane. Secondly, aminoglycosides destroy the cytoplasmic membrane [52]. The ability of aminoglycosides to destabilize the outer membrane by creating holes in the cell wall is probably the most important ability of this class of antibiotics, since bacteria are killed even before protein synthesis is disrupted [52]. The aminoglycosides gentamicin and tobramycin, and the glycopeptide vancomycin are the most commonly used antibiotics in local delivery vehicles [53, 54].

Gentamicin has a broad-spectrum of activity, rapid concentration-dependent bactericidal effect, a low rate of resistance and low cost [52]. Gentamicin is available for intramuscular and intravenous injection, in antibiotic impregnated poly(methylmethacrylate) PMMA beads, in sponge-like collagen implants and as antibiotic component in a coating on intramedullary nails for tibial fracture fixation [53, 54]. Tobramycin and the glycopeptide vancomycin are also used in PMMA bone cement. The aminoglycosides activity is reduced at low pH, at low oxygen-containing environment, in the presence of calcium and magnesium ions, and in the case of hyperosmolarity [52, 55-57]. The suggested mechanism is that an acidic pH impairs gentamicin transport into bacteria, this is because of its larger ionization at lower pH, since the pKa values of the amino groups of gentamicin are between 5.5 and 9 [56].

Vancomycin is active against Gram-positive bacteria such as Staphylococci (including MRSA), Streptococci and Enterococci [2]. Vancomycin interferes with cell wall synthesis in Gram-positive bacteria [58]. It targets the terminal D-Ala-D-Ala of the staphylococcal peptidoglycan stempeptide and inhibits the elongation of the sugar backbone and the crosslinking of the peptidoglycan [59]. The systemic route of administration of vancomycin is oral or intravenous [60]. Vancomycin is often used in the case of β-lactam resistance [59, 61]. Another reason for using vancomycin is when the patient is allergic to β-lactams [17]. Staphylococci, both coagulase negative and coagulase positive are susceptible to vancomycin at levels as low as ≤ 1-5 μg/ml, but susceptibility is drastically lowered in vancomycin-resistant *Staphylococcus aureus* (VRSA) [62].

One potential side effect of high local concentrations of antibiotics is the risk of cytotoxicity. Rathbone *et al.* showed the effect of the concentration of various antibiotic agents on osteoblast cell viability and activity [63]. Gentamicin concentrations of 10 μg/ml resulted in a decrease of less than 25% in alkaline phosphatase (ALP) activity and DNA content. At concentrations between 10 μg/ml and 200 μg/ml, a decrease in ALP activity and DNA content of 25%-50% was reported, while for concentrations higher than 2000 μg/ml more than 75% decrease in ALP activity and DNA content was observed [63]. Isefuku *et al.* used human osteoblast like cells (HOB) to investigate the effect of gentamicin on osteogenesis [64]. ALP activity and DNA content were measured, as well as ^{3}H-thymidine incorporation (measure of cell proliferation) after 4 days of culture. Gentamicin concentrations of 30 μg/ml resulted in no decrease in the aforementioned parameters. Gentamicin concentrations of 100 μg/ml and higher showed a decrease in ALP activity, concentrations of 300 μg/ml resulted in less than 50% decrease of ^{3}H-thymidine incorporation and concentrations of 700 μg/ml showed a decrease in DNA content for all HOB cultures. Haleem *et al.* showed in an *in vivo* study that systemic administration of gentamicin (1.5 mg/kg intramuscularly) or vancomycin (25 mg/kg intraperitoneally), both to simulate therapeutic peak serum concentrations, resulted in 30 minutes serum concentration of 4.5 μg/ml for gentamicin and 35.1 μg/ml for vancomycin and did not impair experimental fracture healing in rats [65].

Tobramycin can potentially be applied locally at higher concentrations (>500 µg/ml *in vitro*) than gentamicin without compromising osteoblast viability [50, 63]. Vancomycin can be released even at concentrations up to 2000 µg/ml with a decrease of less than 25% in ALP activity and DNA content. However, such high concentrations may not be required, since glycopeptides display time-dependent activity, rather than concentration dependent activity.

Thus, in the local delivery of antibiotics such as gentamicin it is therefore not only necessary to reach concentration levels above the minimal bactericidal concentration (MBC) in order to prevent bacterial resistance, but it is also important to keep peak concentration at a level which does not affect bone healing [63, 66]. For instance, local gentamicin levels for prophylaxis against Staphylococcal species (spp.) should be between 128 µg/ml and 200 µg/ml to meet the $MIC_{90\%}$ value and have a reduction of less than 50% in ALP activity and DNA content for osteoblasts as determined *in vitro* [66, 67].

Since antibiotics will act for a short term period when injected locally before being cleared, the design of controlled and sustained delivery system is of equal importance as the choice of antibiotic to be used. In 1970, PMMA bone cement was the first polymeric biomaterial which came to the clinic to serve this purpose [68]. Bone cements are prepared by mixing of a liquid phase and a powder phase containing an initiator, which starts the polymerization process. The liquid phase mainly consists of methylmethacrylate (MMA) monomer. The powder phase consists of either a methylmethacrylate (PMMA) or a methylacrylate (PMA) polymer or a copolymer of one of the two previous compounds with butylmethacrylate (BUMA), ethylmethacrylate (EMA), methylacrylate (MA) or styrene. Furthermore, the powder phase contains an initiator, benzoyl peroxide, which cures the cement upon contact with the activator N,N-dimethyl-p-toluidine (DMpT) from the liquid phase. Radiopacifiers such as $BaSO_4$ and ZrO_2 may also be added to the powder to increase cement visibility in radiographs. A dye may also be added to the powder or to the liquid or to both. Chlorophyllin is most commonly used; however in Cobalt™ G-HV indigo carmine is added. Hydroquinone (HQ) is added to the liquid monomer as a stabilizer in order to prevent premature polymerization during storage [69]. Obviously, the composition of the polymer powder and the monomer liquid will influence the

properties of the resulting bone cement after curing, such as hydrophilicity (water uptake), mechanical strength and porosity (created by air inclusions in the cement dough) [69, 70]. The hydrophilicity of the final polymer components will influence the diffusion of antibiotics from the bone cement into the surrounding tissue and therefore the release rate of the included antibiotic. There are 3 different mechanisms suggested for the release of antibiotics from bone cement *in vitro* [71]. The antibiotics are either released from the bone cement by a surface phenomenon, (1) by bulk-diffusion through pores and connecting capillaries in the bone cement (2) or by a combination of the two mechanisms, the mixed-mode model, where initially antibiotics are released from the surface followed by bulk-diffusion (3) [71]. However, in all PMMA bone cement compositions, the antibiotics are not completely released [69]. A large amount stays encapsulated within the bone cement and is therefore not released during the lifetime of the implant, which would support the theory that antibiotics are mainly released from the bone cement by a surface phenomenon. In fact, Frutos Cabanillas *et al.* performed a release study with CMW1 Gentamicin bone cement which supports this phenomenon [72]. The *in vitro* release was tracked for 8 weeks and it was found that most of the gentamicin was released during the first 2 hours, but then the antibiotic concentration dropped very quickly afterwards to almost no release.

Kühn compared the release of 12 different bone cements *in vitro* over 7 days [69]. Between the different commercial bone cements there was a large difference in release with a several fold higher release of gentamicin [mg/g bone cement] of the bone cements prepared from MMA/MA copolymer compared to PMMA homopolymer, most likely because of the higher hydrophilicity of the copolymer composition. The gentamicin content in different commercial bone cement powders and the amount of gentamicin released after 7 days from several commercial available bone cements can be seen in Figure 2.1 [69].
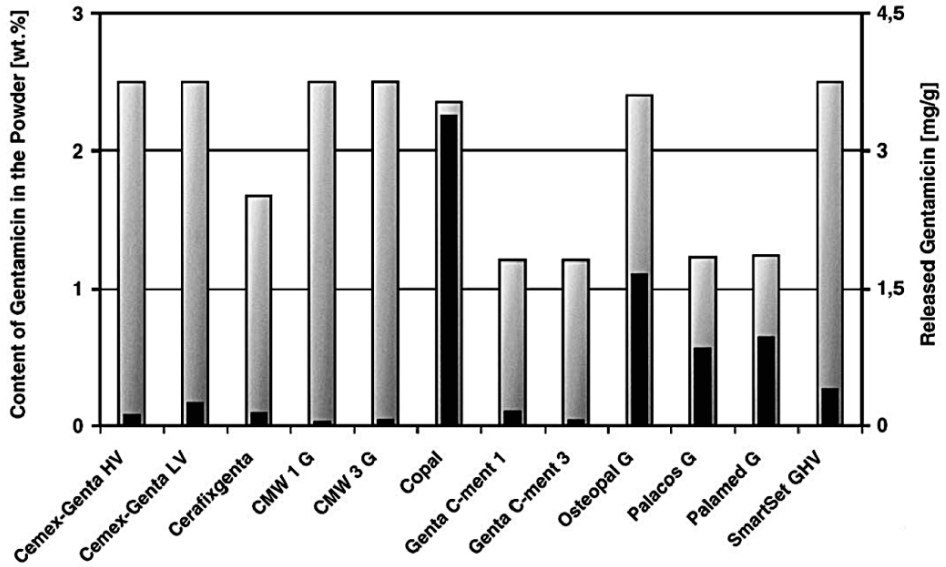
Figure 2.1: Gentamicin content in different commercial bone cement powders (grey bars, y-axis on the left) and the amount of gentamicin released after 7 days in vitro as expressed in mg/g bone cement (black bars, y-axis on the right) [69].
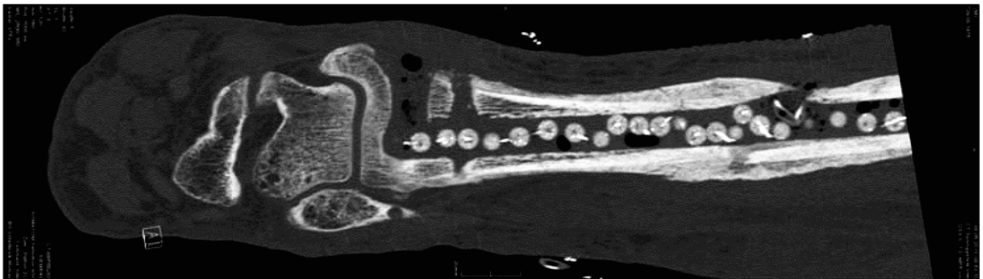

Figure 2.2: Gentamicin loaded PMMA beads, placed after infection of a plated tibial fracture (Image courtesy of Dr. Mario Morgenstern from the BGU Murnau, Germany).

PMMA is used as a carrier material for antibiotics as bead chains (Figure 2.2), blocks (Figure 2.3) and putty. A commercial gentamicin loaded PMMA bead chain is produced by Merck since 1972 under the trading name Septopal®. These bead

chains are primarily used in the treatment of infections but they are also used in prophylaxis [55], although this is not standard practice. They are used for treating prosthetic joint infections, septic arthritis and osteomyelitis. In prophylaxis they may be used when there is an elevated risk of SSI [55].
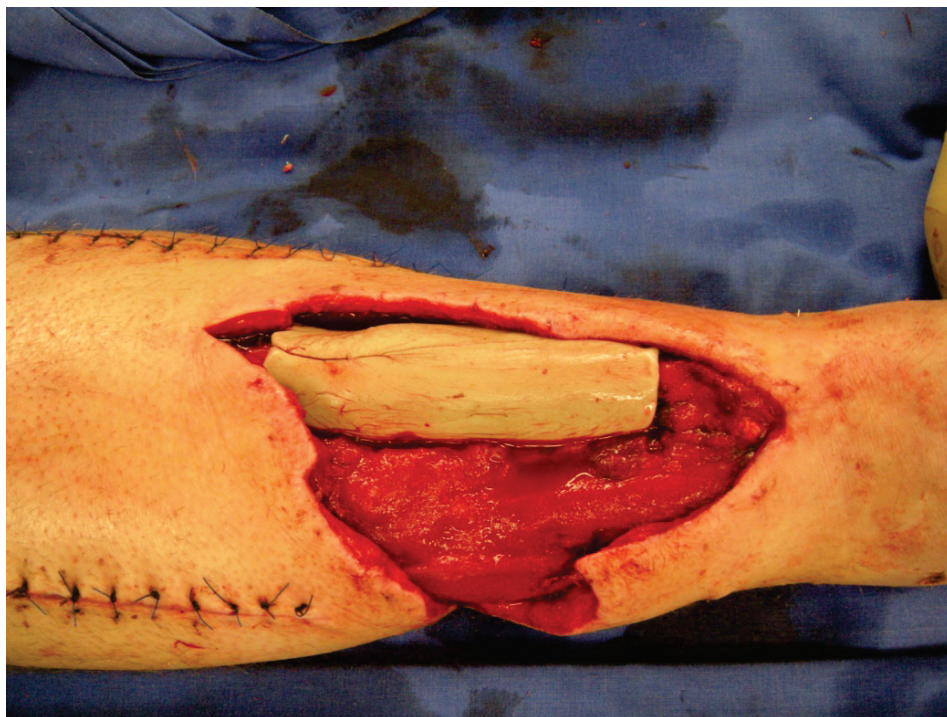

Figure 2.3: Antibiotic loaded PMMA spacer (Image courtesy of Dr. Mario Morgenstern of BGU Murnau, Germany).

The combined action of a systemically administered antibiotic and local delivery using PMMA has been shown to be beneficial in reducing the infection rate upon hip replacement. In a large cohort study of the Norwegian Arthroplasty Register of 22,170 hip replacements, it was shown that local delivery of antibiotics from PMMA bone cement combined with systemic antibiotics had the lowest risk of revision [73]. Patients who received the antibiotics only systemically had a 1.8 times higher revision rate with infection as the endpoint (p=0.01). The systemic antibiotics used were cephalosporins (cephalotin or cefuroxime) or penicillin (cloxacillin or

dicloxacillin). The antibiotics used in the PMMA were gentamicin in Palacos cement or colistin/erythromycin in Simplex™ cement [73].

Polymeric bone cement has also several drawbacks as a local delivery vehicle for antibiotics. The drawbacks are primarily the rapidly reducing local antibiotic levels, and the permanent presence of a foreign body, which may be colonized by bacteria. Low, sub-inhibitory antibiotic levels over an extended period can induce bacterial resistance. For example, studies show increased gentamicin resistance after use of impregnated bone cement in patients receiving a hip implant [67]. In a study published by Weber and Lautenbach, the percentage of bacteria resistant to gentamicin increased from 29% for bacteria which were isolated preoperatively to 41% for bacteria isolated after surgery in which gentamicin-impregnated cement was used [74]. Furthermore, in a study of 34 cases of revision surgery of hip implants, at least one strain of gentamicin-resistant coagulase negative staphylococci (CNS) was found on 88% (30) of the implants in which gentamicin-impregnated cement was used. In 57 cases of revision surgery of hip implants where no gentamicin was included in the cement only 16% (9) grew gentamicin-resistant CNSs [75].

PMMA beads are non-degradable and need to be removed during follow-up surgery, which is an additional risk for acquiring a new intraoperative infection [53]. Unreacted methacrylate monomer can cause toxicity issues, with toxicity directly related to the lipophilicity of the respective methacrylate [76]. Furthermore, the heat generated during polymerization limits the choice of antibiotics: most antibiotics as opposed to gentamicin are heat labile [66]. *In vitro* maximum temperatures reached according to the standards ISO 5833 and ASTM F 451 for curing of bone cement are between 60°C and 80°C. Clinical trials, however, showed lower temperatures between 40°C and 47°C at the bone-cement interface *in vivo* [69]. Although heat necrosis due to high local temperatures, which is suspected to lead to aseptic loosening, might not occur at the interface *in vivo*, the temperature within the curing bone cement might still be much higher than at the interface and affect heat labile antibiotics [69].

Collagen, in contrast to PMMA, is biodegradable. In the body, collagen is degraded by phagocytosis and enzymatic degradation [10]. Collagen fleece loaded with antibiotics is used in contaminated wounds to prevent infection. Several antibiotic-

loaded collagen fleeces are commercially available (Figure 2.4) [77], differing in collagen source, amount of antibiotic loaded and type of antibiotic loaded. Controlled randomized clinical studies in which antibiotic-loaded collagen fleece (1 to 3 sponges per case with 200 – 600 mg of gentamicin sulfate) was used for intra-abdominal-related surgeries or wound infections, had a positive outcome in 95.6% compared to 72.5% for standard therapy, in which patients healed by primary intention or without evidence of post-operative infection [78].

Figure 2.4: *Antibiotic loaded collagen fleece applied at the surgical site before insertion of the implant (Image courtesy of Dr. Mario Morgenstern of BGU Murnau, Germany).*

When using collagen fleece, the antibiotic is released very rapidly [79]. An *in vitro* study with pepsin-treated bovine collagen, 0.5 g of collagen containing 130 mg gentamicin, showed a release of 95% of the antibiotic in 1.5 hours [79]. However, the full resorption of the collagen carrier takes 8 weeks [80]. Antibiotic delivery rates are highly dependent on the fluid supply *in vivo*. Effective local concentrations might be reached during less than 24 hours in well perfused sites and up to 1 week in bony structures [81]. *In vivo*, the mismatch between the rate of antibiotic release and the rate of degradation of the collagen might allow bacteria to attach to the collagen via

microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) [82], serving as substrate for bacterial adhesion.

High water solubility of particular antibiotics might limit the application and the prolonged release from biomaterials. Therefore, research has also focused on formulations with reduced solubility. One approach is to create lipophilic salts of the antibiotic, e.g. for gentamicin: gentamicin sodium dodecyl sulfate (SDS), gentamicin sodium bis(2-ethylhexyl)sulfosuccinate (AOT), gentamicin crobefate,gentamicin laurate, gentamicin myristate and gentamicin palmitate [83-87].

For the use of collagen fleece longer prophylactic antibiotic levels are maintained, when gentamicin crobefate is incorporated in addition to gentamicin sulphate [88]. A prolonged release period (about 12 days *in vivo*) is thereby established, since gentamicin crobefate is hydrophobic as opposed to the sulphate salt of gentamicin which is hydrophilic and thus readily diffuses. However, the degradation rate of the fleeces themselves are not modified [80]. Overall, the main drawback of antibiotic loaded collagen fleece is the non-matching degradation rates and release times. Another drawback is the complicated handling of the collagen fleeces, and the covering of all infected areas by the fleece. Furthermore, although rare, biological responses such as localized hypersensitivity to- and circulation of antibodies against bovine collagen can be elicited [89].

The use of biodegradable synthetic implants avoids these biological responses. For instance synthetic coatings containing antibiotics have been applied on metal implants to prevent infection. Coatings can be applied on a wide variety of materials, turning an implant into a drug delivery device. So far, only poly(D,L-lactide) (PDLLA) coatings have made it to the clinic (Figure 2.5). Clinical trials with metal implants coated with PDLLA with gentamicin incorporated were started in July 2003 [54]. Intramedullary nails with a PDLLA coating containing gentamicin were implanted in the tibia of patients with complex fractures. Indications for use of a gentamicin-containing PDLLA coated intramedullary nail were the presence of a type III open fracture or the performance of a re-osteosynthesis, because the incidence of infection is very high for these cases. In a case study of a 17 year old man with a type III open tibia fracture, this PDLLA gentamicin-coated nail was inserted. All the

wounds healed without complications and after 25 days the patient was fully mobilized. In 2011, this PDLLA gentamicin-coated intramedullary nail was released on the market. *In vitro* release studies in deionized water showed that these PDLLA gentamicin-coated implants released 40% of the gentamicin-sulphate payload within 1h, 70% within 24 hours and 80% within 48 hours [90].
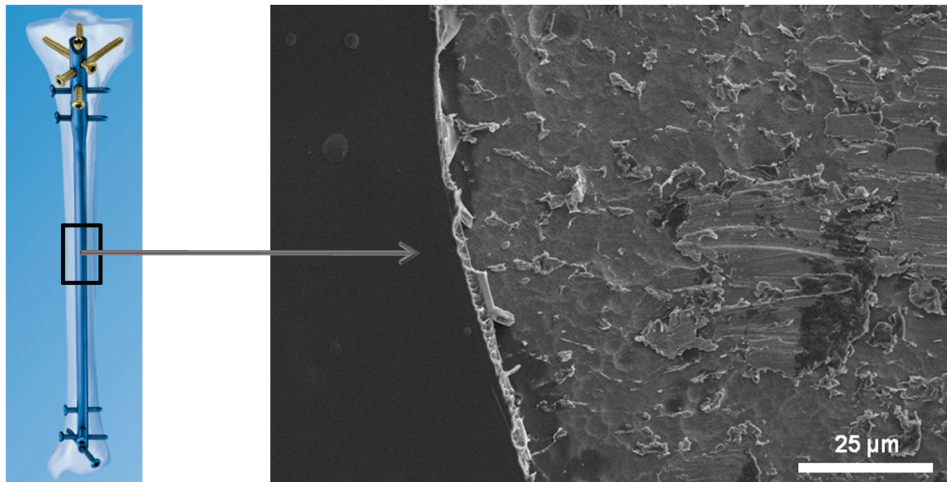

Figure 2.5*: Tibial nail with gentamicin sulphate containing PDLLA coating visualized by SEM (Coating on the left of the image (image available from www.synthes.com) and scratched implant surface revealing coating on metal implant on the right).*

There are several drawbacks related to the use of coated implants. One of the drawbacks is that part of the coating can delaminate during insertion due to applied forces on the implant. Since coating the implant creates a new medical device, the device needs a separate approval before it can be used in the clinic. Furthermore, antibiotic activity and bone healing can be impaired upon release of acidic degradation products of PDLLA which lower the local pH [91]. Low pH values at an implant site might evoke inflammatory foreign body responses with accompanying osteolytic foci in the exposed bone [92]. *In vitro* degradation studies with poly(lactide) based polymers showed pH values between pH 3.0 and pH 7.0 depending on the composition of the polymer, experimental conditions and degradation time [93-95]. The minimum pH is reached at the time that the polymer degrades with the greatest

rate of weight loss. *In vivo*, this pH drop might be less dramatic than that *in vitro,* since body fluids surround the implant have a buffering effect. This has been shown *in vivo* in Wistar rats upon implantation of a PDLLA implant. Whereas *in vitro* the pH dropped by 4 pH units after 4 weeks in Ringer solution, *in vivo* the maximum pH drop was 1 pH unit 1 week after implantation [93]. However, buffering the release of these acidic degradation products might not be possible for large size implants or implants located at anatomical regions with restricted access to body fluids [96]. Thus, the development of a better prophylaxis material for implant surfaces and the optimal local delivery of the antibiotics from these surfaces remain major challenges.

New developments in the local delivery of antibiotics

Different strategies have been pursued by researchers in order to improve systems for the local delivery of antibiotics. For instance, researchers have tried to improve systems already available in the clinics. Besides changing the polymeric carriers, efforts also have been made to incorporate modified antibiotics, to produce polymeric prodrugs based on conventional antibiotics or to entrap silver (nano)particles or to incorporate antimicrobial peptides or antimicrobial peptide elicitors instead of antibiotics. Another new development is the creation of systems that can be applied minimally invasively and have a sustained antimicrobial release profile. And finally, release systems are being investigated which release their antibiotic or antimicrobial compound in response to a biological stimulus related to an infectious state.

Biomaterials loaded with 'new' antimicrobial agents

Many new antimicrobial compounds have been incorporated into biomaterials. For instance, polymeric prodrugs have been developed based upon conventional antibiotics. An example is given by the work of Marcus *et al.* who created a prodrug of gentamicin by grafting poly(ethylene glycol) (PEG) to gentamicin with heterobifunctional linkers, which are cleaved by spontaneous hydrolysis [97]. Upon PEGylation, the half-life of the antibiotic increases *in vivo*. By using reversible PEGylation of gentamicin, PEG$_{20}$-FMS-gentamicin and PEG$_{40}$-Fmoc-gentamicin, the

inactivated gentamicin becomes active again after the linker has been hydrolytically cleaved, whereas irreversibly bound PEG renders the antibiotic inactive [97].

By covalently grafting the antibiotic to a carrier, additionally to increasing the half-life and retarding the time of release of the antibiotic, special ligands can be incorporated to target the complex towards specific functionalities on cells in order to target intracellular infections (Figure 2.6). As an example, Coessens *et al.* synthesized a conjugate of streptomycin linked to poly[N-(hydroxyethyl)-L-glutamine] (PHEG) and dextran with glycerine hydrazide as linker and a mannose-terminated side group for intracellular targeting [98]. Streptomycin was coupled via Schiff base formation. The release of streptomycin from the carrier is a hydrolytic process and dependents on the pH (acidic environments catalyze the hydrolysis of the linker). The authors hypothesized that the lower pH (pH ~5.2) in endosomes and lysosomes could facilitate the intracellular release of the streptomycin from the prodrug by destruction of the Schiff base. The release of streptomycin from the polyglutamine prodrug was tracked at pH 7.4 and pH 5.2. At pH 5.2 approximately 50% of the streptomycin was hydrolyzed from the prodrug, whereas at pH 7.4 about 30% of the streptomycin was released from the prodrug [98].
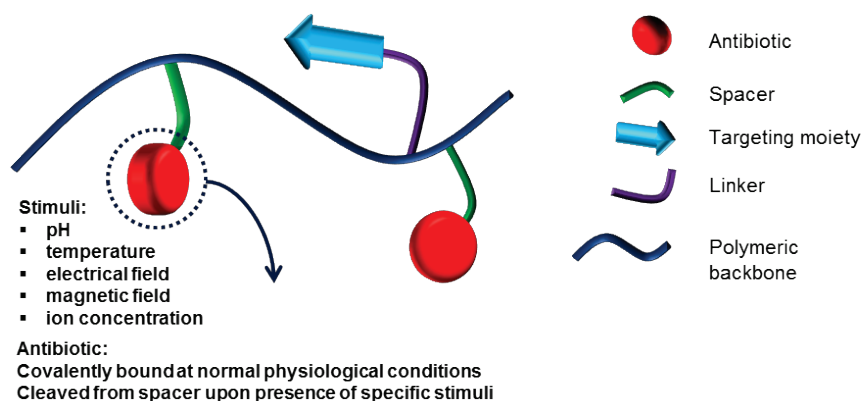

Figure 2.6: Schematic of stimuli-responsive prodrug systems for the targeted delivery of antibiotics.

Polymeric prodrugs also have been investigated by Choi *et al.* with the aim of targeting dendrimer-antibiotic conjugates to bacterial cell walls [99]. Poly(amidoamine) (PAMAM) dendrimers of the fifth generation were tethered with vancomycin at the C-terminus. These vancomycin-conjugated PAMAM dendrimers were able to bind to the surface of even vancomycin resistant bacteria in a synthetic model of the bacterial cell wall. Compared to free vancomycin, the PAMAM dendrimer-vancomycin conjugate bound to these synthetic models of the bacterial cell wall tightly through binding to multiple receptor sites, indicating enhancement in avidity of four to five orders of magnitude. In an affinity study by means of surface plasmon resonance spectroscopy, these PAMAM dendrimer-vancomycin conjugates bound tightly to a vancomycin-resistant surface, which shows only weak (millimolar) affinity to free vancomycin [99].

As multi-resistance has become a problem for the use of conventional antibiotics, the use of silver has received new attention in combination with biomaterials. A comprehensive review on the antimicrobial action of silver ions, silver containing compounds and silver nanoparticles in particular has been published by Rai *et al.* [100]. A book chapter about silver containing biomaterials has been published by Griesser *et al.* [101]. The bactericidal effect of silver is due to their interaction with biomolecules. Proteins, enzymes and cell-membrane components in bacteria contain nucleophilic functionalities, which are capable of coordinating silver cations (Ag^+) [102]. Bacterial cell death is induced by silver cations by reacting and disrupting the function of cell membranes, metabolic proteins and enzymes and also by displacing metal ions, like Zn^+ and Ca^{2+}, which are essential to bacterial cell survival [102]. The antibacterial effect of PMMA bone cement loaded with silver nanoparticles (0.1%, 0.5% and 1.0 %) on *S. epidermidis*, multi-resistant *S. epidermidis* (MRSE) and MRSA was compared with PMMA bone cement loaded with gentamicin sulphate (2%) by Alt *et al.* [103]. PMMA bone cement loaded with gentamicin or silver nanoparticles both inhibited the proliferation of *S. epidermidis*. Silver nanoparticle-loaded PMMA bone cement was able to clear MRSE and MRSA as well, whereas PMMA bone cement with gentamicin was unable to inhibit the proliferation of the resistant strains.

The advantage of using silver nanoparticles over using larger sized particles is the maximization of the active surface of the silver, while keeping the total amount of

silver low [104]. Furthermore, the bactericidal effect seems to be dependent on the shape of the silver nanoparticles as well. Truncated triangular shaped silver nanoparticles were more effective in inhibiting bacterial growth of *E. coli* than spherical silver nanoparticles, which were more effective than rod shaped silver nanoparticles in a study performed by Pal *et al.* [105]. Although silver-related cytotoxicity towards eukaryotic cells can be limited by using silver with nano-dimensions , the preparation of silver nanoparticles is hindered by their tendency to aggregate [106]. To stabilize silver nanoparticles, Travan *et al.* encapsulated the particles in hydrogels composed of Chitlac (lactose substituted chitosan) and alginate [106]. Microspheres prepared of this composite with encapsulated silver nanoparticles eluted only 2.6% of the total amount of silver present in the hydrogel over 5 weeks incubation in saline solution, which did not impair the cell viability of 3 different cell lines (mouse fibroblasts, human hepatocarcinoma cells and human osteosarcoma cells). Whereas Chitlac solutions mixed with silver nanoparticles was found to be cytotoxic to all three cell lines, leading to cell death within 24h. Hydrogels and hydrogel extracts prepared from an alginate-Chitlac mixture with the silver nanoparticles entrapped did not show cytotoxicity towards these cell lines as they are shielded from uptake by mammalian cells [106]. Antibacterial testing was done by adding 20% MH broth as bacterial nutrition to both alginate and Chitlac solutions. The surfaces of alginate-Chitlac gels with and without silver nanoparticles were smeared with 10^6 CFU/ml *S. epidermidis*. Only the silvernanoparticle loaded gels were free of bacterial colonization [106].

The combination of polymers and nanosilver can solve diverse problems related to the use of nanosilver such as: (1) polymers can prevent aggregation of silver nanoparticles, (2) polymers can act as linker for silver nanoparticles in coatings, (3) silver ion release can be tailored by changing the interaction between the polymer and the silver and changing the silver concentration. Several methods for the preparation of polymer/nanosilver composite coatings have been used to incorporate silver in biomaterials as can be found in an extensive review by Guo *et al.* [107]. Because of the multiple mechanisms of antimicrobial action exploited by silver nanoparticles they are less likely to suffer from resistance mechanisms in bacteria

[108], however silver resistance has been observed in clinical isolates from silver exposed sites in patients as well [109].

Antimicrobial peptides (AMPs) have also been incorporated into biomaterials. Antimicrobial peptides are usually rather short peptide sequences, 12 to 100 amino-acids long, positively charged (net charge +2 - +9), are amphiphilic, and have been isolated from single-celled microorganisms, insects and other invertebrates, plants, amphibians, birds, fish and mammals, including humans [110]. A special feature of AMPs is that they target a specific feature of the microbial cellular membrane that distinguishes broad species of bacteria from multicellular plants and animals [111]. Microbial cellular membranes possess negatively charged headgroups on the lipids that form the outermost leaflet of the bilayer which is exposed to the outer world. On the contrary, the outer leaflet of the membranes of plants and animals is composed principally of lipids with no net charge [111]. Although there are large variations in the structure of AMPs, a common feature of cationic AMPs is that they have an amphipatic structure, which allows them to bind to the membrane interface. The interaction with, and finally the disruption of the inner and outer membranes of bacteria leads to the bacterial cell death [112].

Unlike resistance towards antibiotics, acquired resistance towards antimicrobial peptides by sensitive bacterial strains is improbable. However, some bacterial species are resistant by nature, since (1) they lack the appropriate density of acidic lipids to provide peptide-binding sites, (2) Some species produce digestive proteases, which destroy AMPs [111]. Furthermore, degradation by human proteases, unknown toxicity profiles and high costs of production have limited the potential application of AMPs [113]. Only few AMPS have made it to clinical phase testing and none is approved by FDA so far [114]. A promising group of AMPs is the indolicidin-analogues such as MBI-226 (Omiganin) for the treatment of catheter-related infection, which have been used in a clinical phase III trial [110, 114].

Since development of resistance against AMPs is unlikely, recently the potential of functionalizing biomaterials with AMPs has been explored in research. Issues of short half-lives and cytotoxicity can be circumvented by immobilizing AMPs on the surface of biomaterials. The use of a spacer between a biomaterial and an AMP can

influence the orientation of the peptide and hence its bactericidal activity. A comprehensive review on the different strategies for immobilization of AMPs onto biomaterials and its effect on activity, cytotoxicity and long-term stability has been published by Costa *et al.* [115]*.*

Improved polymeric carriers for the passive delivery of antibiotics

In order to overcome the drawbacks of non-degradable antibiotic loaded biomaterials such as PMMA beads and spacers, biodegradable polymer based materials for the delivery of antibiotics have been investigated [116]. To prepare biodegradable antibiotic loaded beads or spacers, either PMMA copolymerized with degradable blocks or resorbable polymers have been used. For the latter a wide variety of polymers, especially aliphatic polyesters prepared by ring-opening polymerization such as PDLLA [117-127], poly(lactic-co-glycolic acid) PLGA [128, 129] and PCL [130, 131] have been used. For example, antibiotic loaded beads from PDLLA and PLGA were prepared by Mader *et al.* [132]. Clindamycin, tobramycin or vancomycin were released in adequate concentrations for at least 30 days from the PLA and PLGA beads, whereas the PMMA beads released this antibiotics in adequate concentrations for only 12 days [132].

Neut *et al.* investigated the use of poly(trimethylene carbonate) (PTMC) discs as a delivery system for gentamicin [133]. The advantage of PTMC is that it degrades without acidic degradation products. PTMC degrades primary by enzymatic degradation. Therefore, the degradation *in vitro* is very slow, whereas the degradation *in vivo* is much faster due to the presence of enzymes [134]. Furthermore, the degradation of PTMC by surface erosion might facilitate a more gradual and sustained release depending on the shape of the delivery system, with the possibly for zero-order release kinetics, whereas other biodegradable materials usually degrade by bulk erosion and lack this control. Neut *et al.* compared the performance of these PTMC discs with conventional gentamicin-containing PMMA beads (Septopal®). PTMC discs loaded with 10 w/w% of gentamicin entrapped were prepared and the *in vitro* release was assessed by immersing the discs in a PBS solution or in a lipase enzyme solution at 37°C. In the lipase enzyme solution PTMC

discs completely degraded in a two week course, whereas the PTMC discs immersed in PBS did not degrade at all. The gentamicin entrapped was released very slowly from the PTMC discs when immersed in PBS solution, with only 10% of the antibiotic being released over two weeks, however in lipase enzyme solution over 50% of the antibiotic was released over two weeks [133]. So, the large difference in degradation and release is the result of the combined enzymatic and hydrolytic degradation mechanism of PTMC. *In vivo* studies of PTMC discs implanted in subcutaneous pockets in the back of rats showed complete degradation of the implant in 1 year [135]. Consequently, in order to find a suitable biodegradable substitute for PMMA beads and spacers, it is important to consider the properties of the material and their mechanism of degradation.

For collagen fleece, most of the research into improvements has focused on sustaining the release of highly water soluble antibiotics. Besides modifying the properties of the antibiotics, the properties of the collagen carrier can be changed to obtain a sustained release. For instance, the release rate of gentamicin from collagen fleece can be modified to a certain extent by varying the porosity of the collagen matrix or by chemical modification, i.e. succinylation of collagen to enhance charge interactions for ionic bonding between the collagen and ciprofloxacin [78, 80, 136].

Instead of modifying the properties of the collagen fleece itself, a collagen composite can be created by incorporation of microspheres within the collagen matrix. The composite collagen fleece contains antibiotic in the collagen matrix, but also antibiotic entrapped in microparticles. PLGA microspheres made of a 50/50 blend of PLGA of molecular weight (MW) of 13.5 kDa and 36.2 kDa respectively, with gentamicin entrapped were prepared by the water-in-oil-in-water double emulsion technique by Schlapp and Friess [81]. These microspheres were dispersed within a collagen matrix to create the composite fleece. A burst release of 55% of the entrapped gentamicin was observed *in vitro* from these fleeces, followed by a more gradual release of the residual gentamicin during the first 7 days upon immersion in PBS at 37°C.

New responsive polymeric carriers for the active delivery of antibiotics

One of the main requirements of a system for the local delivery of antibiotics in prophylaxis is the ability to have a release which is provided over a period of several days. Hydrogels for the delivery of antibiotics combine the advantage of minimally invasive application (injection with needle, through a tube or application of a spray or foam) with the advantage of having a sustained release without the need of frequent re-administration. Hydrogels are polymeric materials with high water content that can be used to entrap antibiotics. The stimuli sensitive hydrogels are a special group of hydrogels that can respond to chemical or physical stimuli as to change their physical properties and cleave attached chemical groups at the site of action. Thermo-responsive hydrogels can respond to changes in the environmental temperature. Thermo-responsive hydrogels are designed to have low viscosity at room temperature, but turn with change of temperature from a viscous state to an elastic state. So, thermo-responsive hydrogels are designed so that they can be injected at room temperature and form a gel at body temperature. Veyries *et al.* used Poloxamer 407 (Pluronic® F-127) to encapsulate vancomycin [137]. Poloxamer 407 is a triblock co-polymer with the poly(propylene oxide) (PPO) as a hydrophobic central block with on both ends a hydrophilic block of poly(ethylene oxide)(PEO). Poloxamer can, when used in concentrations higher than 20 w/v %, gel in-situ because of a sol-gel temperature that is lower than the body temperature. However, there is a risk of lipid metabolism alteration because of high doses of Pluronics. Thus, supermolecular structures of Pluronics combined with cyclodextrins have been investigated to decrease the amount of required polymer for gelation while providing a sustain release [138].

Antibiotics can be mixed into these hydrophilic networks at room temperature and become entrapped at higher temperature. Subsequently the drug is released by several mechanisms: diffusion, swelling and erosion. The antibiotics can diffuse out of the hydrogel depending on the concentration difference across the gel and outside the gel and the path length. Swelling of the hydrogel network allows the entrapped antibiotics to more easily diffuse out of the network. Finally, bulk erosion of the hydrogel also causes antibiotics to be released with the eroded parts and decreases the path length for diffusion from the gel.

Suzuki *et al.* prepared and studied thermo-responsive hydrogels for infection prophylaxis *in vivo,* with hydrophilic blocks composed out of *p*-dioxane (DX) and PEG, the hydrophobic blocks were composed out of PDLLA [123]. This triblock PLA-DX-PEG hydrogel had a MW of 9.8×10^3 Da and a molar ratio of PLA:DX:PEG equal to 5:1:3. This hydrogel degraded in 2-3 weeks in PBS at 37°C. They encapsulated teicoplanin, a glycopeptide antibiotic, within this hydrogel and next to the antibiotic recombinant human bone morphogenetic protein-2 (rhBMP-2). The rationale behind this was to eradicate an infection and at the same time stimulate bone formation. In a release study, 30 mg of the gel with 4 µg of teicoplanin was used to track the release of the antibiotic *in vitro*. Approximately 40% of the encapsulated teicoplanin was released during the first 24 hours and a level above the $MIC_{90\%}$ value for *S. aureus* was maintained for 2 weeks. The polymeric hydrogel was degraded in about 3 weeks. In an *in vivo* experiment of a rat cranial bone defect, the restored area was measured from CT-scans taken 6 weeks upon implantation of the polymeric implant [123]. The implant with rhBMP-2 had a similar size of restored area as compared with the defect, which had been treated with an implant with rhBMP-2 and teicoplanin. The implant with only teicoplanin encapsulated had a smaller restored area as when rhBMP-2 was used, however this area was larger than for the negative control (only the PLA-DX-PEG implant).

Compositions that respond to enzymes have also been investigated. A poly(ester-urethane) based system with covalently bound ciprofloxacin was assessed by Woo *et al.* [139]. The rationale behind this design is that poly(ester-urethane)s have been shown to be susceptible to degradation by hydrolytic enzymes, which are released by leukocytes and macrophages present at the site of trauma and/or implant site. This way a responsive system would be obtained in which ciprofloxacin would be released from the polymer upon these stimuli. The release was tracked from poly(ester-urethane)-ciprofloxacin coated hollow glass tubes in phosphate buffer (pH 7) and cholesterol esterase (CE) (40 unit/ml) solution for 30 days. Although a higher rate of polymer degradation was observed in the presence of the CE enzyme, there was no difference observed in the release of free ciprofloxacin upon immersion in a CE solution as compared with the phosphate buffer solution. So, the data suggest that the enzyme was unable to specifically cleave the polymer segments required to

release free ciprofloxacin, and instead inactive ciprofloxacin with poly(ε-caprolactone) or 1,6-hexane diisocyanate fragments attached were released [139]. Another example of such a responsive system was a poly(vinyl alcohol) (PVA) hydrogel system investigated by Suzuki *et al.* [140]*.* This hydrogel had gentamicin covalently grafted to it by a peptide linker susceptible to cleavage by bacterial enzymes released by *P.aeruginosa*. Several peptide sequences were tested for their hydrolytic activity, and the most efficient one (Gly-(D)Phe-Pro-Arg-Gly-Phe-Pro-Ala-Gly-Gly) was selected [140]. The peptide linker and the gentamicin were grafted to a succinylated PVA hydrogel. In an *in vitro* experiment, it was shown that gentamicin was released upon incubation with exudate of a *P. aeruginosa* infected wound, however exudate of a non-infected wound was not able to cleave the peptide linker and release the gentamicin [140].

Conclusion. The ideal polymeric delivery system for infection prophylaxis

In order for a polymeric biomaterial to be a good candidate for use in infection prophylaxis, the system has to fulfill several requirements. The materials currently available in the clinic contribute to improved infection prophylaxis, by lowering the number of infections in patients. However, all of these materials have their own limitations. For instance PMMA lacks degradability, which is troublesome due to unfavorable release patterns of the antibiotic as well as permanent presence of a foreign body. Collagen on the other hand, can be biodegraded in the human body; however the inability for true sustained release can be an issue for its use. For the PDLLA coatings, the problem lies in the acidic degradation products that hinder bone regeneration and antibiotic action at the same time. Furthermore, all of these systems are rather passive than active in the sense that they are unable to respond and tailor their antibiotic release upon physiological changes in the human body, like temperature or presence of bacterial enzymes. Also the emergence of resistance in bacteria in hospitals worldwide by use and misuse of antibiotics limits the use of delivery systems that are based on conventional antibiotics. Therefore, research has also focused on encapsulating other antimicrobial compounds into biomaterials, such as modified antibiotics, antibiotic prodrugs, silver or antimicrobial peptides. All of

these should have a broad antibacterial spectrum, with coverage of both Gram-positive and Gram-negative organisms.

Finally, a system that can respond to physiological stimuli and therefore can be applied minimal invasively, while sustaining the release of antibiotics over a period of several days can be advantageous. The introduction of linkers in the system, which can be cleaved upon exposure to enzymes produced by bacteria, or other stimuli given by bacterial pathogens, could produce a more effective drug delivery system. In conclusion, there is a wide variety of approaches investigated, all with their specific benefits over traditional delivery systems. But the many requirements for an optimal system make the development of the ideal system a significant challenge.

Acknowledgements

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Chapter 3

Hyaluronic acid derivatives and its polyelectrolyte complexes with gentamicin as a delivery system for antibiotics

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Abstract

Repeated administration of gentamicin is often necessary to have efficacious pharmacokinetics both in infection prophylaxis and treatment. In order to increase the half-time of gentamicin sulfate and minimize the dosage, the antibiotic can be grafted to polymers as a prodrug or encapsulated within polymers. Gentamicin is positively charged and can interact with negatively charged macromolecules to form polyelectrolyte complexes (PECs). The complexation of gentamicin with hyaluronic acid (HA) was investigated here. Subsequently, several hyaluronic acid conjugates were prepared and screened for their thermo-responsive behavior and their ability to form PECs with gentamicin. PEC formation with HA poly(N-isopropylacrylamide) yields an injectable formulation which precipitates out of solution to form micro- or macroscopic gels, depending on its concentration, at temperatures higher than the lower critical solution temperature (LCST).

Introduction

High infection rates in fracture fixation, especially in grade III open fractures, demand urgent improvement of infection prophylaxis methods [1]. The most common pathogens, responsible for implant-associated infection are Staphylococcus aureus (S. aureus) and coagulase negative staphylococci (CNS) [2, 3]. Aminoglycosides are broad-spectrum antibiotics which cover S. aureus and CNS among a wide spectrum of Gram-positive and Gram-negative bacteria [4]. Gentamicin is a frequently used aminoglycoside for infection prophylaxis, as well as for treatment of an infection after removal of the implant [5]. However, the short half-life of gentamicin (depending on multiple factors, e.g. age, weight and gender) makes repeated administration necessary [6]. In order to increase the half-life, extend the release time and minimize the needed dose, gentamicin can be i) grafted to polymers to create a prodrug [7-11], ii) encapsulated in polymers [12-14], and iii) complexed by macromolecules of opposite charge [15-17]. For example, Marcus et al. created a prodrug based on gentamicin, by grafting the antibiotic to poly(ethylene glycol) (PEG) with hetero-bifunctional linkers, degradable by hydrolysis [18]. Gentamicin was then released from the prodrug for up to 66 ± 3 hrs, greatly increasing the half-life of native gentamicin. However, prodrug formation is related with several disadvantages. First

of all the grafted antibiotic should have preferably mono-functional groups, as otherwise network formation will occur. Furthermore, there should be a cleavable leaving group, which does not render the antibiotic inactive or less effective after degradation [19]. The encapsulation of gentamicin sulfate in microspheres is limited by the high hydrophilicity of the sulfate salt, increasing drug loading yields lower encapsulation efficiencies [20]. Complexation of gentamicin by making use of its highly charged nature, would be another approach to increase the half-life and to extend the release time.

Interestingly, as gentamicin possesses 5 positively charged ionisable amino groups [21] (see scheme 3.1) it can interact with negatively charged polysaccharides to form polyelectrolyte complexes (PECs) [22].
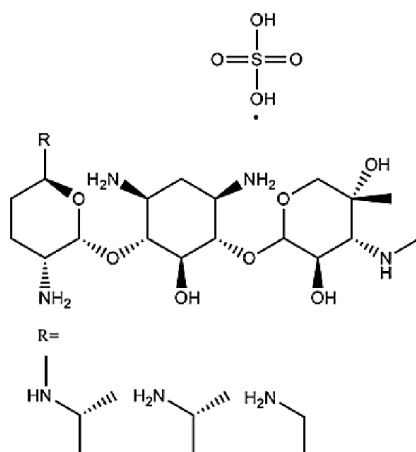
Scheme 3.1: Structure of gentamicin sulfate salt

Hyaluronic acid (HA), composed of repeating dimer units of D-glucoronic acid and D-N-acetylglucosamine, is a linear polysaccharide present in the extracellular matrix of many tissues and involved in a wide range of biological processes, including wound healing [23, 24]. Degradation *in vivo* occurs through the action of 3 enzymes (hyaluronidase, β-D-glucuronic acid and β-N-acetyl-D-hexosaminidase)[25] or through hydroxy radicals produced by phagocytic cells [26, 27]. In physiological conditions, HA is solube and negatively charged because of the presence of

carboxylate groups. Therefore, HA is able to form PECs with positively charged gentamicin. In this paper we investigate the complexation between gentamicin sulfate and HA. Because of the relative instability of PECs between HA and gentamicin, several HA derivatives with or without temperature dependent hydrophilicity/hydrophobicity changes were prepared, and their complexation with gentamicin was investigated to obtain injectable PECs. Finally, the release of gentamicin from these PECs was investigated *in vitro*.

Experimental

Materials

Sodium hyaluronate (HA) from *Streptococcus equi* was purchased from Contipro Biotech s.r.o. (Dolni Dobrouc, Czech Republic), M_n = 170.6 kDa and M_n/M_w = 1.73. Tetrabutylammonium hydroxide solution (TBAOH); 2,2'-azobis(2-methylproprionitrile) (AIBN); methanesulfonic acid (MSA) were purchased from Fluka (Buchs, Switzerland). *N*-isopropylacrylamide (nIPAm) (≥99%); cysteamine hydrochloride (AESH) (≥98% (titration)); *N,N*-dimethylformamide (DMF) anhydrous (99,8%); 1,4-dioxane; deuterium oxide (D_2O); Dowex M-31 cation exchange resin (Dowex CER); 1,1'-carbonyldiimidazole (CDI); dimethyl sulfoxide (DMSO), diethyl ether, gentamicin sulfate salt (GEN), phosphate buffered saline (PBS), sodium tetraborate decahydrate (STDH) (99.5% - 100.5%), ethanol (≥ 99.8 puriss), sulfuric acid (95% - 97%) (H_2SO_4), carbazole (≥ 95% (GC)), hyaluronidase type 1-S from bovine testes (400-1000 units/mg), boric acid (≥99.5%) (Bac), *o*-phthaldialdehyde (≥99.0% (HPLC)) (PTH) and methanol (analytical grade (99.8%)) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Jeffamines (JFMs), amino-terminated polyether copolymers were a gift from Huntsman (Woodlands, TX, USA). The following JFMs were used: M-600 (PO/EO ratio = 9/1, MW = 600 g/mol); M-1000 (PO/EO ratio = 3/19, MW = 1000 g/mol); M-2005 (PO/EO ratio = 29/6, MW = 2000 g/mol); M-2070 (PO/EO ratio = 10/31, MW = 2000 g/mol). Amino-terminated methoxy poly(ethylene glycol) (mPEG-NH$_2$) (MW = 5000 g/mol) was purchased from Jenkem Technology USA (Plano, TX, USA). Sodium hydroxide (≥99%) (NaOH) and 2-mercaptoethanol (99%) were purchased from Carl Roth (Karlsruhe, Germany).

Synthesis of HA derivatives

Sodium hyaluronate was converted into the tetrabutyl ammonium salt (HATBA) by a cationic exchange process [28]. Briefly, sodium hyaluronate was dissolved at 2.73% w/v in deionised water. Dowex CER was rinsed and subsequently soaked in dionised water. Thereafter, the water was removed from the Dowex CER and covered with TBAOH solution (85 g) and mildly stirred for 4 hrs. Then the supernatant was removed from the Dowex CER and replaced with fresh TBAOH solution and the solution was stirred overnight. Then again the supernatant was removed with dionised water until the pH was below 10. Then the resin was moved to a Gooch crucible with vacuum flask and the water was removed from the resin. After removal of the water, the homogeneous sodium hyaluronate solution was passed through the resin in the Gooch crucible into a Büchner flask in about 2 hrs. Finally, the HATBA solution was freeze dried and dessicated under vacuum at 42 °C for 3 days.

Amino-terminated poly(N-isopropylacrylamide) (pN-NH$_2$) polymers with varying molecular weight (11 – 41 kg/mol) were synthesized by radical polymerization of nIPAm in the presence of AESH and AIBN. Different molecular weights were obtained by varying the amount of the radical initiator (AIBN). After degassing by N$_2$ bubbling, 88.4 mmol of monomer was dissolved in 20 ml of DMF. Then, 0.9 mmol of AESH and 0.09 – 3.00 mmol of AIBN were added to the nIPAm solutions, and the radical polymerization was performed for 6 hrs at 70°C under constant N$_2$ bubbling. Then the system was cooled to room temperature (RT). The pN-NH$_2$ polymers were purified by repeated dissolution in 1,4-dioxane and precipitation in diethyl ether. Analysis of the obtained molecular weight, and molecular weight distribution were determined by size exclusion chromatography (SEC) on a multi-detector SEC system with an Alliance 2695 separation module from Waters (Milford, MA, USA) with 2 on-line detectors: a MALS Dawn DSP-F photometer from Wyatt (Santa Barbara, CA, USA) and a 2414 differential refractometer from Waters (Milford, MA, USA) used as a concentration detector. To compensate for their intrinsic temporal delay, the signal of the 2 detectors were aligned. The chromatographic column used was a 2PL gel Mixed C from Polymer Laboratories (Shropshire, UK), using DMF + 0.05M LiCl as mobile phase at a flow rate of 0.8 ml/min at 50°C (Sample injection volume: 218.5 μl, sample concentration: 4 mg/ml – 8 mg/ml).

Finally, HA derivatives were prepared by a direct amidation reaction. HATBA was dissolved at 1.25% w/v in dry DMSO. For the preparation of HApN, respective pN-NH$_2$ were dissolved at concentrations ranging from 4.4% w/v – 13.2% w/v in dry DMSO. For the preparation of HAJFMs, respective JFMs were dissolved at 8.00% w/v in dry DMSO. The HATBA solution was then warmed to 42°C, and 225 µl of MSA and 270 mg of CDI were added. For the preparation of HAmPEG, mPEG-NH$_2$ was dissolved at 9.00% w/v in dry DMSO and the HATBA solution was warmed to 42°C, and 225 µl of MSA and 122 mg of CDI were added to the solution.

All of the solutions were stirred for 1 hr, after which the respective amino-terminated species were added to the reaction vials and the solutions were cooled down to RT and left to react for 48 hrs. All of the reactions were then quenched by adding a saturated NaBr (aq) solution to the reaction, and contents were stirred for 2 hrs. The solutions were dialysed in cellulose tubing (Spectrapor, MWCO = 50 kDa) against demineralized cold water for 5 days to remove unreacted low molecular weight polymers. The final HA derivative solutions were frozen and lyophilized.

Characterization of HA derivatives

Differential scanning calorimetry (DSC): The temperature induced transition from a more hydrophilic to a more hydrophobic conformation of the HA derivatives was assessed by DSC measurements on a Pyris DSC-1 Perkin-Elmer instrument calibrated with indium. HA derivatives were dissolved at 13 % w/w in PBS. 25 µl of HA derivative was added to 50 µl aluminium crucibles, which were sealed thereafter. Measurements were performed under a dry nitrogen atmosphere at a flow rate of 20 ml/min. Each measurement was started with holding the sample at 10 °C for 5 min. Then the sample was heated from 10 °C to 40 °C, and the sample was held for 5 min at 40 °C. Finally, the samples were cooled from 40 °C to 10 °C. This cycle was repeated 6 times for every sample. Both healing and cooling ramps were performed at a rate of 10 °C/min.

Rheological measurements: Rheology was performed on an Anton Paar Physica MCR 302 rheometer with a Peltier controller with plate-plate geometry (25 mm diameter). HA derivatives were dissolved at 13% w/w in PBS. Measurements were

verified to be run within the viscoelastic region, by running amplitude and frequency sweeps prior to the temperature sweep. Storage modulus (G') and viscous modulus (G'') were recorded for these HA derivatives subjected to 0.5% oscillatory strain at 1 Hz while heating from 20 °C to 40 °C, at a rate of 1 °C/min. The borders of the plates were sealed with low viscosity silicone oil, in order to prevent evaporation of water from the hydrogel at the solution-atmosphere interface.

Complexation of HA and HA derivatives and gentamicin

ATR FTIR: Analysis of the complexation of HA derivatives and gentamicin was performed by Fourier transform-infrared spectroscopy (FT-IR) on a Bruker Tensor 27 spectrophotometer equipped with a single reflection diamond attenuated total reflection (ATR) accessory. Spectra (single measurements with 64 repeated scans per IR spectrum) were recorded for HA, gentamicin sulfate, a physical mixture of HA and gentamicin sulfate, and complexes formed between HA and gentamicin dissolved in dionized water at a w/w ratio of 3:1. Scanning electron microscopy (SEM): Samples were dried on alumina stubs and sputter-coated with Au/Pd. Complex morphology was then investigated by SEM on a Hitachi S-4700 field emission scanning electron microscope operated in secondary electron (SE) detection mode, at an accelerating voltage of 3.0 kV – 5.0 kV, and a working distance of 4.3 to 12 mm. UV-Vis spectroscopy: In order to investigate the amount of gentamicin complexed by HA, gentamicin sulfate and HA solutions in MilliQ grade water were mixed at different molar ratios. Immediately after mixing both solutions, precipitation of the complexed material from the solution was observed. After spinning down the vials, the supernatant was collected and new MilliQ grade water was added to the vials, and the vials were vortexed and the second supernatant was collected. The amount of complexed gentamicin in the supernatant was assessed by an absorbance measurements on a spectrophotometer (Multiskan GO, Thermo Scientific) after derivatization with an *o*-phthaldialdehyde (OP) reagent at 332 nm [29]. The amount of HA in the supernatant was assessed by a carbazole absorbance assay at 525 nm [30]. The amounts of gentamicin or HA present within the complex where calculated from the total amount of gentamicin or HA present in the solution prior to complex formation, from which the amounts of gentamicin or HA present in the collected supernatants had been subtracted.

Gentamicin release from polyelectrolyte complexes of gentamicin and hyaluronic acid or its derivatives

Complexes of hyaluronic acid and its derivatives, with gentamicin were prepared in the 2 ml top compartment of a Slide-A-Lyzer MINI dialysis device (MWCO = 10 kDa) (Thermo Scientific). Hyaluronidase was added at 0.5 mg/ml to the complexes. The total volume of the test tube + top compartment was 30 ml. Phosphate buffered saline (PBS) was used as a physiological release medium. PBS was aliquoted and replaced after 0.5, 4, 24, 48, 96 and 120 hrs. The amount of released gentamicin was determined by an absorbance assay after derivatization with *o*-phthaldialdehyde at 332 nm.

Results and Discussion

The amount of gentamicin complexed, or HA bound within a polyelectrolyte complex network was calculated for several molar ratio between HA and gentamicin sulfate (Figure 3.1).

The percentage of complexed gentamicin increased as the molar ratio between gentamicin and hyaluronic acid (GS/HA) was decreased. Simultaneously, the amount of bound HA decreased as the molar ratio between gentamicin and hyaluronic acid was decreased (Figure 3.1). The complexes from HA and gentamicin formed a dense precipitated structure, which imaged by SEM, resembled highly porous networks with macropores (Figure 3.2).
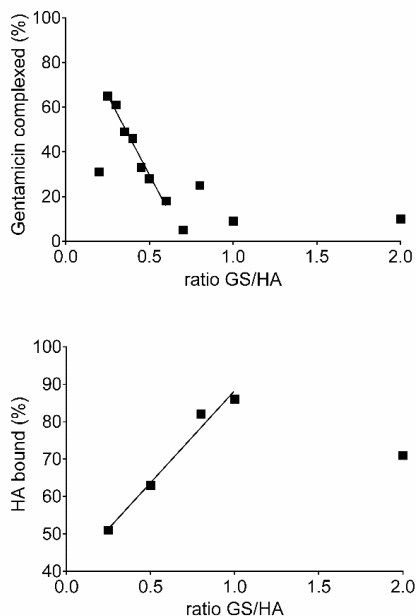

Figure 3.1: *The calculated percentage of complexed gentamicin (GS) and bound HA as function of the molar ratio between gentamicin and HA.*
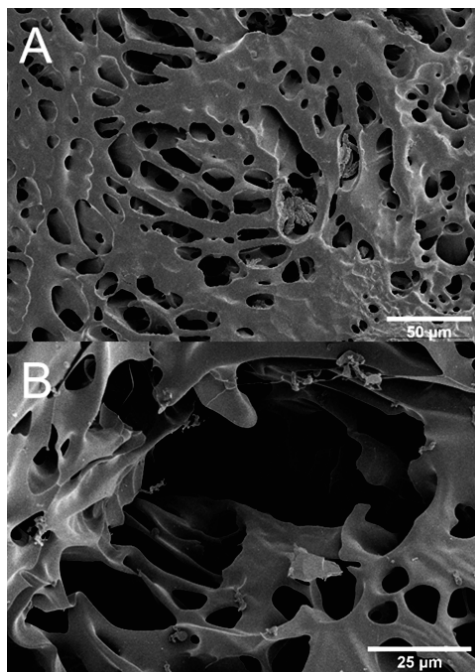
Figure 3.2: *Scanning electron microscopy image of sodium hyaluronate (HA) complexed with gentamicin sulfate at Gen/HA = 0.3 molar ratio. SEM image 2 B is a close up of the network shown in SEM image 2A. Images taken in the secondary electron mode at an accelerating voltage of 3.0 kV and a working distance of 12 mm in vacuum in the absence of water.*

The uniformity of the precipitated structure suggested a microscopically homogeneous complex formation. The precipitate of HA with gentamicin sulfate was then investigated by FTIR (Figure 3.3).
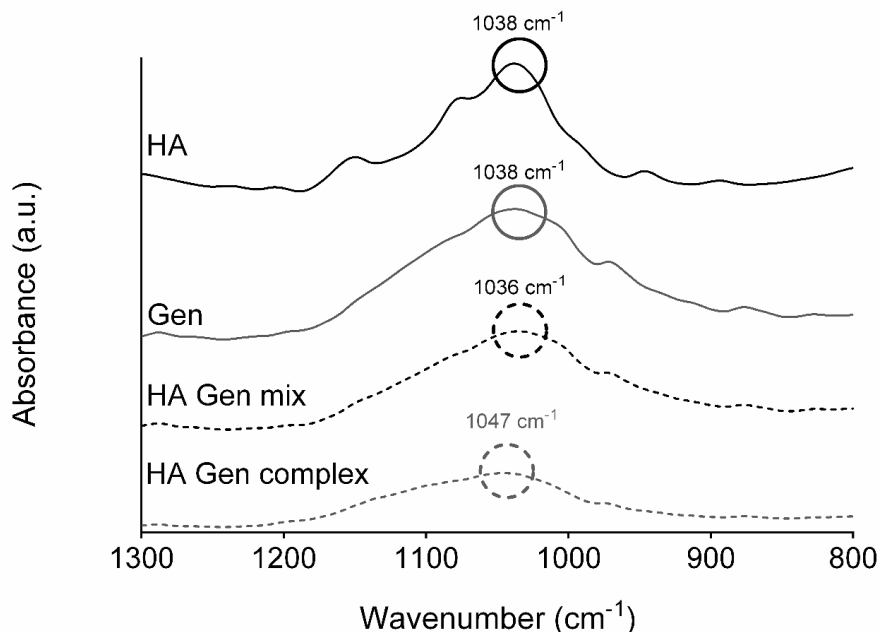
Figure 3.3: *IR spectra of HA, gentamicin (Gen), the physical mixture of HA and Gen (mix Gen/HA) by mixing both compounds in the dry state and after drying upon complex formation in solution (Gen/HA).*

Upon complexation of HA and gentamicin, a shift was observed in the C-O stretch ($v=1260–1000$ cm^{-1}) absorption peak belonging to alcohol groups, both present in HA and gentamicin. The peak for the C-O stretch of the alcohol groups of HA shifted to higher wavenumber in the Ge/HA complex in comparison to the original chemicals ant the physical mixture. This was attributed to increases in the energy for stretching the bond in the Gen/HA, suggesting interaction between the negative charged carboxylate groups and the positively charged amine groups and the formation of hydrogen bonds between the hydroxyl and amine groups.

Synthesis of HA derivatives

The complexation of HA and gentamicin yielded an insoluble complex that precipitated from solution (Figure 3.2). Therefore, the ability of derivatives of HA to complex gentamicin into PEC particles that are stable and stay in solution was investigated. In order to form a depot for gentamicin release after injection, several derivatives of HA with and without temperature dependent hydrophilicity and hydrophobicity were investigated. Poly(*N*-isopropylacrylamide)s with different molecular weights, 4 different poly(ether) copolymers with varying hydrophilicity and methoxy poly(ethylene glycol) (mPEG) were grafted to HA. Thermo-responsive poly(*N*-isopropylacrylamide) was prepared by radical polymerization. In order to create a range of molecular weight, the amount of radical initiator (AIBN) was varied. Number average molecular weights (M_N) were obtained in the range of 11 – 45 kDa, as determined by multidetector-SEC (Figure 3.4).
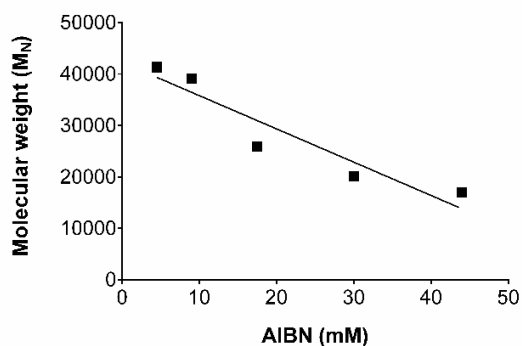

Figure 3.4: *Obtained molecular weight of pN as a function of radical initiator in feed (R^2=0.89).*

Molecular weight values showed a quasi-linear dependence on radical initiator concentration in the molecular weight range of 17 kDa – 41 kDa (Figure 3.4). The PDI (M_N/M_W) does not increase with increasing M_N (PDI = 2.71 for the 17 kDa pN-NH$_2$ and PDI = 2.37 for the 41 kDa pN-NH$_2$). Subsequently, HA derivatives were prepared by a direct amidation of the amino-terminated graft polymers to the HA backbone and grafting assessed using NMR (Figure 3.5).
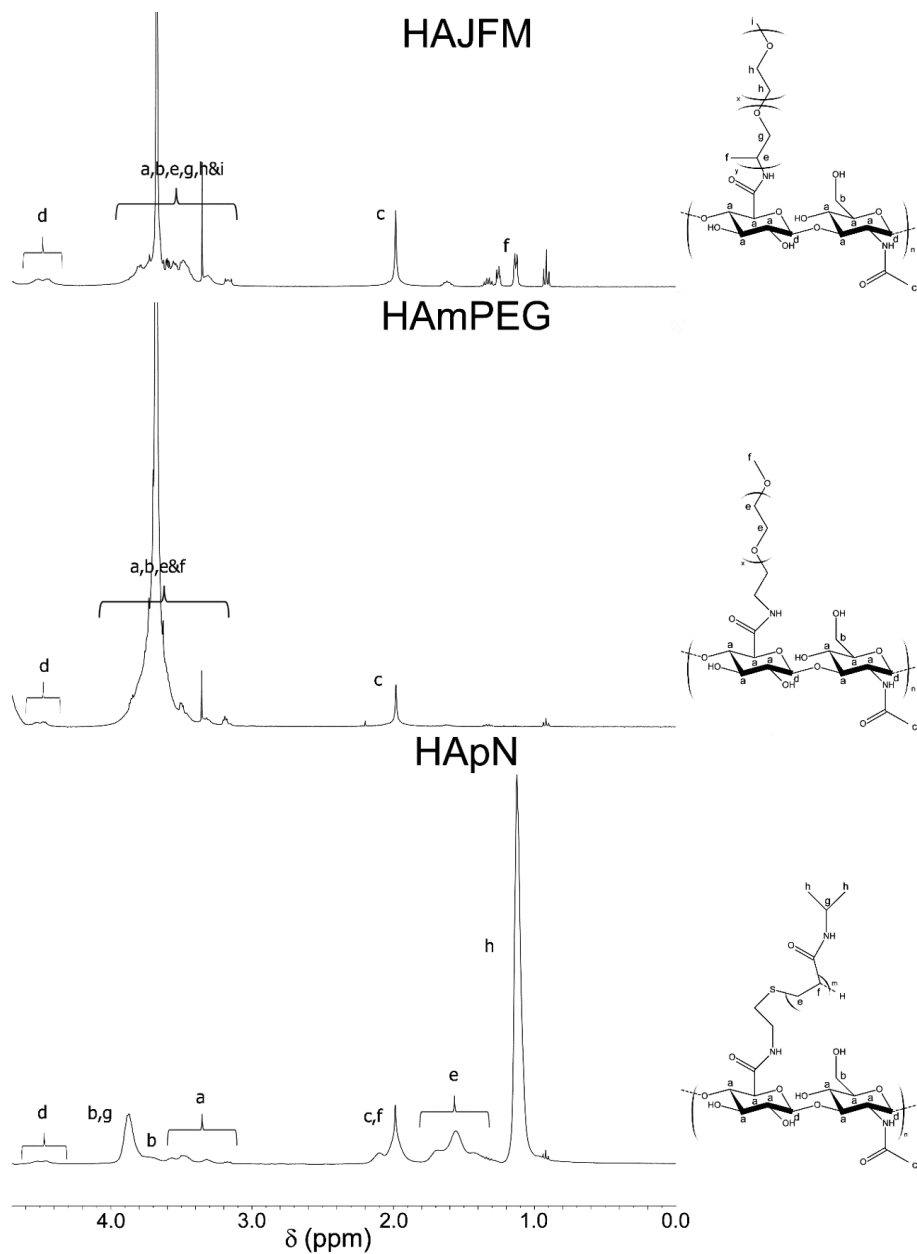
Figure 3.5: *HA derivatives prepared by direct amidation of amino-terminated graft polymers (JFM, mPEG-NH$_2$ and pN-NH$_2$) and their ^{1}H NMR spectrum.*

For each HA derivative synthesis and after dialysis, ^{1}H NMR spectra showed the presence of both signals attributed to HA and graft polymers and those signals were used to assess the degree of substitution (DOS). The DOS values of the synthesized HApN calculated from the ^{1}H NMR spectrum are reported in Figure 3.6.
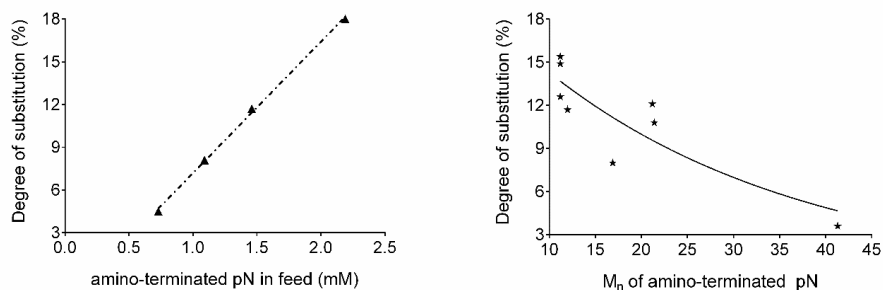

Figure 3.6: Plots of the degree of substitution as a function of pN-NH$_2$ feed for pN-NH$_2$ of M_n = 12 kDa (linear fit, R^2=0.9983) (left) and DOS as a function of the M_n of pN-NH$_2$ constant feed of 1.75g pN-NH$_2$ (right).

A high linear correlation between the amount of pN-NH$_2$ in the feed and the obtained degree of substitution was obtained for a graft polymer of 12 kDa molecular weight within the shown millimolar range. The degree of substitution decreased for a fixed amount of pN-NH$_2$ in the feed with increasing molecular weight of pN-NH$_2$.

The DOS for the other HA derivatives are shown in table 3.1.

Table 3.1: The degrees of substitution for the polyether (co)polymer grafts to HA.

	Degree of substitution	MW graft (kDa)	Ratio PO/EO
HAJFM M600	30	0.6	9
HAJFM M1000	14	1	0.2
HAJFM M2005	42	2	4.8
HAJFM M2070	30	2.1	0.3
HAmPEG	30	5	0

The average degree of substitution values measured by ^{1}H NMR were equal or higher for the HAJFMs and HAmPEG polymers in comparison to HApN. As the molecular weight (M_n) of the JFMs and mPEG were much lower than the pN-NH$_2$ grafts, steric hindrance could be the main cause of the higher grafting efficiency. No correlation was observed between the JFMs molecular weight and DOS in the HA derivatives. Their hydrophobic/hydrophilic characters at the temperature of the amidation reactions, RT, did not significantly influence the DOS values.

Thermo-responsive behaviour of HA derivatives

The thermal behavior of HAJFM derivatives in PBS solution was assessed (Figure 3.7).
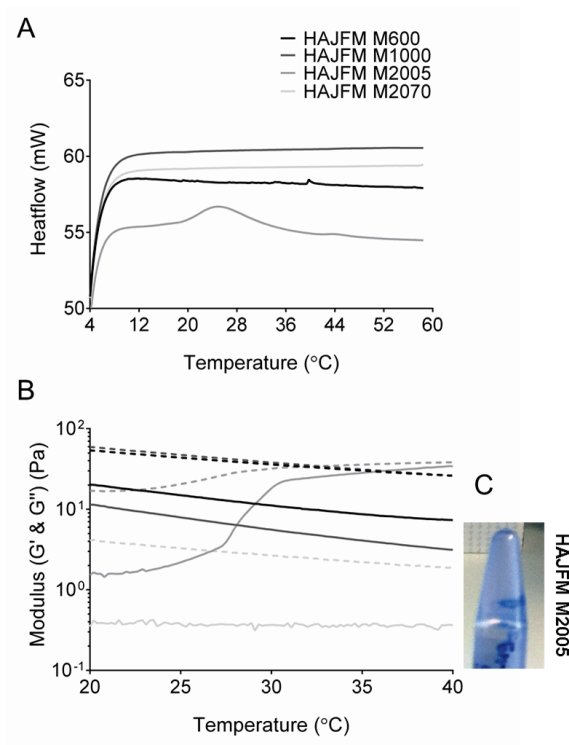

Figure 3.7: Thermo-responsive behavior of HAJFMs. DSC thermogram (A) and rheological temperature sweep (B) for HAJFMs at 13% w/w dissolved in PBS. The vial-inversion test was performed upon heating HAJFM M2005 at 10% w/w to 37 °C (C).

Among the 4 different JFM based HA derivatives prepared; only the HAJFM M2005 derivative did not flow upon vial inversion test at 10% w/w in PBS. This was confirmed by DSC measurements which did not reveal any phase transition in 3 of the 4 JFM based derivatives. The HAJFM M2005 derivative was the only derivative which displayed a small and broad endothermal peak at 25 °C (Figure 3.7). Rheological properties of the HAJFMs during a temperature sweep corresponded well with the calorimetry results and the vial-inversion test. The HAJFM M2005 derivative showed an increase in modulus upon heating to temperatures above 25°C suggesting an increase in hydrophobic interactions, while all other HAJFMs showed a slight decrease in moduli with increased temperature suggesting weakening of hydrogen bond interactions between macromolecules in solution (Figure 3.7).

Finally, HAJFM M2005 was the only JFM based derivative that displayed lower critical solution temperature (LCST) behavior, although it was only relatively weak. Most likely, the length of the amphiphilic brushes reduced the gradient of the transition.

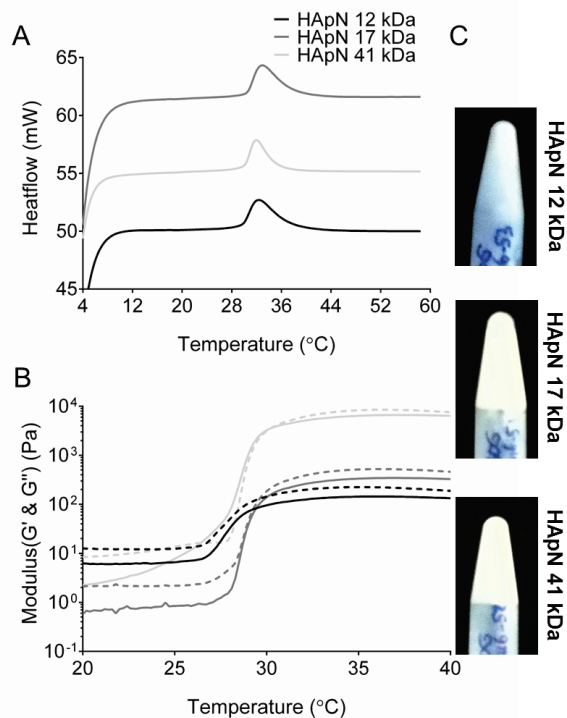The thermal behavior of HApN derivatives in PBS solution was assessed similarly to the HAJFMs (Figure 3.8).

Figure 3.8: *Thermo-responsive behavior of HApNs. DSC thermogram (A) and rheological temperature sweep (B) for HApNs at 13% w/w dissolved in PBS. The vial-inversion test was performed upon heating HApN solutions at 10% w/w to 37 °C (C).*

All HApN derivatives tested presented a LCST behavior which was much more pronounced than for the HAJFMs in the same conditions. In the DSC thermogram of 3 HApNs a peak was observed around 32°C, corresponding with the endothermic transition from sol to gel phase. Repeated heating and cooling cycles show hysteresis of ΔT=5 °C in the thermogram (not shown) for 6 repeated heating and cooling cycles. Upon heating above the LCST an increase in modulus of several orders was observed for all HApNs independently of the pN molecular weight. Although HApN 41 kDa had the lowest DOS, upon gelation it had the highest increase in modulus (Figure 3.8).

Complexation of gentamicin sulfate with HA derivatives

The ability of HA derivatives to complex gentamicin was assessed, as the complexation of HA with gentamicin sulfate resulted in PEC formation. The molecular weight of the HAJFMs, HApNs and HAmPEG was calculated from the ^{1}H NMR data and the molecular weights of pN from the multi-detector SEC. Subsequently, an identical 0.3 Gen/ HA derivative molar complex ratio was used for the HA derivatives with gentamicin as for the Gen/HA complexes (Table 3.2) and PEC formation and stability was assessed in comparison to Gen/HA.

All of the HApN polymers formed visible stable complexes with gentamicin, as seen by a slight increase in turbidity of the solutions. No precipitates were observed for at least 1 week in the solutions kept at RT. Increasing the temperature to 37°C lead to precipitation of the Gen/HApNs. For the HAJFMs, only 2 out of the 4 derivatives formed similar complexes, the more hydrophilic JFM based HA derivatives formed aggregates upon mixing with gentamicin, as observed by a high turbidity of the solution, followed by precipitation of the complex. The HAmPEG derivative solution stayed clear upon mixing with gentamicin at RT and at 37 °C (Table 3.2).

Table 3.2: *Complex formation between HA derivatives and gentamicin at RT.*

	DOS	MW graft (kDa)	Ratio PO/EO	Complexation with gentamicin
Sodium HA	0	NA	NA	Aggregation + precipitation
HApN	12	12	NA	Stable
HApN	8	17	NA	Stable
HApN	4	41	NA	Stable
HAJFM M600	30	0.6	9	Aggregation + Precipitation
HAJFM M1000	14	1	0.2	Stable
HAJFM M2005	42	2	4.8	Aggregation + Precipitation
HAJFM M2070	30	2.1	0.3	Stable
HAmPEG	30	5	NA	Clear solution

The dispersed PECs and precipitates were collected and dried in air before imaging by SEM (Figure 3.9).
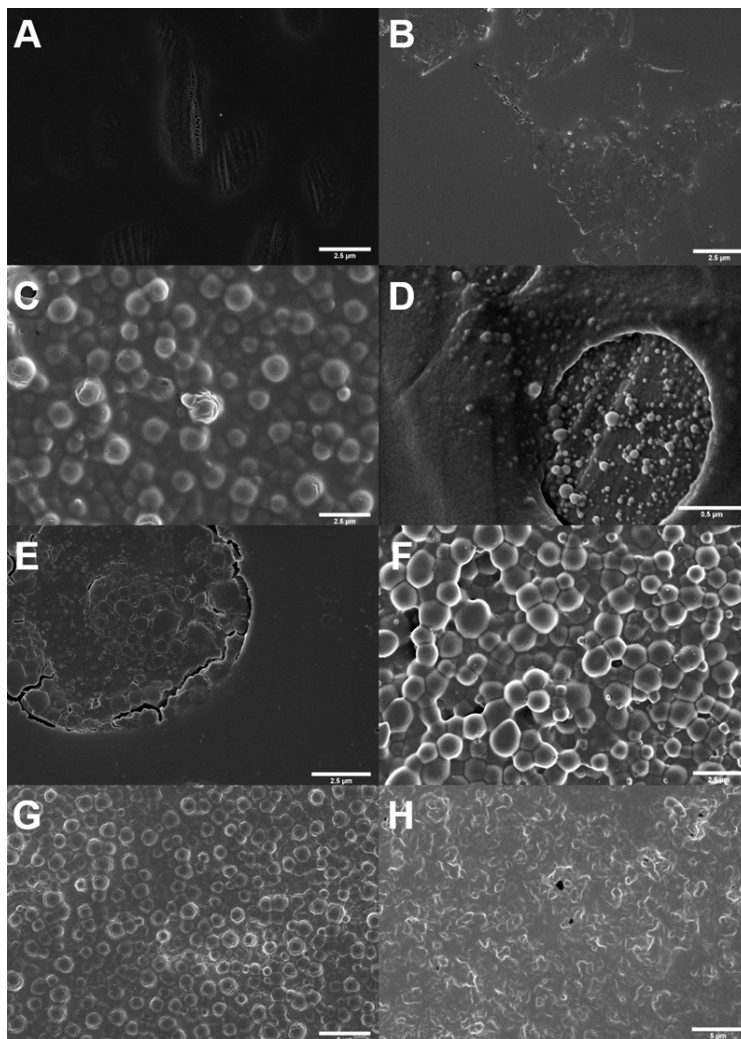
Figure 3.9: Scanning electron microscopy images of HApN polymer (12 kDa pN) (A), gentamicin sulfate (B), complex of HApN (12 kDa pN) and gentamicin (C), complex of HAJFM M-1000 and gentamicin (D), HAmPEG and gentamicin upon mixing (E), HApN (12 kDa pN) and gentamicin (F) and HApN 12 kDa gentamicin complexes before (G) and after (H) heating from RT to 37 °C. Image taken in the secondary electron mode at an accelerating voltage of 3.0 kV – 5.0 kV and a working distance of 4.3 – 5.3 mm.

Spherical structures of several microns were observed for Gen/HApN samples at RT, while at 37°C an aggregate and less defined structure was imaged. These structures

were not observed for solutions that only contained HApN or gentamicin (Figure 3.9A and 3.9B). Upon combining the HAJFM M1000 solution with gentamicin, similar sphere like structures were observed, although in smaller quantities (Figure 3.9D). Upon combination of purely hydrophilic HAmPEG and gentamicin, no structures were observed by SEM (Figure 3.9E), most likely because of an absence of hydrophobic regions within the mPEG grafted HA as opposed to HApN (Figure 3.9F) graft polymers.

Release from poly(electrolyte complexes) of gentamicin and HA derivatives

As HApN presented the most prominent features for the formation of thermo-sensitive PECs, the gentamicin release was measured upon formation of Gen/HApN complexes and compared to the release from Gen/HA in the absence or presence of hyaluronidase in PBS at 37 °C (Figure 3.10).
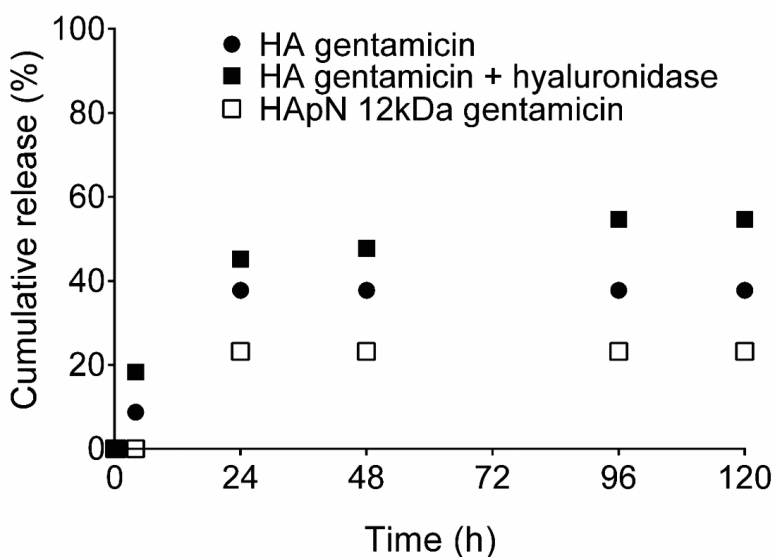

Figure 3.10: Cumulative release of gentamicin from complexes between gentamicin and HA or gentamicin and HApN over a 1 week course at 37°C (hyaluronidase 0.5 mg/ml).

Free gentamicin, as control, readily diffused out of the membrane compartment (data not shown). For Gen/HA and Gen/HApN PECs, burst release followed by a plateau at 48 hrs were observed. Degradation of the HA by hyaluronidase caused a faster

and higher release of gentamicin from the Gen/HA indicating the dependence of gentamicin availability to the degradation of the PEC. The Gen/HApN cumulative release value reached at 48 hrs was lower than the Gen/HA, indicating increased retention of gentamicin in HApN, indicating that the gentamicin/ HA derivative PECs can modulate the availability of the antibiotic.

Conclusions

Gentamicin and hyaluronic acid form complexes when mixed in solution as a result of opposite ionic charges on both molecules. These complexes have reduced solubility and precipitate out of solution. Several thermo-responsive HA derivatives were synthesized and characterized with respect to thermo-responsive character and complexation with gentamicin. The more hydrophilic HAJFMs precipitated out of solution upon complexation.

HApN polymers were able to form stable complexes that stayed in solution at RT while precipitating at physiological temperature, similar to the more hydrophobic polyether copolymer functionalized HA polymers. HA derivative PECs can modulate the availability of the gentamicin and can potentially increase the half-life and extend the release time of the antibiotic.

Acknowledgements

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Chapter 4

Injectable gentamicin-loaded thermo-responsive hyaluronic acid derivative prevents infection in a rabbit model

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Abstract

Despite the use of systemic antibiotic prophylaxis, the surgical fixation of open fractures with osteosynthesis implants is associated with high infection rates. Antibiotic-loaded biomaterials (ALBs) are increasingly used in implant surgeries across medical specialties to deliver high concentrations of antibiotics to the surgical site and reduce the risk of implant-associated infection. ALBs which are either less or not restricted in terms of spatial distribution and which may be applied throughout complex wounds, could offer improved protection against infection in open fracture care.

A thermo-responsive hyaluronic acid derivative (hyaluronic acid- poly(*N*-isopropylacrylamide) (HApN)) was prepared by a direct amidation reaction between the tetrabutyl ammonium (TBA) salt of hyaluronic acid and amine-terminated poly(*N*-isopropylacrylamide) (pN). The degree of grafting, and gelation properties of this gel were characterized, and the composition was loaded with gentamicin. The rheological- and release properties of this gentamicin-loaded HApN composition were tested *in vitro* and its efficacy in preventing infection was tested in a rabbit model of osteosynthesis contaminated with *Staphylococcus aureus*. The gentamicin-loaded HApN composition was able to prevent bacterial colonization of the implant site as shown by quantitative bacteriology. This finding was supported by histopathological evaluation of the humeri samples where no bacteria were found in the stained sections.

In conclusion, this gentamicin-loaded HApN hydrogel effectively prevents infection in a complex wound, simulating a contaminated fracture treated with plating osteosynthesis.

Introduction

Implant-associated infection remains a challenging complication in surgically fixed fractures. On average, 5% of the patients receiving internal fracture fixation implants develop an infection [1]. In closed fractures, where the protective barrier of the skin is

intact, the infection rate is between 0.5%-2%. However, the infection rate can exceed 30% in patients with severe open fractures [2]. Contamination of the fracture site prior to or during the surgical intervention can lead to bacterial adhesion and biofilm formation on tissue and implant surfaces [3, 4]. Once developed, the treatment of implant-associated infection is challenging and expensive, and leads to delayed fracture healing and potential loss of limb function [5, 6].

In order to reduce infection rates in operative fracture treatment, systemic antibiotic prophylaxis is typically administered pre-operatively and may be continued for a number of days depending upon injury severity. Although appropriate antibiotic prophylaxis reduces infection rates, systemic administration may not be able to provide sufficiently high antibiotic concentrations to locations where distribution is limited by vascular damage or edema [7, 8]. This may be the case in many fracture sites or contused soft tissues.

Antibiotic-loaded biomaterials (ALBs) offer the prospect of delivering high local concentrations of antibiotics directly to the site of interest, without requiring an intact vasculature. ALBs, such as antibiotic-loaded poly(methylmethacrylate) (PMMA) cement and beads [9, 10], collagen fleece [11] and PDLLA coatings [12-14] with gentamicin have shown their potential in orthopedic and trauma surgery. Nevertheless, currently available ALBs possess certain suboptimal properties. PMMA cement / beads lack degradability often requiring a removal surgery, have poor release kinetics of antibiotics [10, 15, 16] and their setting can cause heat damage to various antibiotics [10, 17], collagen fleeces have undesirable handling properties, immunogenic potential [18, 19], and coatings of poly(α-hydroxyacid)s form acidic degradation products and have to be approved separately for each type of implant [6]. The complex nature of trauma wounds, *i.e.* the presence of contamination, inaccessible sites, poorly definable wound margins, and involvement of multiple tissues, demands alternative ALBs that are suited for use in these circumstances. The previously described ALBs have confined dimensions at the time of insertion and may have limited distribution in a complex wound.

Hyaluronan (HA) is a biocompatible and degradable polymer with many biomedical applications, notably as vehicle for drug delivery systems [20]. HA and HA derivative-

based aqueous formulations can be injectable and subsequently gelate *in situ,* and are hence good material candidates for delivery of hydrosoluble antibiotics throughout complex wounds [21, 22]. In this study, a direct amidation reaction was performed to graft amine terminated thermo-responsive poly(*N*-isopropylacrylamide) (pN) to HA [23]. The thermo-responsive hyaluronic acid- poly(*N*-isopropylacrylamide) (HApN) hydrogel was loaded with gentamicin and designed to have a low modulus at room temperature (RT) and easy applicability at the surgical site. Once in contact with tissue and body fluids, the HApN solution shifts from a sol state to a gel state as it heats up to temperatures higher than its lower critical solution temperature (LCST). This property of the hydrogel improves the versatility of its application, since it can be applied at any stage before, during or at completion of the surgery and all potentially contaminated surrounding and overlying tissues, implant surfaces and implant cavities can be covered or filled.

In this preclinical study, a gentamicin-loaded thermo-responsive HApN hydrogel was prepared and characterized *in vitro* and *in vivo*. A rabbit humerus model has been established in the past [24], and this model mimics, as close as possible a real trauma case, including an osteotomy and a fracture fixation implant. Additionally, by choosing the humerus the animal's burden can be minimized since rabbits have the opportunity to reduce weight-bearing on the operated limb which is not feasible in their hind limbs. In this study the model was used to assess the efficacy of prophylactic antibiotic delivery.

Experimental

Materials

HA sodium salt from *Streptococcus equi* (HANa) was purchased from Contipro Biotech s.r.o. (Czech Republic), number average molecular weight (M_n) =170.6 kDa and polydispersity (M_w/ M_n) =1.73. Tetrabutylammonium hydroxide solution (TBAOH) (~40% in H_2O, ~1.5 M); 2,2'-azobis(2-methylpropionitrile) (AIBN) (≥98.0% GC); methanesulfonic acid (MSA) (≥99.5%), methanol (analytical grade (99.8%)), ethanol (≥99.8% puriss) and bromophenol blue were purchased from Fluka (Buchs, Switzerland). *N*-isopropylacrylamide (nIPAm) (≥99%); cysteamine hydrochloride

(AESH) (≥98% (titration)), *N,N*-dimethylformamide (DMF) anhydrous (99,8%), 1,4-dioxane (≥99.5%, p.a., ACS); Dowex M-31 cation exchange resin, 1,1'-carbonyldiimidazole (CDI) (reagent grade), dimethyl sulfoxide (DMSO) (≥99.5% for synthesis), sodium carbonate (Na_2CO_3) (puriss., anhydrous, 99.5%-100.5% calc. on the dried substance), potassium sulfate (K_2SO_4) (≥99.0%), gentamicin sulfate salt (GEN) (potency ≥590 µg gentamicin base per mg); boric acid (BAc) (≥99.5%), phthaldialdehyde (≥99.0 by high-performance liquid chromatography) and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Diethyl ether (≥99.5%, p.a.), 2-mercaptoethanol (99%), 2-propanol (≥99.8%), glycerol (≥99%) and sodium bromide (NaBr) (≥99% p.a., ACS) were purchased from Carl Roth (Karlsruhe, Germany). Blood Agar (BA), Mueller-Hinton broth (MHB), Tryptic Soy Agar (TSA) and Tryptic Soy Broth (TSB) were purchased from Oxoid AG (Basel, Switzerland). Gentamicin sulfate loaded-collagen fleece, Gentafleece®, was purchased from Baxter AG (Volketswil, Switzerland). Blank disks (BBL™ Sensi-disc™ blank disc) and disks with gentamicin (BBL™ Sensi-disc™ gentamicin 10 µg) were purchased from BD Diagnostics (Sparks, MD, USA).

Thermo-responsive hyaluronan (HApN) synthesis

Amino-terminated poly(N-isopropylacrylamide) synthesis

DMF was degassed by N_2 bubbling for 15 mins. Ten grams (88.4 mmol) of nIPAm monomer was then dissolved in 20 ml DMF. N_2 bubbling at RT was performed for 2 hrs to remove all oxygen from the solution. Subsequently, 100.2 mg (8.8×10^{-1} mmol) AESH and 492.8 mg (3.0 mmol) AIBN were added to the solution. The system was then heated to 70 °C and the N_2 bubbling was continued during the radical polymerization reaction, which proceeded for 6 hrs. Then the system was cooled down to RT. Hereafter, the poly(*N*-isopropylacrylamide) amine (pN-NH_2) solution was precipitated in diethyl ether at RT.

After precipitation, the product was redissolved in 35 ml of 1,4-dioxane overnight. Then the pN-NH_2 solution was precipitated again in diethyl ether. The redissolution and precipitation steps were repeated 2-3 times until a fine white precipitate was obtained. The precipitate was collected and dried in a vacuum oven at 32 °C until all

residual solvent was removed. The product was characterized by proton nuclear magnetic resonance (^{1}H-NMR) on a Bruker Avance AV 500 NMR spectrometer using deuterium oxide (D_2O) as solvent with 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (TMS) as a standard to set the zero-shift. The NMR spectra were processed and analysed using Mestrenova™ software. The molecular weight of the amino-terminated pNs was determined by multi-detector size exclusion chromatography (SEC). The polymers M_n and polydispersity were determined by a modular multi-detector SEC system with an Alliance 2695 separation module from Waters (Milford, USA) with two on-line detectors: a MALS Dawn DSP-F photometer from Wyatt (Santa Barbara, USA) and a 2414 differential refractometer from Waters (Milford, USA) used as a concentration detector. The different signals from the two detectors were aligned to compensate for their intrinsic temporal delay. Chromatographic column specifications were as follows: Column: 2PL gel Mixed C from Polymer Laboratories (UK); Mobile phase: DMF + 0.05M LiCl; Flow rate: 0.8 ml/min; Temperature: 50 °C; Injection volume: 218.5 μl; Sample concentration: 2 mg/ml.

HApN synthesis

A cationic exchange process was performed to obtain the tetrabutyl ammonium (TBA) salt of HA (HATBA) from HANa [25]. At RT, 1.0 g of HATBA was dissolved in 80 ml of dry DMSO under mild stirring. For the synthesis of HApN, 2.63 g of pN-NH$_2$ was dissolved in 20 ml of DMSO at 25 °C overnight. HATBA solutions were heated to 42 °C and 225 μl of MSA and 270 mg of CDI were added to the reaction vessel. After 1 hr of stirring at 42 °C the pN-NH$_2$ solution was added to the reaction vessel and the constituents were allowed to cool down to 25 °C and react for 48 hrs. A saturated solution of NaBr (aq) was then added to quench the reaction and the solution was stirred at 25 °C for 2 hrs. At this point, the solution containing the HApN was transferred to cellulose dialysis tubing with a molecular weight cut-off (MWCO) of 50 kDa (Spectrapor no 6, 34 mm flat width). The products were dialyzed against demineralized water for 5 days. The HApN solutions were frozen and lyophilized. The samples were finally kept under vacuum for 3 days and sterilized using a cold ethylene oxide cycle and further degassed for 5 days. The degree of substitution of

the HApN was then determined by ^{1}H NMR [23]. Residual TBA content in the final HApN samples was quantified by a Bromophenol Blue Assay [26].

Rheological behavior of gentamicin-loaded HApN formulations

The temperature-induced sol-gel transition of the HApN was assessed by the vial inversion method. HApN was dissolved in phosphate buffer saline (PBS; pH 7.4) at 13% w/w in hermetically closed vials. The flowability of the solutions was assessed visually using a vial inversion test at 22 ± 2 °C and after 5 minutes incubation at 37 °C.

Rheological measurements were performed on an Anton Paar Physica MCR 302 rheometer with a Peltier controller with plate-plate geometry (Ø 25 mm). HApN samples were dissolved at 13% w/w in PBS or in PBS containing 1% w/w gentamicin sulfate. The shear moduli (G') and loss moduli (G'') were recorded as a function of the temperature for these HApN polymers. Solutions were then subjected to 0.5% oscillatory strain at 1 Hz while heating from 20 °C to 40 °C at a rate of 1 °C/min. In order to avoid evaporation of water from the hydrogel composition at the solution-atmosphere interface, low viscosity silicone oil was applied along the border of the plates after sample placement. Measurements were assured to be run within the viscoelastic linear region.

The effect of the sulfate salt of gentamicin on the LCST of HApN was investigated for HApN (derived from high MW HANa; M_n=952.5 kDa, M_w/M_n=1.65) solutions with increasing concentrations of sulfate from gentamicin sulfate dissolved in PBS (0, 26, 52 and 104 mM sulfate). In order to verify that the shift could be attributed to the sulfate salt in the gentamicin sulfate molecule, HApN solutions dissolved in PBS with increasing concentrations of potassium sulfate (K_2SO_4) were also screened. In order to make a valid comparison, the amount of K_2SO_4 that needed to be dissolved to obtain a stoichiometric equivalent of sulfate ions in the final solution was calculated as compared to the HApN solutions with gentamicin sulfate. Shear moduli and viscous moduli were again recorded as a function of the temperature, as described above.

Gentamicin release *in vitro*

Release of gentamicin from the HApN hydrogel was assessed *in vitro*. For the preparation of the gentamicin-loaded HApN, either a 1% w/w or a 2% w/w gentamicin solution in PBS was used to dissolve HApN at 13% w/w. To keep the total gentamicin payload equal (8 mg gentamicin sulfate in total), 800 µl of the 1% w/w gentamicin-loaded HApN or 400 µl of the 2% w/w gentamicin-loaded HApN were injected into 8 ml of pre-warmed PBS (37°C) to form single hydrogel spheres. Vials were then incubated at 37°C in an incubator, while shaking at 20 rotations/min on a shaker plate (New Brunswick Scientific co. Inc. Classic C1, Edison, New Jersey, US). Supernatants (1 ml) were collected after 1 hr, 3 hrs, 6 hrs, 1 day, 2 days, 4 days and 7 days, and 1 ml of fresh PBS was added to the vials. Each condition was performed in triplicate. The amount of gentamicin released was quantified by a modified absorbance assay measured at $\lambda=332$ nm on a spectrophotometer (Multiskan GO, Thermo Scientific) as reported by Sampath and Robinson, after derivatization with *o*-phthaldialdehyde (OPDA) reagent [27]. The detection limit was calculated to be 94 µg/ml for this assay [28].

Gentamicin-loaded collagen fleece (4 cm x 1 cm x 0.5 cm), containing 8 mg gentamicin sulfate, was used as a clinically available comparator in the *in vitro* release study. The release was expressed as cumulative percentage (%) of the total payload, the removal of 1 ml of supernatant and dilution with 1 ml of fresh PBS was accounted for in the calculation.

In vivo assessment of a gentamicin-loaded HApN formulation in a contaminated plated fracture model in rabbits

Study design

An overview is provided with a description of the 3 study groups in Table 4.1. Bacteriological evaluations were performed on 8 animals of each group, and the remaining 2 animals per group were used for histological evaluation.

Table 4.1: in vivo study plan

Group name	ALB	Gentamicin sulfate*
1. Untreated control	-	-
2. Collagen Gen	Collagen fleece	+
3. HApN Gen	HApN	+

*Amount of gentamicin sulfate in ALB is 8 mg, n=10 per study group

Institutional animal care and use committee approvals

The *in vivo* study was approved by the Ethical Committee of the Canton of Grisons, Switzerland (TVB number 28/2014). All procedures were performed in an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) International approved facility and according to Swiss animal protection law and regulations. Thirty skeletally mature, specific pathogen free (SPF) female New Zealand white rabbits (Charles River, Suzfeld, Germany) older than 24 weeks of age and a mean body weight of 3.6 kg ± 0.2 kg were included in this study. All rabbits were assessed by a veterinarian and determined to be healthy prior to inclusion in this study and were allowed to acclimatize to their surroundings for two weeks prior to the start of the study. During the acclimatization period, they were group-housed with a 12 hrs dark/ 12 hrs light cycle, fed with hay, lettuce and supplemental feed for

rabbits (Biomill, Switzerland). After the surgical intervention, the animals were single-housed until euthanasia.

For the first five postoperative days, each animal was checked using a score sheet at least twice daily by a veterinarian or an experienced animal caretaker, thereafter the rabbits were checked at least once a day for the rest of the study duration.

Bacteria

A gentamicin sensitive *Staphylococcus aureus* strain (JAR 060131), isolated from a male patient with a knee prosthesis infection was used in the present study [29]. Bacteria were stored in MHB supplemented with 20% of glycerol for long-term preservation at -20°C.

Bacterial inocula were prepared in PBS as previously described [30], with a target inoculum of 2.0 x 10^6 colony-forming units (CFU) of *S. aureus*. Quantitative culture of each inoculum was performed immediately after preparation to assure the accuracy of the used inocula.

Antibiotic-loaded biomaterials

Gentamicin sulfate loaded collagen fleece was cut into 4 cm x 1 cm pieces using a sterile custom-made steel template. These dimensions were selected in order to allow the fleece to completely overlay the implants used in the *in vivo* study. All the sterile HApN samples were reconstituted at 13% w/w overnight in 1% w/w gentamicin sulfate in sterile PBS at 4°C, 1 day prior to surgery. The HApN hydrogel samples with gentamicin were chilled on ice prior to administration. During the surgery, 800 µl of the gentamicin-loaded HApN hydrogel was injected over the implant. In order to speed up the gelation process, an infrared lamp was used to pre-heat the surgical site. This lamp was removed prior to injection of the hydrogel. The total payload was 8 mg gentamicin sulfate in both the hydrogel and the fleece groups.

Operative procedure

The surgical anesthesia, plating procedure and pre-operative and post-operative analgesia regimens have been described previously elsewhere [24].

Inoculation of the rabbits was performed after creation of the osteotomy with a 0.45 mm Gigly saw, as three separate 34 μl injections into the central screw hole overlying the osteotomy and onto the adjacent proximal and distal screw holes. Subsequently, the wounds were closed without any local antibiotic prophylaxis (group 1), or after gentamicin-loaded collagen fleece was placed (group 2) or HApN hydrogel with gentamicin (group 3) was injected over the 7-hole locking plate (Figure 4.1).
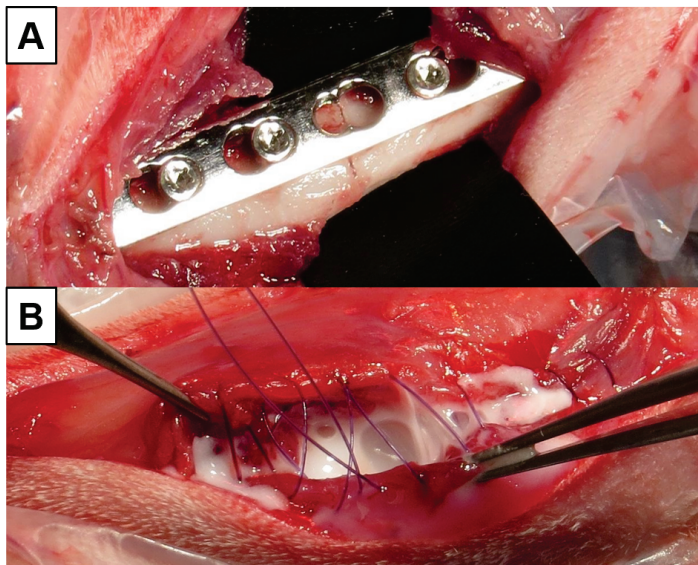

Figure 4.1: *Intra-operative image before (A) and after application of the gentamicin-loaded HApN hydrogel (B) within the surgical field. The HApN hydrogel (white color) fills the surgical field and turns from a sol to a gel state upon contact with the tissue.*

Evaluation of the *in vivo* study

Post-operative care and radiological analyses

Weight was measured at surgery, and 3 and 7 days postoperatively. Radiographs of the operated limb were taken in two planes (lateromedial and caudocranial) after surgery and postmortem to assess proper implant positioning. Animal exclusion criteria were set at a weight loss exceeding 15% of the initial body weight, local infection with severe lameness, persistent swelling and discharge, or signs of systemic infection such as fever, depression and anorexia. One of the rabbits of study group 2, which received the gentamicin-loaded collagen fleece, died during anesthesia due to cardiac arrest and was excluded from the study. The rabbit was intended to be used for histology and was not replaced. Upon completion of the observation period (7 days), all animals were euthanized using intravenously administered Pentobarbital (200 mg/kg, Esconarkon®, Streuli Pharma AG, Switzerland).

Blood analyses

Blood samples were drawn from all included rabbits pre-operatively, 3 and 6 hrs, 1 day post-operatively and then every day until the end of the study. White blood cell (WBC) count was measured at each time point (Vet ABC, Scil animal care, Germany). C-reactive protein (Rabbit CRP Elisa Kit, ICL Inc. Portland, OR, USA) and gentamicin concentrations were measured in serum samples at the same time points. Serum gentamicin levels were quantified by a fluorescence polarization immunoassay (FPIA, lower limit of detection, 0.5 µg/ml) on a Cobas Integra® 400 plus analyzer (Roche Diagnostics AG, Switzerland) using reagents from Roche Diagnostics. Test solutions and calibration standards were diluted with human plasma.

Bacteriology

The soft tissue covering the locking plate and any abscess material were removed using a scalpel, weighed, and then placed into a sterile receptacle containing 10 ml of PBS. The soft tissue samples were then homogenized using an Omni-TH hand-held homogenizer (LabForce AG, Switzerland) with sterile Omni-tip plastic probes. All hardware (plates and screws) were transferred to sterile glass test tubes containing 10 ml of PBS. Plates and screws were sonicated (Bandelin Ultrasonic waterbath RK 510 H, Bandelin, Germany) for 3 min, thereafter they were vortexed for 10 sec. Humerus bone samples were crushed into small fragments with a sterile luer and immediately homogenized in 40 ml of PBS using a Polytron PT3100 homogenizer (Kinematica AG, Switzerland).

All tissue and implant samples were serially diluted (10-fold steps) and plated on BA plates. Agar plates were incubated at 37 °C and colonies were counted after 24 hrs and 48 hrs. The limit of detection of *S. aureus* colonies was 5×10^1 CFU for soft tissue and hardware samples and 2×10^2 CFU for bone samples. All *S. aureus* bacterial growth was tested by a latex agglutination test (Staphaurex™ Plus Latex Agglutination Test, Remel, UK). In case of a negative test result (*i.e.* infection with organism other than *S. aureus*), isolates were identified using a Vitek2 machine (bioMérieux Vitek Inc., Hazelwood, MO, USA). The susceptibility of the *S. aureus* strain used for inoculation and the susceptibility of other isolated organisms to gentamicin were assessed by the disk diffusion method. For *S. aureus* the disk diffusion test was performed on Mueller-Hinton agar (MHA), using *S. aureus* ATCC® 25923 as quality control strain. All zone of inhibition (ZOI) tests were performed in triplicate for each bacterial species. Bacterial suspensions were adjusted to a 0.5 McFarland standard before use. Susceptibility to gentamicin was tested by using discs with 10 μg gentamicin. The bacterial strain was determined to be susceptible, intermediate or resistant according to the clinical and laboratory standards institute (CLSI) guidelines M100-S25 (2015) [31].

Histology

Rabbit humeri, including implant and adjacent soft tissue, were fixed in 70% (v/v) methanol for a minimum of 2 weeks with fresh methanol changes weekly. Contact radiographs (full thickness) were taken using high resolution technical film (D4 Structurix DW ETE, Agfa, Belgium) and a cabinet X-ray system (Model No. 4385A, Faxitron X-ray Corporation, AZ, USA). After fixation, samples were dehydrated by an ascending series of ethanol and were transferred to xylene. Finally, they were infiltrated and embedded in methylmethacrylate (MMA). The polymerized MMA blocks were trimmed using a butcher saw (Bizerba FK 22, Bizerba AG, Switzerland) prior to cutting with an annular diamond saw (Leitz 1600 saw microtome, Leica AG, Switzerland). The samples were glued with cyanoacrylate onto Beracryl holders for sectioning. Contact radiographs of the sections were taken as described previously. Two sections of each sample were selected, which were glued onto opaque Plexiglass® slides, ground and fine polished. Sections were stained with Giemsa-Eosin and histopathological analysis of the slides was performed using a transmission light microscope (BX40, Olympus, Switzerland). Histological findings were described, wherever possible, according to distribution (focal, multifocal, diffuse), morphological character and to severity by a veterinary pathologist.

Results

Preparation and characterization of gentamicin-loaded HApN formulations

The M_n of the pN-NH$_2$ was determined to be 11.2 kDa with a polydispersity index of 3.85, and a recovered mass of 95.2%. The average degree of substitution of pN for the carboxylate group on hyaluronic acid was 15% as verified by integration of the HA proton NMR signals between δ = 3.00 ppm and 3.77 ppm (equivalent to 9 HA protons) and integration of the pN signal at δ = 1.14 ppm (6 protons, -CH$_3$) [23]. Residual TBA content in the HApN polymer was low and quantified to be 0.1%.

A vial inversion test of the HApN composition in PBS showed that at concentrations above 9% w/w the composition flowed easily at RT, upon heating to 37 °C the composition did not flow anymore. A 13% w/w concentration was selected as it has the required temperature dependent gelation for this study. Rheology was performed on the 13% w/w HApN, dissolved in PBS alone or in PBS with 1% w/w gentamicin sulfate (Figure 4.2A).

The 13% w/w HApN composition in PBS (Figure 4.2A, black circles) was found to be a flowing sol at RT, with moderate shear and viscous moduli below 1 Pa. Upon heating above the lower critical solution temperature where the shear modulus G' over take the viscous modulus G", the shear moduli of all the HApN compositions (with and without gentamicin) increased more than 3 orders of magnitude up to 10 kPa at 37 °C. The onset of gelation for the HApN without gentamicin added, was approximately 28 °C. Upon addition of 1% w/w gentamicin sulfate the LCST decreases to 25 °C.

As can be seen from Figures 4.2B and 4.2C, the shift in LCST is proportional to the gentamicin sulfate concentration in the PBS, with the largest shift observed for the highest concentration. The same effect is observed when HApN is dissolved in K$_2$SO$_4$ at stoichiometric equal concentrations. However, the effect is stronger for K$_2$SO$_4$ than for gentamicin sulfate.
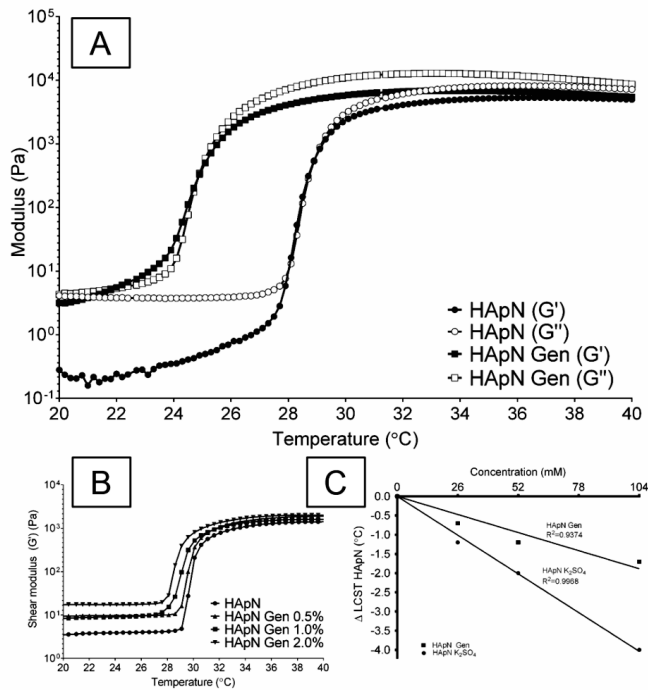
Figure 4.2: *Viscoelastic shear moduli of 13% w/w HApN solution in PBS, without or with addition of 1% w/w of gentamicin sulfate (A). Shear moduli of a 13% w/w HApN solution in PBS with increasing gentamicin sulfate content (B). Concentration dependent LCST shift (ΔT) for HApN (13% w/w) solutions in PBS with increasing sulfate content from gentamicin sulfate (0 – 104 mM sulfate) or increasing stoichiometric equal concentration of sulfate ions from potassium sulfate (K_2SO_4) (C).*

In vitro gentamicin release

The cumulative release of gentamicin sulfate from collagen fleece and HApN hydrogels with 1% and 2% w/w gentamicin over a 1 week period were measured *in vitro* at 37 °C (Figure 4.3).

An initial burst release of gentamicin was observed for all the tested ALBs (Figure 4.3). After 1 hr, 67% of the gentamicin incorporated in the collagen fleece was released, whereas for the HApN hydrogel with 1% w/w gentamicin and 2% w/w, 47%

and 62% of the total gentamicin payload was released, respectively. After 24 hrs, 88% of the total amount of gentamicin was released from the collagen fleece, and 86% and 96% respectively for the 1% w/w and 2% w/w gentamicin containing HApN hydrogels. After 7 days incubation in PBS, all ALBs had released their entire gentamicin payload. The HApN hydrogel (800 µl) with 1% w/w gentamicin sulfate was chosen for the *in vivo* study as it matches more closely the volume of the applied collagen fleece in the *in vivo* study (2 cm^3) and because of its slightly slower initial release of gentamicin as compared to the 2% w/w gentamicin sulfate containing HApN hydrogel.
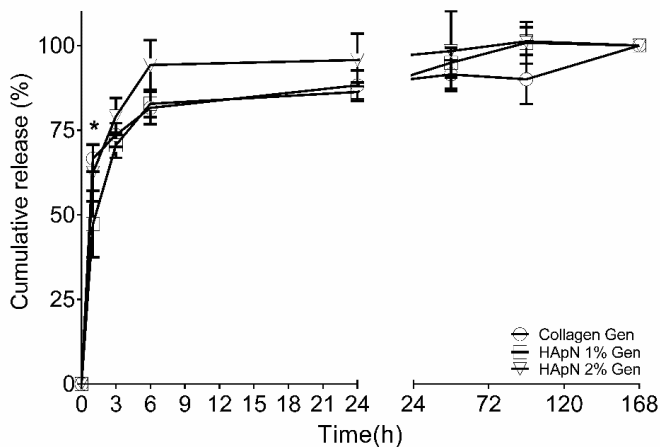

Figure 4.3: *Gentamicin cumulative release profile of collagen-gentamicin fleece and HApN hydrogels loaded with 1% w/w and 2% w/w gentamicin incubated at 37 °C for 1 week in PBS. n=3, error bars represent standard deviation.*

In vivo observations

Between the rabbits from the 3 study groups no difference in weight development was observed over the course of the observation period. Rabbit weights were 3.4 kg (±0.2 kg) (untreated control group rabbits), 3.5 kg (±0.2 kg) (gentamicin loaded collagen fleece treated rabbits) and 3.4 kg (±0.3 kg) (gentamicin loaded HApN hydrogel treated rabbits) after 1 wk, respectively.

C-reactive protein & white blood cell count

After surgery the C-reactive protein (CRP) levels in plasma were elevated in all 3 study groups (Figure 4.4).
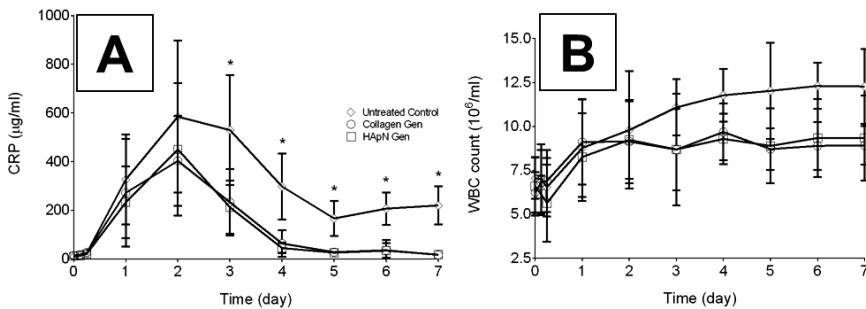

Figure 4.4: *Plasma C-reactive protein (CRP) levels (A) and white blood cell (WBC) values (B) for the untreated group, gentamicin-loaded collagen fleece (Collagen Gen) treated group and the gentamicin-loaded HApN hydrogel (HApN Gen) treated group. Values are expressed as mean value per group (n=10) ± standard deviations. Statistical analysis on the CRP levels was performed by Mann-Whitney test (*, P≤0.05).*

The peak in CRP production is found 2 days after surgery, after which CRP levels started to decrease for all 3 study groups. For the 2 groups treated with either the Collagen Gen or the HApN Gen, CRP levels returned to base line values. However, after day 3 the CRP levels of the untreated group stayed significantly elevated until the end of the study (Figure 4.4). A similar trend is observed for the white blood cell (WBC) counts in the rabbit plasma, where 7 days after surgery rabbits of the untreated group have 12.3 x 10^6 WBCs/ml as opposed to 8.9 x 10^6 (Collagen Gen) and 9.3 x 10^6 (HApN Gen) WBCs/ml in the treated groups (Figure 4.4).

In vivo systemic gentamicin concentrations

The plasma concentration of gentamicin sulfate released from both HApN and Collagen fleece was measured over a 1 week period (Figure 4.5).
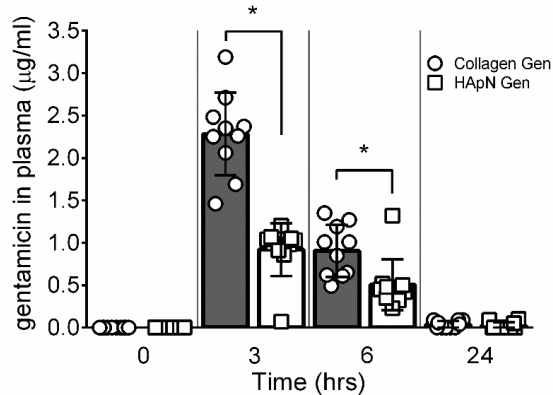

Figure 4.5: *Plasma concentrations of gentamicin after application of antibiotic loaded ALBs in vivo: Collagen Gen and HApN Gen at 0, 3, 6, 24 hrs. Values for the plasma concentrations of gentamicin are expressed as the mean value per group (n=10) ± standard deviations. Statistical analysis on the gentamicin concentrations was performed by Mann-Whitney test (*, P≤0.05).*

Systemic gentamicin concentrations at 3 hrs upon administration were found to be 2.3 µg/ml for Collagen Gen, and 0.9 µg/ml for the HApN Gen, respectively. After 24 hrs no gentamicin could be traced in the plasma from both ALBs (Figure 4.5).

Bacteriology

Quantitative culture of each inoculum, showed that CFU counts ranged from 1.2 x 10^6 CFU - 3.5 x 10^6 CFU for the inocula administered to the rabbits. Bacteriological quantification for harvested tissues and implant is shown in Figure 4.6.
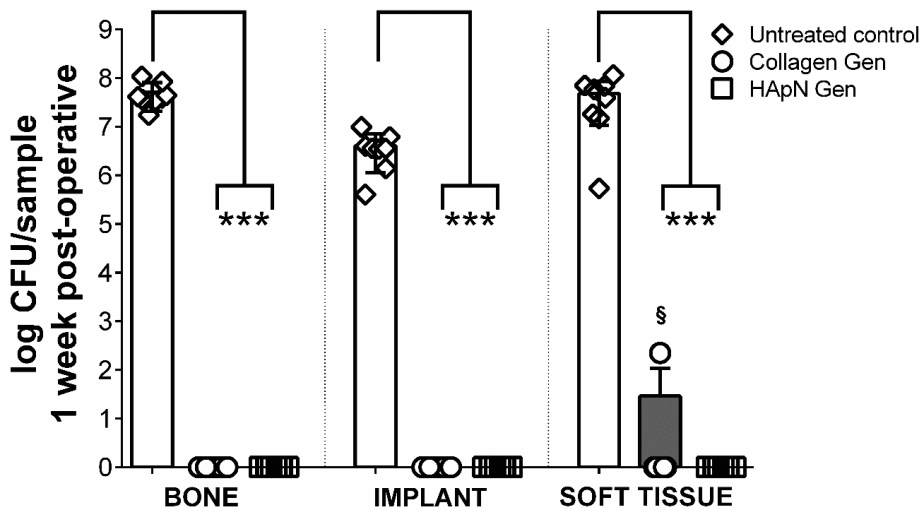
Figure 4.6: Bacteriological quantification of harvested tissues and implant 1 week post-operatively from: Untreated control group (Untreated Control); Collagen fleece with gentamicin (Collagen Gen); HApN hydrogel with gentamicin (HApN Gen). § indicates causative organism other than S. aureus. n=8, error bars represent standard deviation. Statistical analysis on the number of CFUs was performed by Mann-Whitney test (***, P≤0.001).

All rabbits (8/8) that did not receive any antibiotic treatment (untreated control) developed an infection (Figure 4.6). Bone, soft tissues and implant material of these rabbits were all colonized by *S. aureus*, as proven by a positive latex agglutination test for *S. aureus.* The *S. aureus* strain used in this *in vivo* study was found to be susceptible to gentamicin (average zone diameter for *S. aureus* JAR 060131 was 20 mm and for the quality control strain, the average zone diameter for *S. aureus* ATCC® 25923 was 22 mm indicating the test was performed correctly). In the group of rabbits treated with the collagen fleece with gentamicin (Collagen Gen), all tissues and implant material, except for the soft tissue of one rabbit (1/8), stayed free from bacterial colonization. The soft tissue sample of the infected rabbit was negative for the *S. aureus* latex agglutination test and was identified as *Streptococcus salivarius* (*S. salivarius*). This strain was at least intermediately susceptible to gentamicin as determined by the disk diffusion method (average zone diameter was 19.5 mm ± 0.9

mm). None of the rabbits (0/8) of the group that received the HApN hydrogel with gentamicin (HApN Gen) developed an infection. Bone, soft tissue and implant were free of bacteria.

Histological evaluation

Images of Giemsa Eosin stained sections of the operated humerus, including implants and surrounding soft tissues, are shown in Figure 4.7.
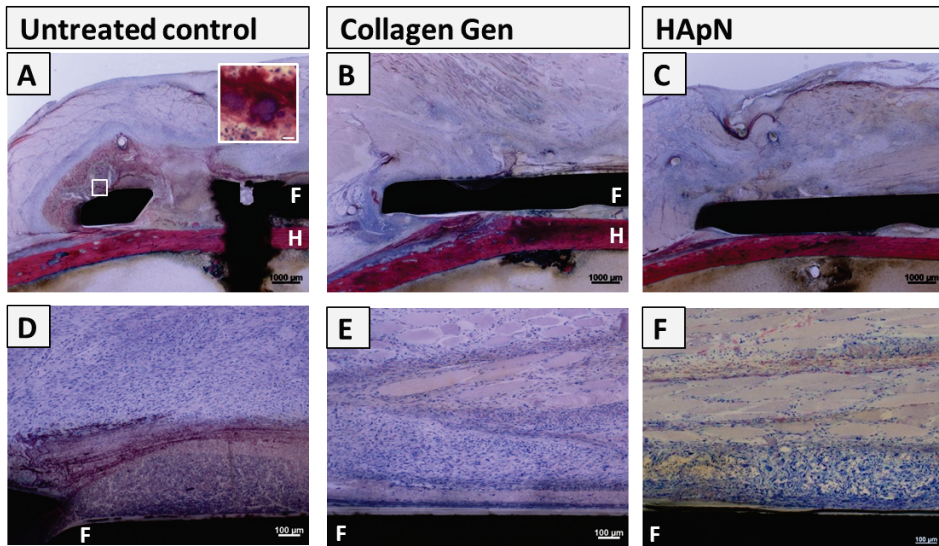

Figure 4.7. *Microphotographs of histological humerus samples (incl. stainless steel implant and soft tissue (A,B,C)) at day 7 after inoculation with S. aureus (Giemsa-Eosin stained thick-sections; objective lens [A, B, C]: 1.25x; [D, E, F]: 10x; [inset Figure A]: 100 x oil immersion). Fracture fixation implant (F) and the cortex of the humerus (H) are indicated in the image. The inset in figure A represents a magnification of the area within the white bordered box. The scale bar in the inset in figure A represents 10 μm. Histological changes: see text.*

All three groups displayed signs of surgery-induced damage at the 7-day euthanasia time point, such as fibrin formation and muscle necrosis. However, some distinctions between treated and untreated groups were evident. In the untreated control group (Figure 4.7, left column), obvious suppurative inflammation was observed surrounding the implant. At high magnification, Giemsa-positive coccoid bacterial

micro-colonies were identified in the untreated control group, and seen to be embedded in fibrin and surrounded by polymorphonuclear granulocytes at the periphery of a micro-abscess (Figure 4.7 D, inset).

In comparison, the Collagen Gen and HApN groups (center and right columns, respectively) displayed a more physiological response at the implant interface and there were early signs of muscle regeneration (Figure 4.7 E and F).

Discussion

Open fracture treatment has been shown to benefit from the local application of ALBs [12, 32]. However, improvements in the development of ALBs may offer the possibility of further reducing the infection rates associated with operative fracture care [2, 33]. Hydrogels are attractive ALBs as they offer the prospect of a less invasive application than non-injectable materials such as PMMA or collagen fleece. Hydrogels also offer versatility in that they can be applied as an anti-infective adjunct to any fracture fixation system and can protect the entire surgical site regardless of the size or extent of tissue damage and regardless of the 3D geometry of the repaired fracture [34-36]. Furthermore, hydrogels based on biodegrabable polymers do not require removal. Coated implants or bone cement beads may not achieve these particular performance targets, especially in cases where tissue damage and contamination extends beyond the immediate vicinity of the implant/ALB.

Previously described hydrogel systems that were based on hydrated polymer matrices with already formed networks, are applied as highly viscous compositions prior to surgery to coat the implant [37]. Therefore, there is a risk of retaining only a low percentage of the starting amount of gel on the implant during the insertion procedure. This high viscosity may also result in a suboptimal dispersion within the surgical field. The gentamicin-loaded HApN formulation characterized in this study showed liquid-like behavior at RT, enabling distribution throughout the contaminated surgical site. Once placed within the wound, the gentamicin-loaded HApN dispersed throughout the wound, gelated soon thereafter, and effectively prevented bacterial infection. The treatment was also shown to prevent infection-induced CRP increases

over time in all rabbits and prevented the appearance of suppurative abscess formation adjacent to the implant.

The addition of gentamicin sulfate to the HApN resulted in a reduction in LCST (from 28°C to 25°C). The temperature-induced phase transition of thermo-responsive polymers is influenced by the presence of co-solutes, such as salts [38], and their position in the Hoffmeister series [39]. Of all anions, sulfate has the strongest salting-out potential [38]. In addition, anions like SO_4^{2-} have shown to increase the viscosity of polysaccharide solutions, as observed as an increase in G'' at temperatures below the LCST for HApN [40]. As the temperature of tissues in the extremities of the body is ordinarily much lower than 37°C, the shift in LCST is beneficial for their application to obtain fast gelation in traumatic wounds.

The HApN Gen showed an initial and rapid burst release of antibiotic, followed soon thereafter by complete exhaustion of the antibiotic from the material. This release profile favorable in terms of reducing toxicity concerns, but also in terms of optimal pharmacodynamics of gentamicin, which is a concentration dependent antibiotic. *In vitro,* the gentamicin released at 1 hr was significantly lower for the HApN Gen hydrogel than the collagen fleece, and this was also seen in the plasma measurements of rabbits three and six hours after surgery. This may indicate a slightly lower burst release of gentamicin upon application of the thermo-responsive HApN Gen than for the Collagen Gen. This is, however, difficult to verify as the half-life of gentamicin in the blood circulation of the rabbit is very short and accurate assessment of the pharmacokinetics with such few time-points is therefore a challenge [41, 42]. Nevertheless, the peak plasma gentamicin levels measured in both groups are much lower than the commonly observed maximum concentration (C_{max}) of gentamicin in the human serum (18 µg/ml) upon intravenous administration of a conventional dose of 6 mg gentamicin/ kg body weight [43], indicating systemic toxicity is not a concern after application of either ALB. Clearly, both the thermo-responsive HApN hydrogel and collagen fleece with gentamicin provided adequate protection against infection, despite the low total amount of gentamicin applied, highlighting the fact that local delivery is a highly effective prophylactic strategy.

Although numerous publications have described hydrogels as antibiotic or antifungal delivery systems, few hydrogels have been tested for their efficacy in *in vivo* models [36, 37, 44-51], and those that have done so present varied success in their primary goal of infection prevention. Giavaresi *et al.* tested a non-responsive HA hydrogel with poly-D,L-lactic acid grafted as side groups, and loaded with vancomycin in an *in vivo* model with methicillin-resistant *S. aureus* (MRSA) in the rabbit femur. The vancomycin loaded hydrogel reduced, but was not able to clear the MRSA infection [37], possibly due to the high bacterial load of MRSA (10^4 CFU and 10^6 CFU) used in the study. Jia *et al.* tested a platelet-rich plasma derived leucocyte gel in a rabbit tibia model with *S. aureus* inoculation without the presence of a fracture fixation implant. Again, however, 4 out of 9 treated rabbits became infected [47], although as a non-conventional antibacterial agent, this result is nevertheless of interest. Taken together, none of these *in vivo* studies showed complete clearance of the bacterial burden, as opposed to the present study, in which the bacterial burden is completely cleared. Soon after inoculation of bacteria into the surgical site, the innate immune defenses will be activated and begin to combat the contaminating bacteria. Although the amount of bacteria killed by the innate defense system relative to those killed by the antibiotic cannot be estimated, in the absence of antibiotic, this dose of bacteria will consistently achieve a longstanding infection for up to at least ten weeks in this model [24]. This indicates that the antibiotic delivery is a critical feature in the eradication of infection by the hydrogel. In using the HApN hydrogel for prophylaxis, we prove that a flowable degradable thermo-responsive hydrogel loaded with gentamicin can eliminate a single species *S. aureus* contamination. As such, the efficacy against other pathogens such as Gram-negative or resistant bacteria is not shown. Furthermore, the bacteria in this model are planktonic and so are more easily targeted than for example bacteria within a biofilm. However, gentamicin does retain activity against many Gram-negative pathogens, and gentamicin has even been selected as the antibiotic of choice for the only commercially available antibiotic coated trauma implant on the market, which is used prophylactically in the same clinical context as intended use for the HApN.

Should the HApN hydrogel be required to treat an infection, it would present a much more formidable challenge, considering that the bacteria will likely have formed

biofilm on implanted hardware rendering them highly resistant to antibiotics. Additionally, the local tissues will likely be inflamed, with granulation tissue and abscess formation possible. The antibiotic distribution and concentrations required to achieve eradication in this situation may well require greater control over, or greater amounts of release from, the hydrogel than is achieved with the tested formulation. Therefore, the data presented should not be extrapolated to any claim of efficacy in the treatment of established infection. Overstreet *et al.* investigated the efficacy of a gentamicin loaded poly(*N*-isopropylacrylamide-co-dimethyl-γ-butyrolactone acrylate-co-Jeffamine® M-1000 acrylamide) (G-PNDJ) viscous hydrogel as treatment of established infection in a rabbit model [36]. The G-PNDJ hydrogel, with an average 30 mg gentamicin, was able to treat the infection in combination with debridement. Future work with the HApN hydrogel described here may involve evaluating the efficacy as a treatment option in a similar *in vivo* model.

Collagen fleece is a benchmark delivery product used for the local delivery of gentamicin in infection prophylaxis and was chosen as an ideal comparison for the hydrogel in the absence of any commercially available, gentamicin loaded hydrogels. The systemic gentamicin concentration observed in the collagen fleece group is in accordance with previous published work by Mehta *et al.* [52], where collagen fleece resulted in an average systemic gentamicin concentration of 3.2 µg/ml. The single rabbit in the collagen gen group that was found to have a superinfection with a streptococcus (of intermediate gentamicin resistance [53, 54]), is believed to be due to the rabbit licking the wound and delivery of oral microflora after the initial gentamicin is released from the collagen. This highlights the importance of proper post-surgical wound care and the need to protect the surgical field until wound closure, rather than as a particular failing of the collagen fleece. Several clinical cases have been published where collagen fleece with gentamicin was applied to prevent infection: e.g. after major or minor amputations in patients with diabetes or diabetic complications; before insertion of plate osteosynthetic hardware; and at the site of the removed disc after discectomy in spinal surgery [55]. In a case series report of 35 patients with open fractures, the efficacy of applying the gentamicin-loaded collagen fleece during the plating procedure was investigated. The majority of the fractures were considered to have united 40 weeks post-surgery. Local wound

complications were observed in 10% of the patients, and 7% of the patients had deep tissue infections following the procedure [55]. In general, the wound healing time was shorter after application of the gentamicin-loaded collagen fleece and no patient progressed to non-union or implant failure during long-term follow-up.

Although similarly effective in preventing infection, the collagen fleece lacks many of the handling features of HApN. HApN may therefore be expected to be equally effective as collagen fleece, however, with greater ease of use and greater distribution throughout even complex wounds. Furthermore, there is the possibility to reconstitute the HApN hydrogel with different antibiotics for loading. This may eventually result in greater clinical uptake, should such a material become clinically available. Prior to clinical implementation however, the effect of the HApN hydrogel with gentamicin on the bone healing process should be investigated.

Conclusions

The described gentamicin-loaded HApN hydrogel is designed for complex trauma cases, where contamination may have spread throughout complex wound sites. The gentamicin-loaded HApN hydrogel was effective in preventing *S. aureus* infection in a rabbit prophylaxis model in the presence of osteosynthesis material. The HApN hydrogel offers comparable prophylaxis against a current clinical product, yet promises greater versatility in terms of application.

Acknowledgements

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Chapter 5

Local application of a gentamicin-loaded thermo-responsive hydrogel allows fracture healing upon clearance of a high *Staphylococcus aureus* load

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Abstract

Antibiotic loaded biomaterials (ALBs) have emerged as a useful adjunctive antimicrobial measure for the prevention of infection in high risk cases, such as the surgical fixation of open fractures. A biodegradable thermo-responsive poly(*N*-isopropylacrylamide) grafted hyaluronic acid (HApN) hydrogel loaded with gentamicin has recently been shown to prevent implant-related infection in an osteosynthesis model in the rabbit. The aim of this study was to determine the influence of this HApN hydrogel on bone healing. A rabbit humeral osteotomy model with plating osteosynthesis was used to compare fracture healing in rabbits receiving the hydrogel versus the controls, both with and without bacterial inoculation. Outcome measures were mechanical stability and histological score.

Application of the HApN hydrogel did not significantly influence mechanical stability or histological evaluation of fracture healing in the non-inoculated group relative to controls. In the inoculated groups, control animals receiving the bacterial inoculum without hydrogel were culture positive at euthanasia and were found to display weaker mechanical stability and higher histopathological scores in comparison with equivalents receiving the gentamicin-loaded HApN hydrogel who cleared the bacterial load.

Therefore, the presented hydrogel is an effective antibiotic carrier that does not affect fracture healing. Addition of an osteopromotive compound in the hydrogel may accelerate fracture healing upon successful prophylaxis.

Introduction

Infection is one of the most common complications associated with operative fracture fixation [1-3]. As the number of patients receiving implants increases year on year [4], the number of implant associated infections is also set to increase. Systemic administered prophylactic antibiotics have been shown to reduce the number of infections. In the primary fracture fixation of both closed [5] and open fractures [6] antibiotic prophylaxis reduced the infection rate in comparison with patients receiving either a placebo or no antibiotic. However, the fact that infections still occur indicates systemic antibiotic prophylaxis is not completely effective in all cases. The failure of

systemic antibiotic therapy may be due to compromised vasculature, tissue deconstruction and edema preventing antibiotic delivery when applied intravenously [7]. The delivery of antibiotics directly to the intended site of action using antibiotic loaded biomaterials (ALBs) is an attractive option to overcome this problem [8]. Degradable ALBs offer advantages over some of the conventional ALBs such as antibiotic loaded bone cement beads or spacers due to the need for surgical removal of the latter.

A thermo-responsive formulation of a hyaluronic acid (HA) derivative loaded with gentamicin, which is injected and gelates *in vivo*, could successfully prevent bacterial colonization of a contaminated implant site in rabbits in a one-week study [9]. The physically crosslinked hydrogel is composed of HA, a linear polysaccharide, that degrades enzymatically in the body. Although its relatively simple primary structure, it is involved in a plethora of biological processes [10-13] and its derivatives have been widely used as injectable carriers for drug delivery [14, 15].

A critical feature for such an ALB that may be added directly to a fracture site is the effect on fracture healing. Few reports are available on bone healing after delivery of antibiotics by a biodegradable ALB and clearance of a bacterial load [16-18]. In particular, bone healing data in the context of an appropriate release profile and subsequent or simultaneous degradation of the delivery vehicle is lacking. The foreign material itself, the time to degrade and the effect of high local concentrations of antibiotics may potentially influence fracture healing [19, 20]. In addition, the dead bacterial contaminants will still have some potential to induce bone resorption due to upregulation of osteoclast activity [21, 22].

Bone healing in the presence of an ALB has also been investigated by Peng *et al.* and by Ambrose *et al.*, however these were not prophylactic studies, but rather treatment studies investigating the treatment of osteomyelitis with antibiotic-loaded non-degradable poly(methylmethacrylate) (PMMA) and biodegradable thermosensitive hydrogel nanoparticles or polyester microspheres, respectively [23, 24]. Upon establishment of an acute infection and subsequent debridement and treatment with either antibiotic loaded PMMA beads or an mPEG-PLGA based hydrogel, no histological differences in bone healing were observed between the two

ALBs four and eight weeks post-operatively. However, a higher degree of bone healing in the defect treated with the antibiotic-loaded biodegradable polyester microspheres was observed at four weeks compared to animals treated with antibiotic loaded PMMA. In a prophylaxis model, biodegradable antibiotic-loaded injectable microspheres composed of a blend of poly(lactic-co-glycolic acid) with poly(ethylene glycol) dry-mixed with β-tricalcium phosphate and subsequently mixed with 0.5% carboxymethyl cellulose in saline to make a paste, were shown to effectively prevent infection in a large animal model [17]. However, the lack of positive and negative control groups prevented the assessment of bone healing. None of these studies included a quantitative measure of bone healing, such as bending stiffness.

In this study, the effect of the injectable thermo-responsive physical crosslinked hydrogel on the bone healing process was assessed. A rabbit humeral osteotomy model with plating osteosynthesis was used to compare fracture healing in animals receiving the hydrogel versus the controls, both with and without bacterial inoculation. Outcome measures in assessing new bone formation were given by the mechanical stability and histological scores. Bacterial colonization and potential foreign body reaction were assessed by quantitative bacteriology and histopathological analysis, respectively.

Experimental

Materials

Hyaluronic acid sodium salt (HANa) from *Streptococcus equi* was purchased from Contipro Biotech s.r.o. (Dolni Dobrouc, Czech Republic), number average molecular weight (M_n) = 170.6 kDa and polydispersity (M_n/M_w) = 1.73. Tetrabutylammonium hydroxide solution (TBAOH) (~40% in H_2O, ~1.5 M), 2,2'-azobis(2-methylpropionitrile) (AIBN) (≥98.0% GC), methanesulfonic acid (MSA) (≥99.5%), methanol (analytical grade (99.8%)) and ethanol (≥99.8% puriss) were purchased from Fluka (Buchs, Switzerland). *N*-isopropylacrylamide (nIPAm) (≥99%), cysteamine hydrochloride (AESH) (≥98% (titration)), *N,N*-dimethylformamide (DMF) anhydrous (99,8%), 1,4-dioxane (≥99.5%, p.a., ACS), deuterium oxide (D_2O) (99.9 atom% D), Dowex M-31 cation exchange resin, 1,1'-carbonyldiimidazole (CDI) (reagent grade),

dimethyl sulfoxide (DMSO) (≥99.5% for synthesis), gentamicin sulfate salt (Gen) (potency ≥590 µg gentamicin base per mg) and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Diethylether (≥99.5%, p.a.), glycerol (≥99%) and sodium bromide (NaBr) (≥99% p.a., ACS) were purchased from Carl Roth (Karlsruhe, Germany). Blood Agar (BA), Tryptone Soya Agar (TSA), Tryptic Soy Broth (TSB) and Mueller-Hinton broth (MHB) were purchased from Oxoid AG (Basel, Switzerland).

In vivo study design

Four study groups were included in this study, both non-inoculated and inoculated, with the relevant control group. Comparison groups involved bacterial inoculation or not, and groups with or without (antibiotic loaded) hydrogel. The 4 study groups within this study are listed in Table 5.1.

Table 5.1: List of the in vivo study groups, including presence of inoculum, thermo-responsive hydrogel and gentamicin sulfate.

Group name	*S. aureus*	HApN	Gentamicin sulfate
1. Non-inoculated control	-	-	-
2. Non-inoculated + HApN	-	+	-
3. Inoculated control	+	-	-
4. Inoculated + HApN Gen	+	+	+

n = 10 per study group. Target inoculum was 2.0 x 10^6 colony forming units (CFU) per rabbit. Volume of HApN hydrogel applied is 800 µl (13% w/w) per rabbit. Total payload of gentamicin sulfate in the hydrogel is 8 mg per rabbit.

Preparation of gentamicin-loaded thermo-responsive hydrogel

Amino-terminated poly(*N*-isopropylacrylamide) ($pN-NH_2$) was prepared and characterized with respect to its molecular weight as described previously [9]. The molecular weight (M_n) of the $pN-NH_2$ obtained was between 21.2 – 21.4 kg/mol, the polydispersity index (M_w/M_n) was between 2.32 and 2.63, and the recovered mass was between 82.2% and 83.4% for the two batches synthesized under the same condition, which were subsequently used in the synthesis of the 2 HApN batches used during the *in vivo* study.

Subsequently, NaHA was converted into its tetrabutyl ammonium salt (HATBA) by a cationic exchange process described by Bellini and Topai [25]. Subsequently, $pN-NH_2$ was grafted to the HATBA in a direct amidation reaction [9]. The degree of substitution of pN-NH for the carboxyl group on the HA backbone was then determined by proton nuclear magnetic resonance (^{1}H-NMR) by integration of the HA proton NMR signals between δ = 3.00 ppm and 3.77 ppm, equivalent to 9 protons belonging to the HA dimer and integration of the pN signal at δ = 1.14 ppm, deriving from 6 protons on the two methyl groups of pN [26]. The degree of grafting of pN to HA for the HApN batches used in this study was calculated to be 11% – 12%.

In vivo study

The *in vivo* study was approved by the Ethical Committee of the Canton of Grisons, Switzerland (TVB numbers 2015_06F and 2015_13E). All procedures were performed in AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International) approved facility and according to Swiss animal protection law and regulations. Forty skeletally mature, pathogen free (SPF) female New Zealand white rabbits (Charles River Laboratoris International, Inc, Sulzfeld, Germany) older than 24 weeks of age and a mean body weight of 3.9 ± 0.4 kg were included in this study.

Animal welfare

All rabbits were assessed by a veterinarian and determined to be healthy prior to inclusion in this study. Rabbits were group-housed and allowed to acclimatize to their surroundings for two weeks prior to the start of the study. After the surgical intervention, animals were single-housed until euthanasia. After surgery, each animal was checked at least twice daily for the first five postoperative days by a veterinarian or an experienced animal care taker. Thereafter, the animals were checked once daily at day six and seven postoperative and finally bi-weekly for the remainder of the study. Five of the forty rabbits used in this study had to be excluded during the observation period, and were replaced.

Pre-operative bacterial inoculum and hydrogel preparation

A gentamicin sensitive *S. aureus* (JAR 060131) isolated from a 68-year old male patient with an infected total knee arthroplasty was used in the present study [27]. Bacteria were stored in MHB supplemented with 20% of glycerol for long term preservation at -20°C. Bacterial inocula were prepared in PBS as previously described [28], with a target inoculum of 2.0 x 10^6 colony forming units (CFU) of *S. aureus*. Quantitative culture of each inoculum was performed immediately after preparation to assure the accuracy of the used inocula.

The sterile HApN samples were reconstituted at 13% w/w 1 day prior to surgery. For study group 2 (non-inoculated + HApN), reconstitution was in sterile PBS, whilst for study group 4 (inoculated + HApN + Gen), reconstitution was in sterile PBS containing 1% w/w gentamicin sulfate. The total payload of the antibiotic loaded HApN hydrogel was 8 mg gentamicin sulfate in 800 μl of the hydrogel, which was the volume injected over the implant.

Surgical procedure

Surgical anesthesia, pre- and post-operative analgesia were administered to the operated rabbits and the plating procedure was performed as described elsewhere [29]. Rabbits belonging to study group 1 (non-inoculated control) and study group 2 (non-inoculated + HApN) did not receive a bacterial inoculum, whereas rabbits belonging to study group 3 (inoculated control) and study group 4 (inoculated + HApN + Gen) received a bacterial inoculum after creation of the osteotomy with a 0.45 mm Gigli saw. The inoculum consisted of three separate injections of 34 µl bacterial suspension into the empty screw hole overlying the osteotomy, and onto the adjacent proximal and distal screw holes. Rabbits belonging to study group 2 (non-inoculated + HApN) received a HApN hydrogel injected over the 7-hole locking plate, but no inoculum. Rabbits of study group 4 (inoculated + HApN + Gen) received the gentamicin-loaded HApN hydrogel after obtaining the inoculum. The wounds were closed immediately after application of the hydrogel. A schematic overview of the rabbit humerus osteotomy model including fracture fixation hardware is found in figure 5.1.
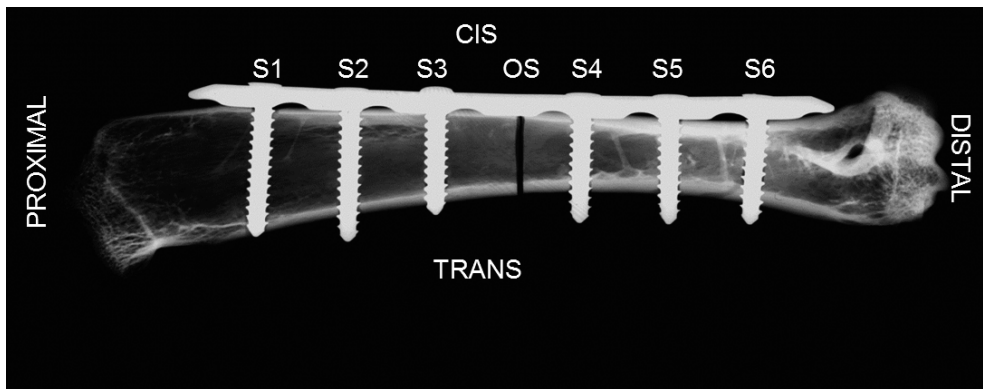

Figure 5.1: *Contact radiograph depicting the rabbit humerus osteotomy model including: screw postions (S1-S6), osteotomy (OS), inoculation points is performed on top of S3,S4 and in the OS, the HApN hydrogel spreads around the fracture fixation plate and in the osteotomy gap.*

Observation

Blood samples were withdrawn from all included rabbits pre-operatively, 3 hrs, 6 hrs, then once daily until day 7 post-operatively, and then on a weekly basis until the end of the study. White blood cell (WBC) count was measured at each time point (Vet ABC, Scil animal care, Viernheim, Germany). C-reactive protein (CRP) concentrations were quantified by ELISA (Rabbit CRP Elisa Kit, ICL Inc. Portland, OR, USA) for each time point until day 7 post-operatively. Serum gentamicin levels were quantified by a fluorescence polarization immunoassay (FPIA, lower limit of detection, 0.5 µg/ml) on a Cobas Integra® 400 plus analyzer (Roche Diagnostics AG, Risch, Switzerland) using reagents from Roche Diagnostics. Test solutions and calibration standards were diluted with human plasma. Weight of the rabbits was measured at surgery, 3 days and 7 days post-operatively and then weekly for the duration of the study. Radiographs of the operated limbs were taken in two planes (lateromedial and caudocranial) from all operated rabbits directly after surgery and then weekly until the end of the study. Animal exclusion criteria were: signs of systemic infection such as fever, depression and anorexia, local infection with severe lameness, persistent swelling and discharge or a weight loss exceeding 15% of the initial body weight. All rabbits were euthanized at the end of the observation period using intravenously administered Pentobarbital (Esconarkon®, Streuli Pharma AG, Switzerland).

Bacteriology

The soft tissues including all large muscles covering the implant, and any abscess material if present, were removed using a scalpel, weighed, and then placed into a sterile receptacle containing 10 ml of PBS. These samples were then homogenized using an Omni-TH hand-held homogenizer (LabForce AG, Switzerland) with sterile Omni-tip plastic probes. All implant samples were transferred to sterile glass test tubes containing 10 ml of PBS. Implant samples were then sonicated (Bandelin Ultrasonic waterbath RK 510 H, Bandelin, Germany) for 3 min, thereafter they were vortexed for 10 sec. Further processing of the samples for quantitative bacteriology was performed as described elsewhere [9].

Mechanical testing of rabbit humeri

After removal of the plate and screws of the operated humeri, the bending stiffness of the bone was determined via a non-destructive four-point bending test according to ISO 9585 (1990), using a material testing machine (Instron 5866, Instron, Norwood, MA, USA) equipped with a 1 kN load cell. Axial compression along the machine axis was applied to the bone by two loading pins of 10 mm diameter and 25 mm distance in between them, attached to the machine actuator. With the posterior side of the humerus facing towards the machine actuator, each specimen was supported by two pins of 10 mm diameter connected to the machine base at 50 mm distance between each other. The supporting points were defined by letting each specimen settle according to its anatomical landmarks.

The setup with a specimen mounted for biomechanical testing is shown in figure 5.2. Prior to testing the specimens, the load necessary to fracture intact non-operated rabbit humeri was determined for cadaveric humeri of age-matched rabbits (circa 400 N). Both the operated humerus and the contralateral humerus of each rabbit included in the study were then loaded to 100 N at a rate of 1 mm/min. Axial stiffness of the rabbit humeri was derived from the ascending linear slope of the load-displacement curve in the interval between 50 N and 100 N. Finally, the stiffness was expressed as the normalized stiffness of the operated humerus compared to the stiffness of the intact contralateral humerus of the same rabbit.
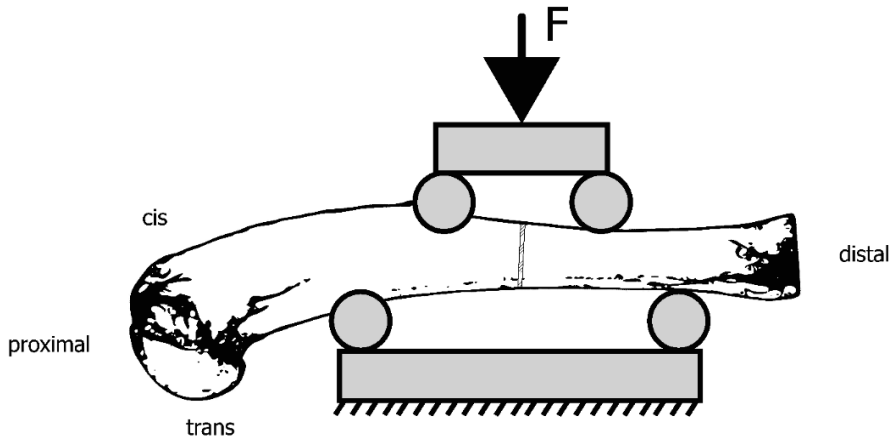
Figure 5.2: *Mechanical testing setup showing the positioning of the mounted operated rabbit humerus. Distance between the two loading pins (Ø=10 mm) attached to the actuator was 25 mm, and between the two loading pins (Ø=10 mm) at the machine base was 50mm. The relative stiffness was calculated as the stiffness of the operated humerus divided by the stiffness of the contralateral humerus of the same rabbit.*

Histopathological evaluation

Full thickness contact radiographs were taken in two planes (lateromedial and caudocranial) using high resolution technical film (D4 Structurix DW ETE, Agfa, Belgium) and a cabinet X-ray system (Model No. 4385A, Faxitron X-ray Corporation, USA) to document the state of the humeri after biomechanical testing. Rabbit humeri were then fixed in 70% (v/v) methanol for a minimum of two weeks, with fresh methanol changes weekly. After fixation, samples were dehydrated through an ascending series of ethanol and transferred to xylene and finally the samples were infiltrated and embedded in methylmethacrylate (MMA). Polymerized MMA blocks were trimmed using a butcher saw (Bizerba FK 22, Bizerba AG, Switzerland) and after that cut with an annular diamond saw (Leitz 1600 saw microtome, Leica AG, Switzerland). The samples were glued with cyanoacrylate onto Beracryl holders for sectioning. Contact radiographs of the sections were taken as described previously. The two sections to be stained were selected from as close as possible to the midline

of the humerus, including the empty screw holes. Sections were glued onto opaque plexiglas slides, ground and fine polished. One of the sections of the humeri was stained with Giemsa-Eosin, and the other was stained with Brown and Brenn for bacteria. The Giemsa-Eosin stained sections were used for semi-quantitative histopathological evaluation with a focus on tissue repair and/or fracture healing. Histological findings were described according to distribution, morphological character and severity using a semi-quantitative grading scheme (5-point scale) [29, 30]. The graded parameters were specially selected for this model by a veterinary pathologist. The Brown and Brenn stained sections were used to confirm the presence of bacteria. The histopathological analysis included osteotomy gap filling, endosteal and periostal callus formation, presence of inflammation, presence of multinuclear histiocytes and presence and amount of Brown and Brenn positive coccoid bacteria.

Statistical analysis

Statistical evaluation was performed using IBM SPSS Statistics software, vs 23. Results are presented as mean of each group with standard deviation (SD). Data was screened for normality of distribution with the Shapiwo-Wilk test. An independent T-test was run to compare the values of the bacterial inoculum in the two inoculated groups. One-way Analysis of Variance (ANOVA) test with Tukey post-hoc correction for multiple comparisons was conducted to compare the outcome measures for quantitative bacteriology data, normalized stiffness and periosteal callus formation between the four study groups. Finally, two-way ANOVA test with Tukey post-hoc correction was performed to detect significant influence of the treatments on the hematological values and weight change data in the four study groups. Level of significance was set to $*p<0.05$ for all statistical tests.

Results

Prevention of infection by gentamicin-loaded HApN hydrogel at 4 weeks

Total viable counts of the bacterial inocula given to each rabbit in both inoculated groups (group 3 and 4) are shown in Figure 5.3 A. There was no statistically significant difference in the inocula given to the two groups. The implant and fibrous capsule samples from rabbits belonging to study groups 1 & 2 (both non-inoculated) were all culture negative, and none of the humeri contained any visible abscesses. The implant and fibrous capsule samples of rabbits belonging to study group 4 (inoculated + HApN+ Gen) were culture negative for bacteria (Figure 5.3 B). In 1 out of the 10 rabbits from study group 4, small abscesses were observed close to the osteotomy site, however these abscesses were found to be culture negative.
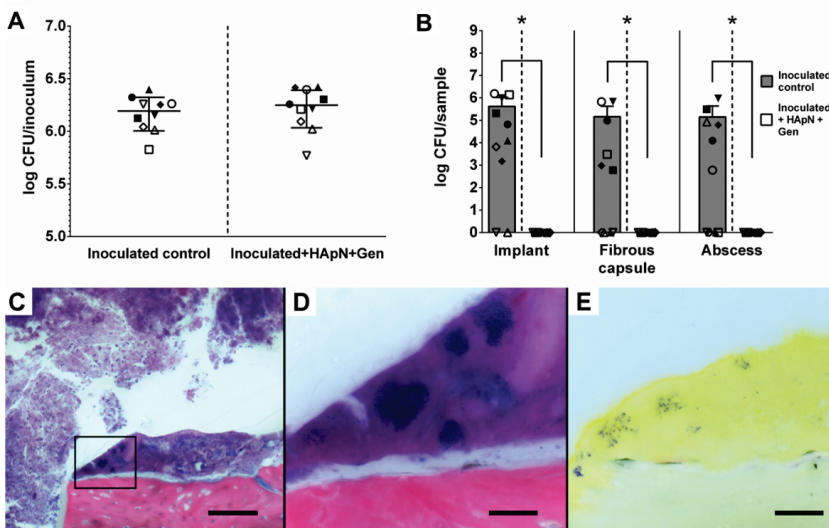

Figure 5.3: *A) Quantification of bacterial inocula used during rabbit surgeries B) Bacterial quantification of implant, fibrous capsule and if present of abscess material, n=10 per study group, error bars indicate standard deviation (SD). Each of the 10 rabbits per study group is represented by a different symbol (5 closed and 5 open symbols) C) Microphotographs of Giemsa-Eosin (C, D) and Brown and Brenn-stained (E) humeri sections at the trans side near the osteotomy of an infected rabbit (study group 3). The blue dots visible in both Giemsa-Eosin and Brown and Brenn sections are Gram-positive bacteria. Figure D and E are close ups of the area within the square in figure C.*

One rabbit belonging to study group 3 was culture negative for bacterial growth, however a low amount of Gram-positive bacteria were found in the Brown and Brenn stained section of this animal. Generally, the stained sections of the humeri from rabbits from the inoculated control group showed high amounts of bacteria visible in Giemsa-Eosin (Figure 5.3 C, 5.3 D) and Brown and Brenn stained sections (Figure 5.3 E). Furthermore, empty osteocytic lacuna were observed in the cortex (focal osteonecrosis near the osteotomy) and a large amount of acellular debris was observed in the bone marrow (diffuse necrotizing osteomyelitis) (Figure 5.3 C). The presence of bacteria was accompanied by large amounts of inflammatory cells. Most bacteria were found in the proximity of the S3 screw (proximal screw, next to osteotomy site) and in the osteotomy, locally where the bacterial inoculation had been performed.

Rabbits from the inoculated control group that were negative for the Brown and Brenn staining, showed Gram-positive bacterial growth for the quantitative bacteriology.

In none of the humeri from rabbits that belonged to the group of rabbits that received a bacterial inoculum and subsequently the gentamicin-loaded HApN hydrogel (inoculated + HApN + Gen) Gram-positive bacteria were detected microscopically.

CRP concentrations, WBC counts and weight of the rabbits were quantified and the lymphocyte/granulocyte ratios were calculated throughout the observation period and are shown in Figure 5.4. Rabbits from the inoculated control group and the inoculated + HApN + Gen group in this study had normal pre-operative CRP levels. Seven days post-operative CRP levels for these groups in this study were matching with historical data (Figure 5.4) [9].

For the rabbits belonging to the non-inoculated control group and the non-inoculated + HApN group, CRP levels increased to a maximum value at 2 days post-operatively and returned back to pre-operative levels.

The lymphocyte/granulocyte ratio was significantly lower for the inoculated control rabbits in comparison to the ratio in the rabbits that successfully cleared the bacterial load (inoculated + HApN + Gen group) at day 28 (Figure 5.4). There was no

significant difference in lymphocyte/ granulocyte ratio between rabbits belonging to the 2 study groups that did not receive an inoculum (Figure 5.4).

The gentamicin concentration in the plasma of the rabbits was assessed 3 hrs post-operative, which has previously been shown to display an approximate peak serum concentration [9]. The rabbits from the inoculated + HApN +Gen group had a 3 hrs post-operative gentamicin plasma concentration of 1.11 ± 0.32 µg/ml.
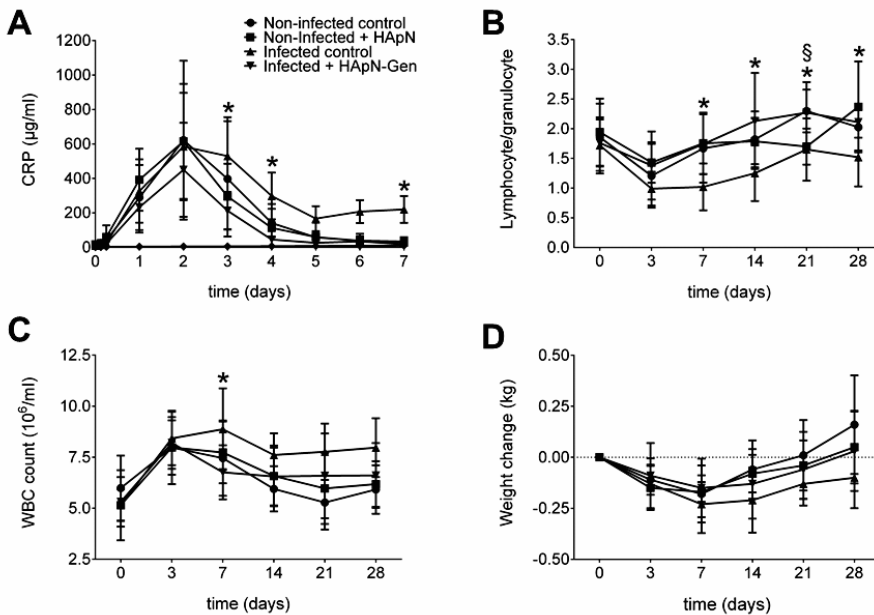

Figure 5.4: *Haematological and clinical observations of rabbits from non-inoculated control group, non-inoculated + HApN hydrogel group, inoculated control group and inoculated + HApN + Gen group. Presented is the CRP concentration in rabbit serum (data from inoculated control group and inoculated + HApN + Gen was collected in a previous in vivo study [9] ; the ratio of lymphocytes to granulocytes; WBC count and weight change in comparison to pre-operative weight. In all cases n=10, and data is represented as mean ± SD. (§-sign represents significance between the non-inoculated groups 1 and 2, *- sign represents significance between the inoculated study groups 3 and 4).*

A similar trend as for the lymphocyte/granulocyte ratio could be observed for the WBC count. At day 7 the inoculated control rabbits had a significantly higher WBC count than rabbits from the inoculated + HApN + Gen group.

Fracture healing in the presence of the gentamicin-loaded HApN hydrogel at 4 weeks

Bone healing of the operated rabbit humeri was visualized by contact radiographs of the humerus sections, as well as by staining sections of the operated humeri with Giemsa-Eosin (Figure 5.5). In the non-inoculated control group, there was lots of new periosteal callus formation around the osteotomy gap, leading to the closure of the osteotomy gap (Figure 5.5 & 5.6). Bone was formed both at the cis and trans site of the bone, though somewhat more on the trans site than on the cis site because of the presence of the fracture fixation plate. The presence of the fracture fixation plate leads to local stress shielding and diminishes formation of new callus at the cis site of the bone [31, 32]. Also new bone formation was observed in the screw holes proximal and distal to the osteotomy site leading to high osteo-integration of all screws. Bone was formed by osteochondral ossification, as can be seen by small amounts of bluish stained cartilage tissue in the osteotomy where the bone is bridging (Figure 5.5) in the non-inoculated control rabbits. The humeri from the rabbits that received the HApN hydrogel in the absence of bacterial inoculation (Figure 5.5) showed also a large amount of new periosteal callus formation, both at the cis and trans site of the bone and in the screw holes proximal and distal of the osteotomy site, however the amount of endosteal and periosteal callus formation was slightly lower, especially at the cis site (Figure 5.6).
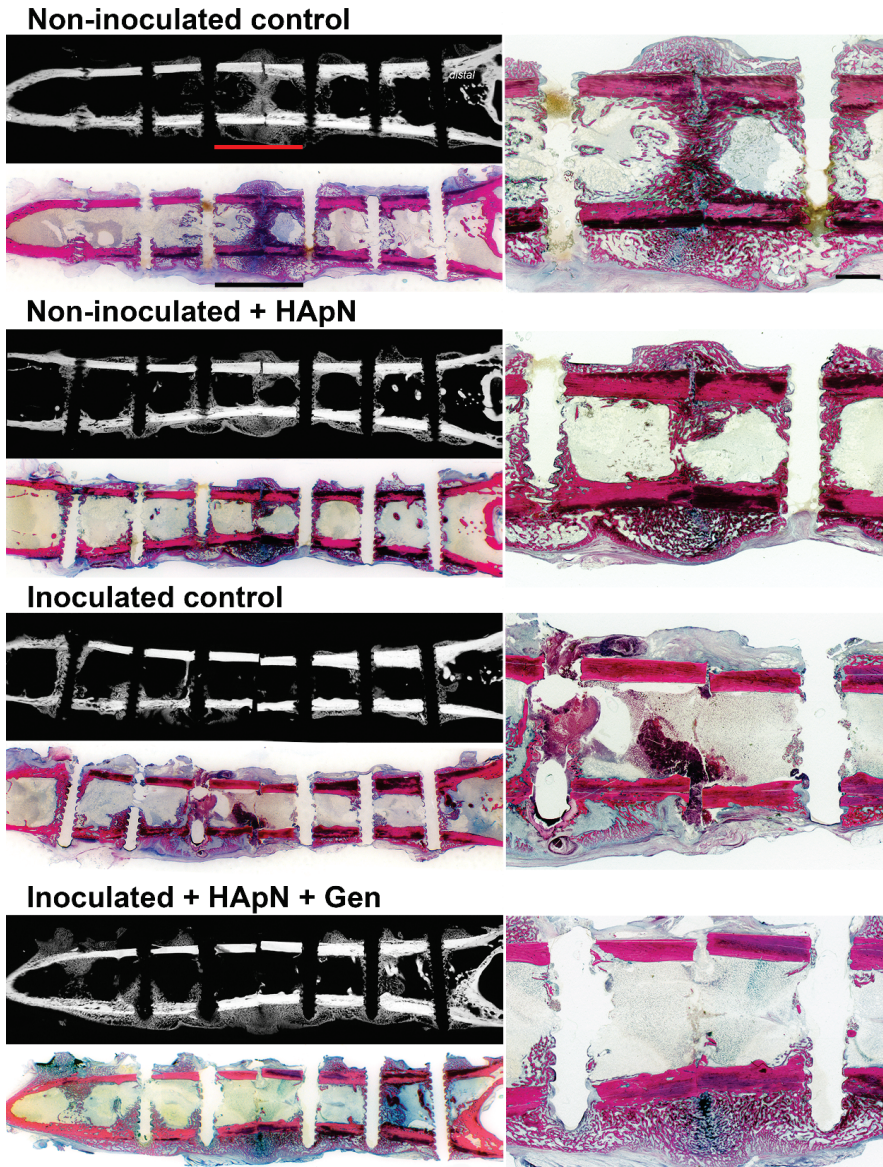
Non-inoculated control

Non-inoculated + HApN

Inoculated control

Inoculated + HApN + Gen

Figure 5.5: *Contact radiographs and Giemsa-Eosin-stained overview microphotographs (left, scale bar represents 10 mm) of the complete operated rabbit humeri depicting the osteotomy site (OS) and all six screw holes (S1 – S6, 4 weeks post-operative, longitudinal cutting plane, fracture fixation implants removed, situation after mechanical testing), as well as magnified details of the humerus osteotomy site (right, scale bar represents 2 mm) including the two adjacent screw holes (S3, left and S4, right).*

In the case of an established localized infection in the humerus, as was the case for rabbits from the inoculated control group, there was no callus formation and no bone formation is seen at the anatomical sites where the inoculation was performed (in the osteotomy and the S3 and S4 screw holes) (Figure 5.5). Large amounts of necrotic tissue can be seen in the medulla at the osteotomy site and at the S3 screw. Screws more distant to the inoculation site, showed better osteo-integration. Humeri from rabbits that received the inoculum, but that were treated with the HApN hydrogel with gentamicin, showed new callus formation (Figure 5.5). Opposed to the inoculated control rabbits, no necrotic tissue was observed. The new formed callus was localized at the trans site of the humerus and at the screw holes, while the cis callus and the endosteal bone formation at the osteotomy site was relatively low (Figure 5.6).
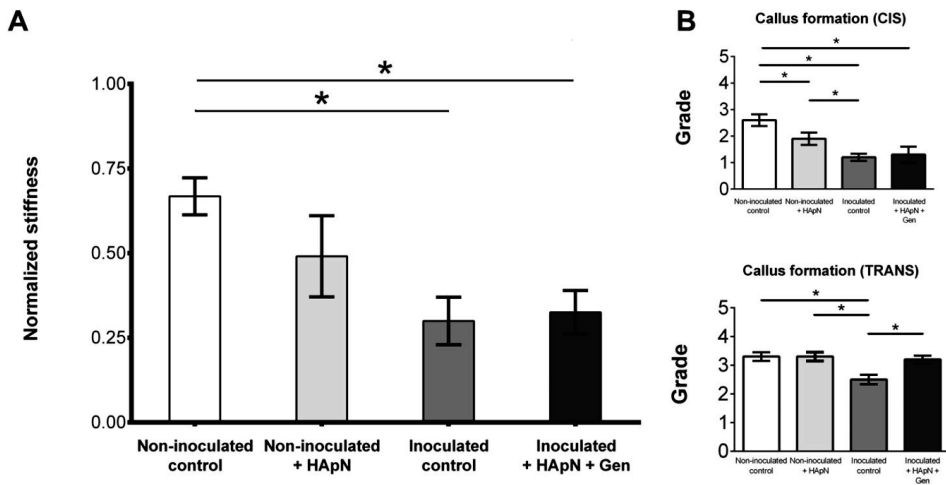

Figure 5.6: *Results of mechanical testing by four-point bending test of operated and contralateral humeri (A) and of histopathological scoring for new callus formation, at the cis and at the trans site of the humerus (B).*

As the presence of remaining biomaterial upon unfinished degradation can delay fracture healing, histopathological evaluation of the rabbit humeri sections also focused on localizing any reaction to remaining hydrogel components.

In the sections of the rabbits from both groups receiving the hydrogel (Figure 5.7), there are large amounts of mono- and multinucleated histiocytes with foamy bluish cytoplasm present. Cells with similar morphology are absent in sections from the rabbits of other groups which did not receive the HApN hydrogel (Figure 5.7). Furthermore, similar to Figure 5.7 large amounts of bony callus formation could be observed in the humeri from both non-inoculated groups at the osteotomy site and fibrous incapsulaton of the implant was observed. Soft tissue near the osteotomy in the inoculated control group showed acellular necrotic debris, fibrin and a mixed population of inflammatory cells due to inflammation below the plate which was not observed in the inoculated rabbits that were treated with the gentamicin-loaded HApN hydrogel.
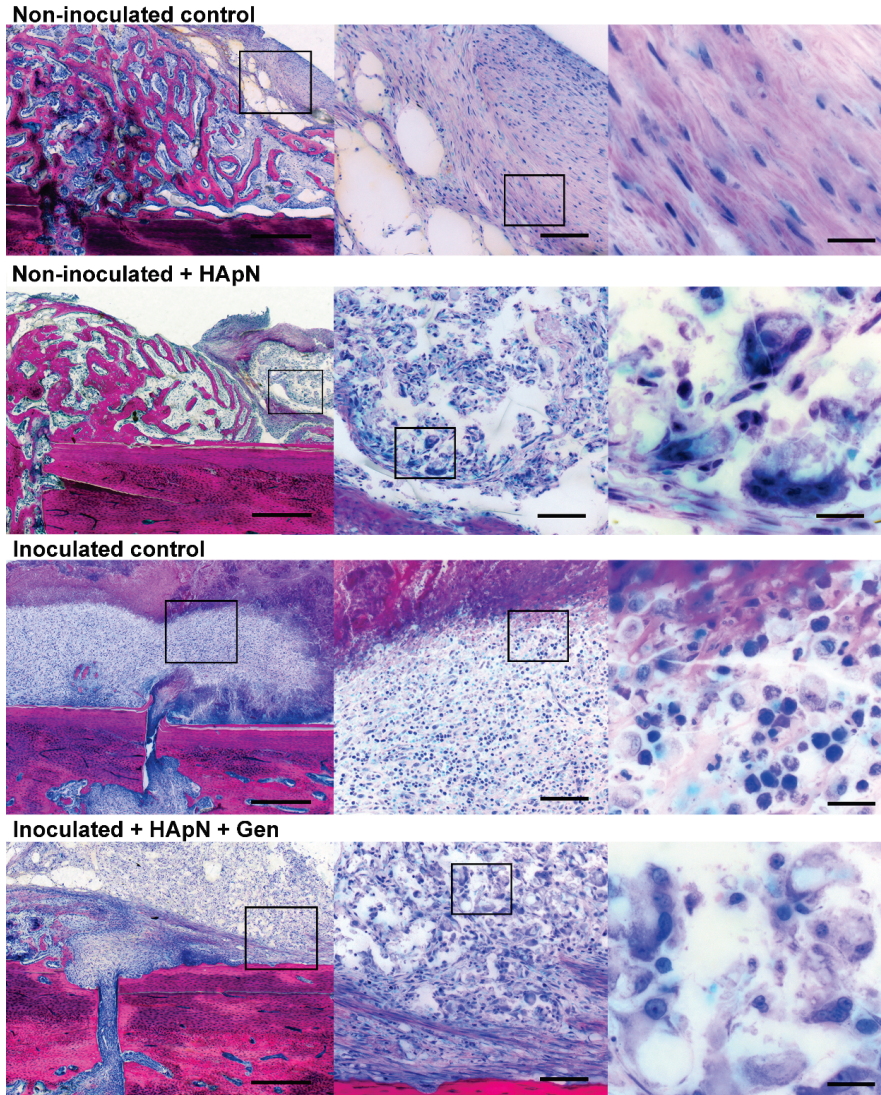
Figure 5.7: *Microphotographs of Giemsa-Eosin stained sections of the rabbit humeri at the cis site of the osteotomy site (left column, scale bar represents 500 µm), cellular details of the tissues below the plate (marked by black frames) are shown (middle column: scale bar represents 100 µm; right column: scale bar represents 20 µm). No callus formation is recorded in the inoculated control group which instead shows necrosis and a mixed-cellular inflammation. The two HApN-treated groups (with and without gentamicin) both show formation of multinucleated histiocytes with foamy bluish cytoplasm.*

The normalized stiffness value of the operated humeri was 67% of the stiffness of the contralateral humerus in the non-inoculated group without hydrogel, indicating that fracture healing has not yet achieved complete healing at 4 weeks, while complete healing was achieved 10 weeks post-operatively [29]. There was no statistical difference between the non-inoculated control and the non-inoculated + HApN group. Still, the bone stiffness was still lower for the latter, 49% of the stiffness of the contralateral humerus, in comparison to the rabbits that were only operated (Figure 5.6).

The humeri from rabbits that received the bacterial inoculum, whether they were treated with the HApN hydrogel with gentamicin or not, showed to have significantly lower stiffness than the two non-inoculated groups. The inoculated control group having a mean normalized stiffness of 0.30 and the inoculated + HApN + Gen group 0.33 of the stiffness of the contralateral humerus.

Discussion

The osteotomy-model including fracture fixation implant used in this *in vivo* study represents a realistic simulation of fracture healing after trauma, by incorporating all the factors that might influence bone healing or occurrence of an infection (i.e. antibiotics, bacteria, fracture and implant) [29]. In this model, the gentamicin-loaded HApN hydrogel successfully prevented infection during a 4 week post-operative period.

It is known that the created osteotomy in the presence of plating fixation heals in approximately 10 weeks [29, 33, 34]. In this study we used the model to assess the influence of a degradable ALB on bone healing, both under sterile and contaminated conditions at 4 weeks as the largest variations between the study groups were anticipated in this early bone healing phase. Both mechanical testing and histopathological evaluation of the operated humeri showed that the presence of the HApN hydrogel does not significantly influence bone healing in comparison to rabbits that only had surgery.

Four weeks upon surgery rabbit humeri that only underwent surgery (non-inoculated control group) partially healed and gained back 67% of the stiffness in comparison to

the non-operated contralateral humeri. In the histological sections this was observed as the formation of callus, both on the cis and trans site of the bone, as well as periost around the osteotomy.

The thermo-responsive HApN hydrogel was injected as a flowable sol with low viscoelastic moduli at room temperature, and upon heating above the lower critical solution temperature it forms a gel over the fracture fixation plate and all the contaminated tissues in the surgical site. The presence of the HApN hydrogel did not significantly affect bone healing in terms of relative stiffness. The relative stiffness of the humeri that received the HApN hydrogel was 49%, 4 weeks post-operatively, and not significantly reduced in comparison to humeri that only had surgery. In the histological sections callus formation was observed at both cis and trans site of the humerus, although the amount of callus formation was slightly decreased at the cis site.

Whereas in mammals degradation of HA usually takes 0.5 day up to several days depending on the anatomical location [35], conjugation of moieties to the HA dimer may alter its degradation behavior [36] and prolongs its presence *in vivo* [37] and the delivery of the entrapped drugs [38]. Indeed it is known that chemically crosslinked methacrylated hyaluronan hydrogels with differential *in vitro* enzymatic degradation profile modulate the formation of bone *in vivo* [39]. The HA formulation used in this *in vivo* study was rendered thermo-responsive by poly(*N*-isopropylacrylamide) (pN) grafts. Moderate chain length pN, as the 20 kDa graft used in this *in vivo* study does not decrease cell viability *in vitro* [40]. Presence of remnants of the HApN hydrogel, suggested by the occurrence of moderate amounts of mono- and multinucleated histiocytes where the hydrogel was applied, suggests ongoing and incomplete resorption of the HApN hydrogel 4 weeks post-operatively. The CRP values, WBC counts and also the final body weight change in the non-inoculated rabbits that received the HApN hydrogel were similar to the rabbits that only had surgery.

In the rabbits that received the *S. aureus* inoculum, but where no treatment was provided (inoculated control) no bone healing was observed. Implants and fibrous capsule samples from 9 out of the 10 rabbits were culture positive for *S. aureus*. Whereas in all the other study groups, new bone formation was observed around the

osteotomy, as well as in the S3 and S4 screw holes, no bone formation was observed in the humeri from the inoculated control rabbits. An inflammatory reaction to staphylococcal infection leads to disruption of the homeostatic balance of bone matrix production and resorption leading to bone loss [41, 42]. CRP values and WBC count were elevated in the rabbits that established a localized infection in the humerus.

Implant and fibrous capsule samples of rabbits that received the HApN hydrogel with gentamicin after inoculation were all culture negative. Also no bacteria were found in the Brown and Brenn stained images. CRP levels and WBC count were comparable to the values observed in the plasma of non-inoculated rabbits. Callus formation around the osteotomy was predominantly located at the trans site of the humerus in the rabbits that had received the HApN hydrogel with gentamicin. Large amounts of mono- and multinucleated histiocytes could be observed at the cis site of the humerus near the osteotomy suggesting ongoing HApN hydrogel clearance, similar to rabbits in the study group that only received the hydrogel. Osteoblast viability can be lowered by high concentrations of aminoglycosides when exposure takes place over an extended time *in vitro* [19, 20]. However, *in vivo* such an effect is not necessarily seen [43] and systemic gentamicin levels 3 hrs post-operatively in this study were low, as was intended by local application of the hydrogel, and were comparable to previously measured values [9].

Operated humeri from the rabbits that cleared the infection by the HApN hydrogel with gentamicin had a significant lower stiffness than the rabbits that only had surgery. Although these rabbits had a significantly larger amount of new callus formed at the trans site of the humerus as observed in infected rabbits, a limited amount of bone was formed at the site of the humerus where the HApN hydrogel with gentamicin had cleared the bacterial load. This limited callus formation at the cis site of the humerus concealed the difference in healing status between humeri from the gentamicin-loaded HApN treated and infected rabbits in the mechanical testing data.

As the bacterial load in this study was high (2×10^6 CFU), and much higher than the normal amount of contamination found in wounds, a large amount of dead bacteria might remain in the inoculated area. The presence of this dead bacteria, killed by the

gentamicin released from the HApN hydrogel might influence bone healing, as was shown in mice [44, 45]. In mice, this surface-associated material from *S. aureus* cell wall preparations stimulates the production of TNFα and IL-6, leading to increased bone resorption and osteoclast formation [44, 45].

However, on the long term fracture healing is anticipated as after the clearance of the high bacterial load with the gentamicin-loaded HApN hydrogel callus formation was observed which might make bridging of the osteotomy possible on the long term. A potential solution to speed-up the bone healing process could consist of the addition of osteopromotive factors [46-49].

Conclusion

In this *in vivo* fracture model including osteotomy, plate fixation and bacterial inoculum, the gentamicin-loaded HApN hydrogel was successful in preventing an infection. The HApN hydrogel itself did not evoke any systemic response as was shown by the absence of differences in hematological values between the rabbits that were only operated and the rabbits that were additionally injected with the HApN hydrogel. Bone formation is not significantly hindered by application of the HApN hydrogel itself as can be seen from the mechanical testing results, as well as from the evaluated stained humeri sections. However, there is a delay in bone healing which might likely be resolved at a later time point. In the group where rabbits got inoculated with *S. aureus* and which received the HApN hydrogel with gentamicin, additional factors such as the presence of dead bacteria might further slowdown bone healing. However, on the long term were the infected humeri showed lots of necrosis and a total absence of callus formation round the osteotomy and the S3 and S4 screw holes, it is to be expected that the humeri of the inoculated rabbits that got treated with the HApN hydrogel with gentamicin would heal. Thus, the thermo-responsive HApN hydrogel is an effective delivery system for prophylactic antibiotics which does not affect fracture healing itself, and will be evaluated in a large animal model in the future.

Acknowledgements

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Chapter 6

Preparation of gentamicin dioctyl sulfosuccinate loaded poly(trimethylene carbonate) matrices intended for the treatment of orthopedic infections

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Abstract

Infection is a common problem in trauma- and orthopaedic surgery. Antibiotic-loaded biomaterials are used locally to clear infections as an adjunct to systemic antibiotics. Gentamicin-sulphate (GEN-SULPH) is commonly used in antibiotic-loaded biomaterials, although it displays high water solubility resulting in quick diffusion from the carrier. The objective of this study was the preparation of a lipophilic derivative of gentamicin to reduce solubility and obtain a slower release. Subsequently, this lipophilic gentamicin was entrapped within poly(trimethylene carbonate) (PTMC) matrices. Hydrophobic ion-pairing was used to prepare lipophilic gentamicin (GEN-AOT). The susceptibility of *Staphylococcus aureus* NCTC 12973 and *Staphylococcus epidermidis* 103.1 for GEN-AOT was tested and the viability of fibroblasts upon exposure to GEN-AOT was assessed. GEN-AOT was then loaded into PTMC films. GEN-AOT was successfully prepared as confirmed by FTIR-spectroscopy. GEN-AOT was bactericidal for *S. epidermidis* and *S. aureus* at 0.5 µM and 8.5 µM, respectively. At 1.1 µM GEN-AOT no reduction in fibroblast viability was observed. At 11 µM the reduction was ~50%. PTMC discs loaded with GEN-AOT were prepared by compression molding. Therefore, it could be concluded that lipophilic GEN-AOT was at least as potent as GEN-SULPH. For *S. epidermidis* it was even more potent than GEN-SULPH. More than 50% fibroblast cell viability was maintained at bactericidal concentration for both bacterial strains.

Introduction

The incidence of infection seriously threatens modern-day advances in the field of orthopaedics and traumatology. Most infections related to implanted fracture fixation devices and orthopaedic implants are caused by Staphylococci [1,2]. Bacteria present in a biofilm on the surface of an implant, necrotic bone or living tissue can enter a state of reduced growth and become less susceptible to killing by growth-dependent antimicrobials [3]. Treatment of these infections is therefore difficult, and usually involves systemic administration of antibiotics over an extended time of approximately 6 weeks. Antibiotic-loaded biomaterials are used as an adjunct treatment for clearing these infections. Two types of antibiotic-loaded biomaterials are used in the clinic: Non-degradable poly(methyl methacrylate) (PMMA) blocks and

beads, which need to be removed after the treatment has finished [4] (these PMMA based materials have a poor antibiotic release profile [5]), and degradable biomaterials such as calcium phosphate cements [6] or collagen fleece [7]. The latter may be advantageous as they do not need removal. However, usually antibiotic release is not simultaneous with degradation [8,9]. Often this leads to a suboptimal antibiotic release profile and the presence of a biomaterial without antibiotic action.

Many antibiotic-loaded biomaterials used in the clinic are loaded with gentamicin [10]. However, the sulphate salt of gentamicin, gentamicin-sulphate (GEN-SULPH) is highly soluble, which results in fast diffusion from the carrier [9]. A less soluble salt, gentamicin crobefate, has been combined with collagen fleece, extending the release kinetics, but simultaneous resorption of the carrier and antibiotic release was not achieved [8].

Poly(trimethylene carbonate) (PTMC) as opposed to other biodegradable polymers synthesized by ring-opening polymerization (ROP), degrades by enzymatic surface erosion [11,12]. Other biodegradable polymers, like poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and poly(caprolactone) (PCL) and their copolymers degrade by bulk erosion into acidic degradation products. So, PTMC has the advantage of degrading in a more controlled fashion which could be beneficial for the release profile of antibiotics from the material. PTMC based carriers will gradually reduce their thickness when degrading, so the diffusion pathway for antibiotics entrapped closer to the core of the carrier will be become shorter, leading to a more gradual release as compared to release from a bulk degrading matrix. Furthermore, the degradation products of PTMC are non-acidic (predominantly cyclic TMC monomer, cyclic and linear oligomers of TMC and 1,3-propanediol) [13], so these will not interfere with aminoglycoside activity and bone healing, which are impaired at low pH.

Therefore, it was hypothesized that a delivery system based on PTMC, and entrapping a lipophilic gentamicin complex, could be advantageous for a sustained antibiotic release and would be able to achieve this in the absence of negative stimuli that impair antibiotic action and bone healing.

Hence, a lipophilic complex of gentamicin was prepared by hydrophobic ion-pairing with dioctyl sodium sulfosuccinate (tradename Aerosol OT (AOT)) and characterized by means of Fourier Transform Infrared Spectroscopy (FTIR). Minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) for relevant bacterial species, as well as cell viability upon exposure, were assessed for this lipophilic gentamicin. High molecular weight linear PTMC was prepared by ROP and characterized by means of gel permeation chromatography (GPC). Finally, PTMC films loaded with GEN-SULPH and GEN-AOT were prepared by compression molding, demonstrating the ability to produce PTMC membranes homogeneously loaded with lipophilic gentamicin for a sustained and controlled antibiotic release.

Experimental

Materials:

TMC monomer was obtained from Huizhou Foryou Medical Devices (China). Stannous octoate (Sn(Oct)$_2$)$_,$ dioctyl sodium sulfosuccinate, gentamicin sulphate salt and sodium chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium acetate, potassium chloride, calcium chloride dehydrate and tyrode buffer saline solution (TBSS) were purchased from Fluka (Buchs, Switzerland). Dimethyl sulfoxide (DMSO) (≥99.5%) was purchased from Carl Roth (Karlsruhe, Germany). *Staphylococcus Aureus (S. aureus)* NCTC 12973 was purchased from the National Collection of Type Cultures (NCTC). *Staphylococcus epidermidis (S. epidermidis)* 103.1 was obtained as clinical isolate from a patient with an infected plate osteosynthesis at the BGU Murnau, Germany. Tryptone soy agar (casein soy bean digest agar, CM0131) and Mueller Hinton (MH) broth (CM0405, LOT# 970220) were purchased from OXOID (Basel, Switzerland). Cation adjusted Mueller Hinton (CAMH) broth was prepared by adding Ca^{2+} and Mg^{2+} to the MH broth to obtain a final cation content of 20-25 mg/L Ca^{2+} and 10-12.5 mg/L Mg^{2+}. The cation containing solutions were sterile filtered before addition to the broth. Dulbecco's modified Eagles medium (DMEM), L-glutamine, sodium pyruvate, foetal calf serum (FCS) and Trypsin/Ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco Life

Technologies (Waltham, MA, USA). Cell titer blue® was purchased from Promega (Madison, WI, USA).

Preparation of lipophilic gentamicin-dioctyl sulfosuccinate (GEN-AOT)

AOT was used for hydrophobic ion pairing (HIP) with GEN-SULPH. Equal volumes of GEN-SULPH in buffer (10 mM sodium acetate, KCl and $CaCl_2$ (pH 5) (0.40% w/v)) and AOT in CH_2Cl_2 (1.25% w/v) were mixed by vigorous stirring for 3h and left for 0.5h to separate the 2 phases. GEN-AOT was isolated from CH_2Cl_2 by evaporation of the solvent. Both structures for GEN-SULPH and GEN-AOT are depicted in figure 6.1.
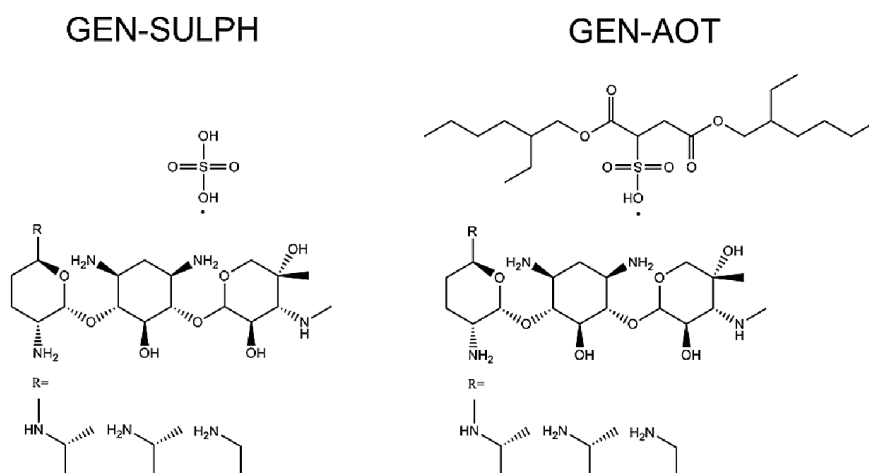

Figure 6.1: Structure of gentamicin sulfate (GEN-SULPH) and gentamicin AOT (GEN-AOT)

Characterization of gentamicin-dioctyl sulfosuccinate (GEN-AOT)

The successful hydrophobic ion pairing of GEN-SULPH with AOT was confirmed by Fourier Transform Infrared Spectroscopy (FTIR) on a Bruker Tensor 27

spectrophotometer equipped with a single reflection diamond attenuated total reflection (ATR) accessory. Spectra (single measurements with 128 repeated scans per IR spectrum) were recorded for both starting compounds, GEN-SULPH and AOT, and for the GEN-AOT isolated from the CH_2Cl_2 layer after the HIP process. Additional to the previous stated compounds, also the spectrum for a physical mixture of GEN-SULPH and AOT was recorded.

Antimicrobial susceptibility testing

2 clinical relevant bacterial strains were selected for susceptibility testing of GEN-AOT. The used strains were: *S. aureus* NCTC 12973 (*SA*) and *S. epidermidis* 103.1 (*SE*). Antibiotic solutions were prepared in CAMH broth by doubling dilution in a concentration range from 271µM – 0.06 µM. The minimal inhibitory concentrations (MIC), the concentration for which bacterial growth is inhibited and the minimal bactericidal concentrations (MBC), the concentration for which bacteria are killed, were determined according CLSI guidelines M7-A9 (2012) [14]. All concentrations of the antibiotic solutions were measured in triplicate, as well as the positive control (no antibiotic + inoculum), DMSO control (10% v/v DMSO in broth + inoculum) and the sterility control (no antibiotic + no inoculum). For the susceptibility testing, GEN-SULPH and AOT were solubilized and diluted directly in CAMH broth. In order to solubilize GEN-AOT and to prepare the dilution range, GEN-AOT was first dissolved in DMSO at 10x the final concentration, after which 10 µl of this solution was added to 90µl CAMH broth in the wells of a 96-wells plate. Solubilisation of GEN-AOT in 10% v/v DMSO did not impair viability of the bacterial strains (data not shown). Although, an end concentration of 5% v/v DMSO in the wells did not inhibit bacterial growth, it cannot be excluded that there is a synergistic effect between GEN-AOT and DMSO [15]. Finally, 100 µl inoculum ($5x10^5$ CFU/ml) of the respective bacterial strain was added to every well, to reach a final volume of 200 µl/ well.

Viability testing for fibroblast cell line

The hTERT fibroblast BJ-1 cell line was used for viability testing upon exposure to the antibiotics following the basic rules as prescribed in ISO-10993-5 [16]. Viability was assessed after 24h and 72h of exposure of the cells to the antibiotics. An

antibiotic concentration range from 0.53 µM – 2.12 mM was prepared for viability testing. After 24h and 72h respectively, cell media containing the antibiotics was removed from the wells of the 96-wells plate and the cells were incubated for 2.5h with cell titer blue after which the fluorescence intensity (FI) was measured ($\lambda_{excitation}$ = 560nm, $\lambda_{emission}$ = 590 nm). FI was measured with a Perkin Elmer Victor 3 1420-012 multi-label microplate reader. All sample concentrations, including the controls, were measured in quadruplicate. Viability was expressed as relative percentage with respect to the untreated control. Viability = (FI_{sample}-$FI_{negative\ control}$)/ ($FI_{positive\ control}$-$FI_{negative\ control}$) x 100.

Polymer synthesis and characterization:

High molecular weight (HMW) PTMC was synthesized by ROP of trimethylene carbonate in the presence stannous octoate ($Sn(Oct)_2$) (2×10^{-4} mol $Sn(Oct)_2$ per mol TMC) as catalyst (Figure 6.2). No initiator was added to obtain a high molecular weight polymer. Polymerization was performed in the melt in dry vacuum sealed silanized glass ampoules for 48h at 130°C. Number average molecular weight (M_n) and intrinsic viscosity (IV) were determined by gel permeation chromatography (GPC, Viscotek (US)). Chloroform was used as eluent at a flow rate of 1.0 ml/min. The GPC system was equipped with ViscoGEL I-guard-0478, ViscoGEL I-MBHMW-3078 and ViscoGEL I-MBLMW-3078 columns in series and a TDA 302 triple detector array with refractometer-, viscometer-, and light scattering detectors to determine the absolute molecular weights.
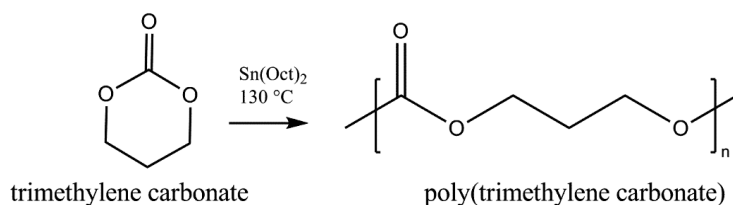

Figure 6.2: *Ring opening polymerization of trimethylene carbonate*

Preparation of GEN-AOT loaded PTMC discs

PTMC was dissolved in CH_2Cl_2 at a concentration of 4% w/v. Then either no antibiotic or 10% w/w GEN-SULPH or 10% w/w GEN-AOT was added to this solution. GEN-SULPH was dispersed through the PTMC solution by ultrasonication for 5 min. Since GEN-AOT is soluble in the PTMC solution it was not sonicated. Subsequently, the GEN-SULPH or GEN-AOT containing PTMC solutions were stirred for 24h. The GEN-SULPH containing solutions were then precipitated in diethyl ether and the GEN-AOT containing solutions were poured in petri-dishes after which the CH_2Cl_2 was allowed to evaporate for 1 week. Finally, PTMC, PTMC with 10% w/w GEN-SULPH and PTMC with 10% w/w GEN-AOT were compression moulded in stainless steel molds at 70°C and 300kN force with a laboratory press (Fonteijne THB008, The Netherlands). The antibiotic-containing polymer films were cooled to room temperature using cold water. 500 µm thick films were obtained, and ø 8 mm discs were punched out of these films.

Characterization of GEN-AOT loaded PTMC discs

Differential Scanning Calorimetry was performed on a Pyris DSC-1 Perkin-Elmer instrument calibrated with indium. PTMC, PTMC-GEN-SULPH and PTMC-GEN-AOT samples were weighed in hermetically closed aluminium pans. Measurements performed in triplicate, were carried out under a dry nitrogen atmosphere at a flow rate of 20 mL/min. Each measurement was run heating the sample from -30°C to 100°C. The sample was held for 1 min at 100°C and cooled to the initial temperature. Heating and cooling ramps were run at a rate of 5°C/min.

Optical microscopy

Optical microscopy was performed to assess the antibiotic distribution throughout the GEN-SULPH and GEN-AOT loaded PTMC films. Images of the films were captured using a Zeiss CCD camera (Axiocam HRc, Jena, Germany) attached to an optical microscope in diffuse illumination mode with a macrolens (Leica, Macrofluo, Wetzlar, Germany) using Zeiss Axiovision Vs40 (v4.6.3.0) image acquisition software. Gray values (GVs) of circular areas of the specimens with 3200 µm radius were recorded

and analysed. Average GVs and standard deviation were calculated for the PTMC, PTMC-GEN-SULPH and PTMC-GEN-AOT films for this area.

Results

The composition of the product of the HIP process of GEN-SULPH and AOT was determined by FTIR. The FTIR spectrum of GEN-SULPH, AOT and the product of the HIP (GEN-AOT) can be found in Figure 6.3. An absorption band at 1734 cm^{-1} (Figure 6.3) in the FTIR spectrum can be both observed for AOT and GEN-AOT. This band can be attributed to the stretching of the C=O groups of the ester of the AOT molecule. The absorption band between 1620 cm^{-1} and 1610 cm^{-1} (Figure 6.3) present in both GEN-SULPH and GEN-AOT can be attributed to the primary amine groups present on the gentamicin molecule (Figure 6.3). Vibrational bands attributed solely to GEN-SULPH and AOT can be identified in the spectrum of GEN-AOT. As GEN-SULPH is not soluble in CH_2Cl_2, these features suggest the successful ion-paired GEN-AOT complex formation. Furthermore, the absorption wavenumber for the stretching vibration for the amine groups present in the gentamicin-sulphate complex is located at 1620 cm^{-1}. The same stretching vibration in the physical mixture of GEN-SULPH and AOT was observed. For the amine groups present in the GEN-AOT complex this vibrational band is located at 1608 cm^{-1}.
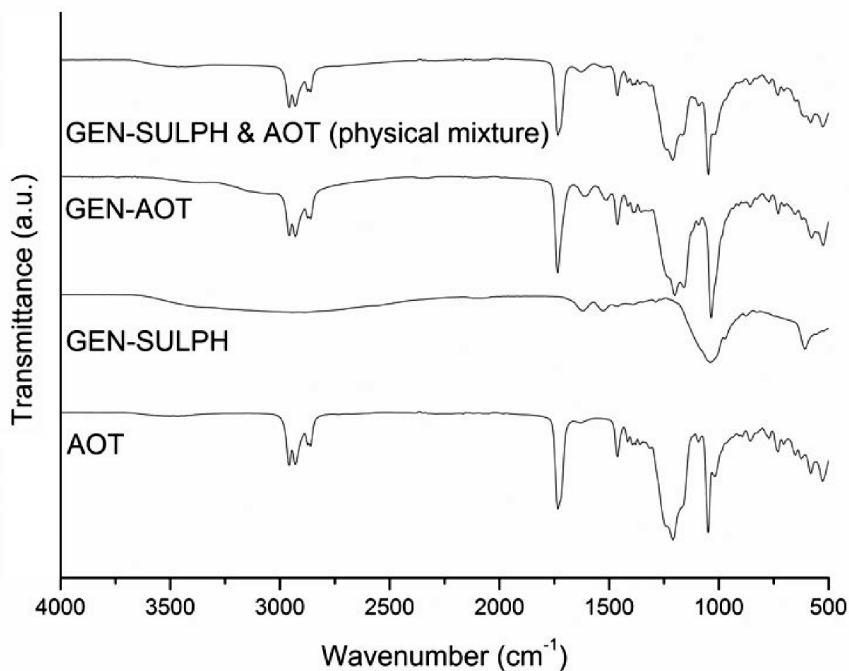
Figure 6.3: FTIR spectra of GEN-SULPH, AOT, physical mixture of GEN-SULPH and AOT and GEN-AOT.

The susceptibility of *SA* and *SE* for GEN-AOT and GEN-SULPH as represented in MIC and MBC values can be found in Table 6.1.

Table 6.1: Susceptibility of S. aureus and S.epidermidis for GEN-SULPH, GEN-AOT and AOT.

	Gram-positive			
	S. aureus NCTC 12973		*S. epidermidis* 103.1	
	MIC (µM)	MBC (µM)	MIC (µM)	MBC (µM)
GEN-SULPH	2.1	8.5	0.5	4.2
GEN-AOT	2.1	8.5	0.5	0.5
AOT	544	544	544	>544

As can be seen from Table 6.1, GEN-AOT was at least as effective as GEN-SULPH in inhibiting and killing both *Staphylococcal* species. Towards *S. epidermidis* 103.1 it is more bactericidal than GEN-SULPH. AOT itself has no antimicrobial effect as can be seen from the high MIC and MBC values.

The cell viability data reported in Figure 6.4 indicated that GEN-SULPH did not impair fibroblast viability. Even at high concentration of 2.1×10^3 µM GEN-SULPH, 75% fibroblast cell viability was observed. For GEN-AOT, concentrations up to 1.1 µM did not negatively influence fibroblast cell viability. At 11 µM GEN-AOT fibroblast cell viability was reduced to ~50%. For free AOT, a sharp decrease in viability is seen when the concentration in the medium is increased from 11×10^2 µM to 21×10^2. This decrease in fibroblast cell viability appears at a higher concentration than for GEN-AOT. HMW PTMC with M_n= 272kg/mol and IV= 2.3 was obtained, which was used for preparation of the antibiotic loaded PTMC discs. PTMC discs, PTMC discs with GEN-SULPH and PTMC discs with GEN-AOT prepared by compression molding were prepared (Figure 6.5). Both the PTMC discs and PTMC discs with GEN-AOT were transparent while the PTMC discs containing GEN-SULPH were opaque indicating a dispersion of the salt in the polymer matrix.
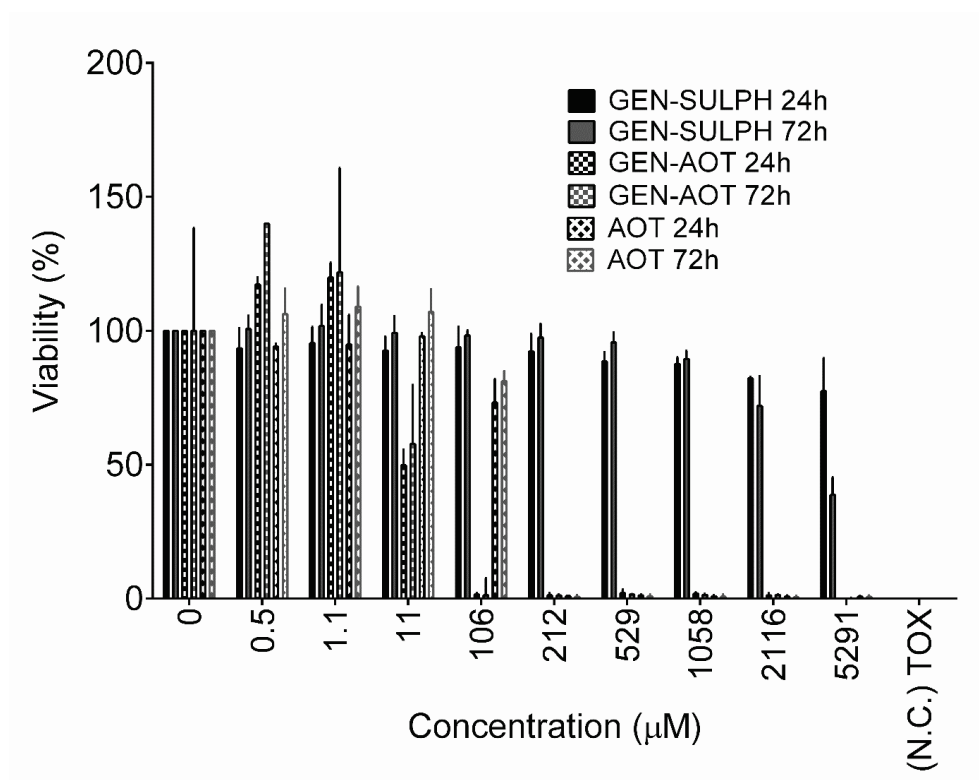
Figure 6.4: *hTERT fibroblast cell viability upon 24 h and 72 h of exposure to GEN-SULPH and GEN-AOT (n=4 ± SD, and N.C. is negative control).*

Figure 6.5: *ø 8 mm PTMC discs (left 4), PTMC discs with GEN-SULPH (middle 4) and PTMC discs with GEN-AOT (right 4) prepared by compression molding.*

Table 6.2: *Glass transition temperature (T_g) for PTMC, PTMC-GEN-SULPH and PTMC-GEN-AOT samples.*

	Peak (T_g)	
Specimen	Heating ramp (T(°C))	Cooling ramp (T(°C))
Pure PTMC	-14.2	-16.1
PTMC + GEN-SULPH	-12.5	-13.7
PTMC + GEN-AOT	-12.4	-14.5

Figure 6.6: *Gray values obtained for PTMC discs, PTMC discs with GEN-SULPH and PTMC discs with GEN-AOT by optical microscopy in diffuse illumination mode.*
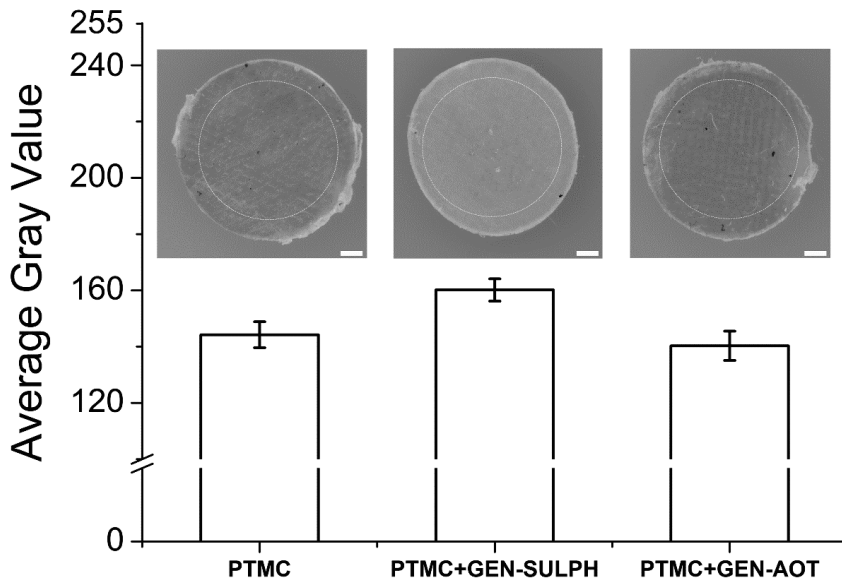
The glass transition temperature (T_g) for the PTMC, PTMC-GEN-SULPH and PTMC-GEN-AOT discs can be found in table 6.2. The GEN-SULPH and GEN-AOT loaded PTMC samples show a slight decrease of the glass transition temperature, which could be explained by the presence of a relatively high concentration of gentamicin

(10% w/w). This decrease is due to a possible enhanced interaction between the PTMC chains, established in the presence of the gentamicin.

The homogeneous dispersion of GEN-SULPH and GEN-AOT through the PTMC matrix was assessed by optical microscopy. Average gray values were recorded for all the specimens (Figure 6.6). A significant higher gray value (more reflection due to scattering of larger antibiotic particles) was observed for the PTMC-GEN-SULPH specimens. For all of the films, the standard deviation of the GVs was low, indicating a homogenous dispersion/dissolution of the antibiotic through the films.

Discussion and conclusion

GEN-SULPH and GEN-AOT inhibit *S. aureus* and *S. epidermidis* and kill *S. aureus* at the same molar concentration. GEN-AOT kills *S. epidermidis* even at lower molar concentration than GEN-SULPH. This indicates that the ion pairing with dioctyl sodium sulfosuccinate does not affect the potency of the antibiotic. Even more, in the case of *S. epidermidis* GEN-AOT seems to be more effective than the sulfate salt of gentamicin; potentially it is more effective in destabilizing the bacterial membrane. Besides the susceptibility of relevant bacterial species for the hydrophobic modified gentamicin, its toxicity towards eukaryotic cells should be taken into account. The modified gentamicin should kill relevant bacterial species, but at the same time it should not impair cell viability. GEN-SULPH has limited effect on fibroblast viability. Only at very high concentration (2.1×10^3 µM) viability is reduced to ~75% compared to the untreated control. AOT has an effect on fibroblast cell viability when the concentration is increased from 11×10^2 µM to 21×10^2 µM. In the presence of 11 µM GEN-AOT, cell viability was reduced to ~50%. Reduction of fibroblast cell viability is observed at lower concentration for GEN-AOT than for AOT. Possibly, the effect of AOT on cell viability is combined with the lipophilic character of GEN-AOT which makes it possible for the GEN-AOT to cross the cell membrane and have a stronger effect on cell viability than AOT alone. However, GEN-AOT killed *S. epidermidis* at a molar concentration of 0.5 µM and killed *S. aureus* at 8.5 µM, which is below the concentration for which cell viability was reduced with 50%. HMW PTMC was successfully synthesized and was used for the preparation of GEN-SULPH and

GEN-AOT loaded discs. In the PTMC the GEN-AOT was well solubilized, while the GEN-SULPH was dispersed through the polymeric matrix**.**

Acknowledgements

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Chapter 7

Polymer microparticles with low dispersity loaded with gentamicin sulfate and hydrophobic gentamicin

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Abstract

Extended local release of antibiotics is required for the treatment of infection. Biodegradable polymeric microparticles can extend the release of hydrophilic gentamicin sulfate, therefore it was incorporated in microparticles. Furthermore, the hydrophilicity of gentamicin was also lowered by a hydrophobic ion pairing process to potentially further extend the release, as well as to be able to incorporate the antibiotic in the same phase as the polymer during the encapsulation process. Membrane emulsification yielded monodisperse gentamicin AOT loaded PDLLA and PTMC microparticles when the polymer and antibiotic were dissolved in the same solvent as dispersed phase in the cross flow membrane emulsification process. These monodisperse antibiotic loaded particles could be interesting for the sustained controlled release of antibiotics.

Introduction

The treatment of bone infections involves systemic administration of antibiotics over an extended time frame, usually a 4-6 weeks period. It is complemented by the application of ALBs to increase further the local concentration of antibiotic without reaching toxic systemic concentrations and clear the affected tissues from contamination [1, 2]. The high hydrophilicity of several antibiotics, such as gentamicin sulfate commonly used in ALBs limits the time of antibiotic release when encapsulated in injectable hydrophilic polymeric networks, such as hydrogels or nano- or microgels as the antibiotic readily diffuses out. Therefore, these systems usually allow for short term release of antibiotics, suitable for infection prophylaxis, but not for treatment purposes. In order to extend the release and keep the advantages of an injectable system, a so called 'plum pudding' composite hydrogel can be prepared by the incorporation of the antibiotic in hydrophobic biodegradable polymer micro- or nanoparticles dispersed in the hydrogel network [3]. Entrapment of drugs or antibiotics is commonly performed by emulsion solvent evaporation or emulsion solvent extraction processes [7-10]. However, applying these conventional techniques leads to a wide size distribution of the microparticles.

For example, poly(ε-caprolactone) (PCL) microparticles loaded with gentamicin sulfate have been prepared by an emulsion solvent evaporation technique. PCL microparticles produced with a range of initial PCL concentration in the oil phase showed an increase size and large size distribution (Figure 7.1).
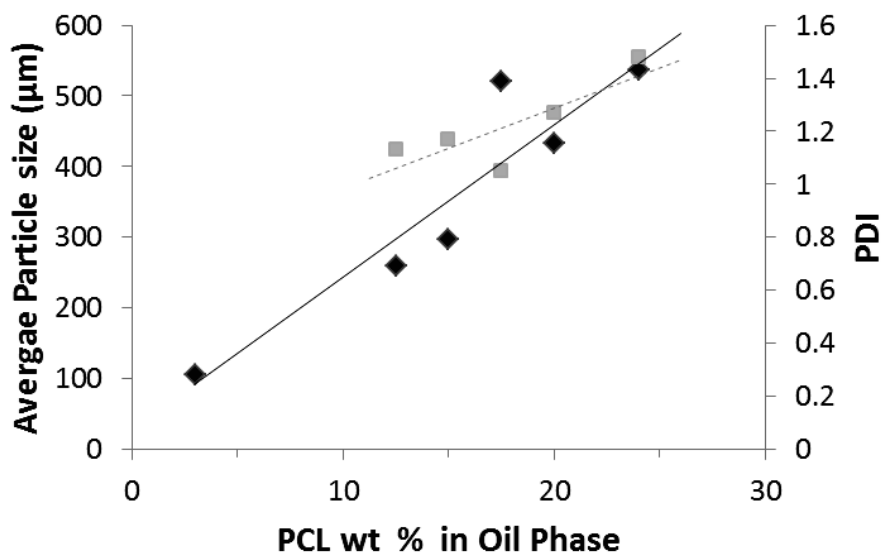

Figure 7.1: *Plot of the average microparticle size and polydispersity (PDI) as a function of PCL (Mn=80.000 g/mol (GPC)) concentration in the oil phase of a single emulsion. PCL was dissolved in 9 ml of dichloromethane (DCM). Subsequently, the emulsion was injected at a rate of 0.5 ml/min in 250 ml PBS with 0.5% w/w PVA, under magnetic stirring. After addition of the total volume of emulsion, stirring was continued for 1 hr, during which the DCM was allowed to evaporate and the particles could harden out. Finally, particles were washed with ultrapure water and spun down (3x), collected and lyophilized. Data point at 3 wt % PCL is from [4].*

Importantly, the gentamicin sulfate can only be dispersed at low concentration, circa 0.3% w/w in the PCL/DCM solution. A gentamicin sulfate dispersion in the PCL microparticles is obtained with relatively low encapsulation efficiency of 39.5 % [4]. The relatively high PDI will together with the heterogeneous dispersion of gentamicin sulfate, result in different release rates of the incorporated antibiotic, with a faster release from smaller particles due to the larger surface area of the particles and the shorter diffusion pathways of the antibiotic from the particles [5]. These lead to a

release profile consisting in a burst release followed by a sustain release of 30 days [4]. Improved dispersion of gentamicin can potentially be achieved using a water in oil in water emulsion process [6].

In order to better control the size of, and hence the release from the microparticles, efforts have been made to develop techniques which yield monodisperse microparticles such as obtained by membrane, microchannel and microfluidic emulsification devices [5, 7, 8]. In addition, increasing the availability of antibiotic over time can also be achieved by hydrophobic ion pairing, extending its availability via a reduced water solubility of the antibiotic [9, 10]. Besides extending the release, the hydrophobic modification also simplifies the microencapsulation process of the antibiotic in the hydrophobic polymeric matrix, as only a single emulsion has to be prepared [9], increases drug encapsulation efficiency and drug loading and minimizes the burst release [11].

Here we first describe the preparation of a hydrophobic gentamicin dioctyl sulfosuccinate. Then, poly(D,L-lactide) (PDLLA) and poly(trimethyl carbonate) (PTMC) microparticles with low size dispersion using a microfluidic emulsification device is reported. The degradation rate of PCL was deemed too slow, up to years *in vivo* and therefore was not assessed further [12]. The influence of the gentamicin sulfate added via a double water/oil/water emulsion process and the hydrophobic salt of gentamicin, gentamicin dioctyl sulfosuccinate, solubilised directly in the PDLLA and the PTMC oil phase, on the produced microspheres size and PDI is finally reported.

Experimental

Materials

Stannous octoate (92.5%-100%), gentamicin sulfate (potency ≥590 µg gentamicin base per mg), dioctyl sodium sulfosuccinate (AOT) and poly(vinyl alcohol) (fully hydrolysed), sucrose (≥99.5% (GC)) were obtained from Sigma-aldrich (St. Louis, MO, USA). D,L-lactide was purchased from Purac Biochem (Gorinchem, The Netherlands). Trimethylene carbonate was a gift from Huizhou Foryou Medical Devices (Huizhou, China). Sodium acetate, potassium chloride and calcium chloride dehydrate were purchased from Fluka (Buchs, Switzerland). Dichloromethane (DCM) was purchased from Carl Roth (Karlsruhe, Germany).

Synthesis and characterization of polymers for encapsulation

Linear poly(trimethylene carbonate) (PTMC) and poly(D,L-lactide) (PDLLA) were prepared by ring opening polymerization of the corresponding monomers under argon at 130°C and using stannous octoate as catalyst. The obtained polymers were purified by dissolution in chloroform and subsequent precipitation in ethanol. Finally, the polymers were dried at RT under vacuum. Number average- and weight average molecular weights (M_N and M_W) and polydispersity (M_W/M_N) of the polymers were determined by gel permeation chromatography (GPC) (Viscotek, USA) using chloroform as the eluent at a flow rate of 1.0 ml/min. The GPC setup was equipped with a series of ViscoGEL columns and a TDA 302 Triple Detector Array including refractometer-, viscometer- and light scattering detectors for the determination of absolute molecular weights. The obtained molecular weights, were Mn = 274 kg/mol with a PDI value of 1.35 for PTMC and Mn = 187 kg/mol with a PDI value of 1.38 for PDLLA.

Preparation of hydrophobic modified gentamicin

Dioctyl sodium sulfosuccinate (AOT) was used for the hydrophobic ion pairing with gentamicin sulfate. Equal volumes of gentamicin sulfate in buffer ((10 mM sodium acetate, KCl and $CaCl_2$ (pH 5) (0.40% w/v)) and AOT in DCM (1.25% w/v) were mixed by vigorously stirring for 3 h and left for 0.5 h to separate the two phases. Hydrophobic modified gentamicin (gentamicin AOT) was isolated from the DCM fraction by evaporation of the solvent. Fourier Transform Infrared Spectroscopy (FTIR) was performed on a Bruker Tensor 27 spectrophotometer equipped with a single reflection diamond attenuated total reflection (ATR) set-up. Spectra were recorded for gentamicin sulfate, AOT and the gentamicin AOT isolated from the DCM layer, as a control the spectrum for a physical mixture of gentamicin sulfate and AOT was recorded.

Preparation of antibiotic loaded microparticles

For the preparation of monodisperse antibiotic loaded microparticles a membrane emulsification technology (microsieve™) of Nanomi monosphere technology (Oldenzaal, The Netherlands) was applied. Shortly, a 1 % w/w PTMC or a 1 % w/w PDLLA solution in DCM were prepared and given into the feed as the dispersed phase. The dispersed phase was injected through the microsieve™ membrane at a flowrate of 4 ml/h by a syringe pump. The continuous phase consisting of ultrapure water with 2% w/w of PVA, was flushed through the collection compartment below the membrane at a flowrate of 63 ml/h by a peristaltic pump (Figure 7.2).
In order to prepare hydrophobic antibiotic loaded microparticles, 20% w/w gentamicin AOT with respect to the polymer content was dissolved into the DCM solution containing the polymer before injection through the membrane.

Gentamicin-sulfate loaded microparticles were prepared by creating a first emulsion prior to membrane emulsification. By mixing a small volume of gentamicin-sulfate dissolved in ultrapure water in a larger volume of polymer dissolved in DCM by ultrasonication with an ultrasonic probe.
Finally, microparticles were collected in a beaker glass and allowed to harden out during mild stirring. Finally, the collected dispersions containing the hardened microparticles were freeze dried in the presence of 5% sucrose, as a cryoprotectant.
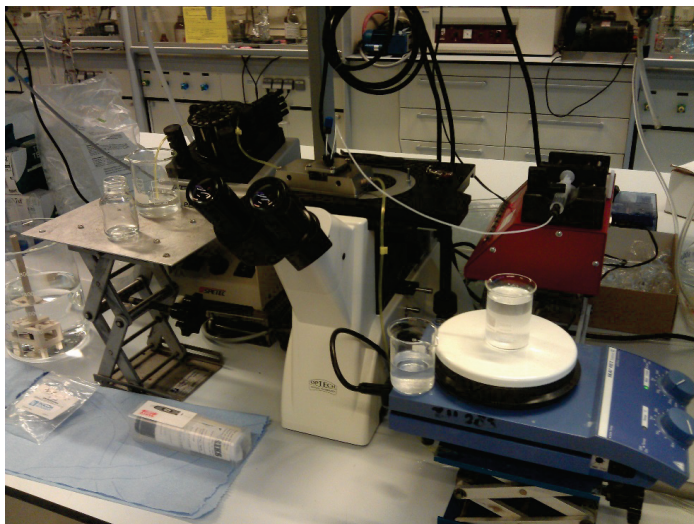
Figure 7.2: *General setup for microparticles production with a membrane emulsification device. The dispersed phase is injected through the microsieve membrane by an injection pump and the continuous phase is flow through the channel below the membrane by a peristaltic pump.*

Characterization of antibiotic loaded microparticles

Microparticles were imaged using a light microscope (Nikon Eclipse Fluorescence Microscope). Microparticle sizes were determined with ImageJ software, a public domain, Java-based image processing program developed by the National Institutes of Health (Bethesda, MD, USA). The average size and size distribution of the microparticles prepared by the emulsion solvent evaporation method was determined by measuring 10 spheres each time for the different concentration of PCL used. Finally, PDLLA and PTMC microparticle sizes obtained by membrane emulsification were determined by measuring 100 particles for each of the applied conditions. The mean diameters (number mean (D_n), volume mean (D_v) and polydispersity (PDI)) of the microparticles were calculated according to the following equations:

$$D_n = \frac{\sum_{i=1}^{k} n_i D_i}{\sum_{i=1}^{k} n_i} \qquad D_v = \frac{\sum_{i=1}^{k} n_i D_i^4}{\sum_{i=1}^{k} n_i D_i^3} \qquad PDI = \frac{D_v}{D_n}$$

Results

Preparation of hydrophobic modified gentamicin

The successful hydrophobic ion pairing was confirmed by FTIR measurements. An absorption band at 1734 cm^{-1} was observed for both AOT and gentamicin AOT. This band belongs to the energy of stretching the C=O group of the ester of the AOT molecule. Between 1620 cm^{-1} and 1610 cm^{-1} an absorption band is observed associated with the primary amine groups present both in gentamicin sulfate and gentamicin AOT. In the spectrum vibrational bands solely associated with gentamicin sulfate or AOT could be identified in the spectrum of gentamicin AOT. Since gentamicin sulfate is not soluble in DCM, these had to derive from the ion paired complex. Furthermore, a shift in absorption wavenumber for the stretching of the amine group present on the gentamicin molecule has been observed from 1620 cm^{-1} for gentamicin sulfate to 1608 cm^{-1} for gentamicin AOT.

Polymeric microparticles fabrication using a membrane emulsification method

PDLLA particles were prepared with low PDI by the membrane emulsification method. Incorporating hydrophobic modified gentamicin in the same phase as the polymer yields smaller particle sizes. Encapsulating gentamicin sulfate in another water phase results in bigger particles again, as well as increase in polydispersity. PTMC particles were prepared, incorporating gentamicin AOT resulted in a drop in particle size as well as PDI. Again, incorporation of gentamicin sulfate increase microparticle size (Table 7.2). Gentamicin AOT loaded monodisperse microparticles are shown in figure 7.3.

Table 7.2: *Microparticle size and polydispersity of microparticles prepared by microsieve membrane emulsification*

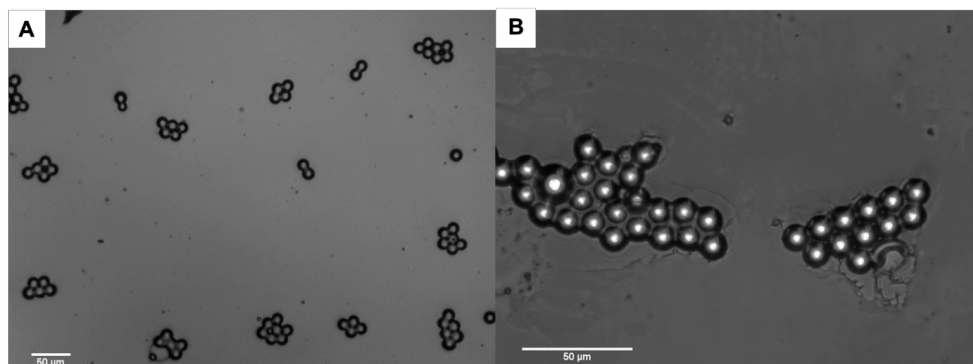| Microparticle composition	D_n (µm)	D_W (µm)	PDI
PDLLA	20	21	1.06
PDLLA + Gentamicin AOT	11	11	1.04
PDLLA + Gentamicin sulfate	20	31	1.52
PTMC	40	62	1.56
PTMC + Gentamicin AOT	11	12	1.06
PTMC + Gentamicin sulfate	20	24	1.24

Figure 7.3: *Gentamicin AOT loaded PDDLA microparticles (A) and gentamicin AOT loaded PTMC microparticles (B) were prepared with low dispersity (PDI < 1.1).*

Discussion

Generally, the size distribution for microparticles obtained by the classical emulsion solvent evaporation method is broad. By increasing the polymer concentration, and hence the viscosity of the oil phase, larger particles are obtained. Wide size distributions limits the predictability of the release, as the surface area of the particles and the diffusion pathway of the antibiotic from the particle differs between microparticles with different sizes. Furthermore, antibiotic particles dispersion in the polymeric oil phase leads to much larger diameter than the average microparticle size and might cloak injection needles when trying to decrease particle size. Membrane emulsification allowed for the preparation of monodisperse PDLLA and PTMC microparticles. However, when a first water phase containing the gentamicin sulfate was mixed with the oil phase to obtain gentamicin-sulfate loaded microparticles higher PDI values were obtained independently of the polymer used. The reason for the larger PDI for the gentamicin-sulfate containing microparticles could be an improper size of the emulsion droplets of the first emulsion flowing through the pores of the membrane, leading to a variety of particle size and shapes. Incorporation and solubilisation of gentamicin AOT directly in the oil phase creates significantly smaller particles with conserved low PDI, in comparison to the polymers only microparticles. This can be hypothetically attributed to a decrease in the viscosity of the polymer solution loaded with gentamicin AOT in the dispersed phase.

Conclusion

Preparation of monodisperse PDLLA and PTMC microparticles was achieved by membrane emulsification. By hydrophobic modification of water soluble gentamicin sulfate using ion pairing, the antibiotic was soluble in the oil phase (DCM) of the emulsion in which also the polymer was dissolved. By this method monodisperse gentamicin loaded microparticles of around 10 microns diameter were obtained. These microparticles are suitable for the development of an injectable controlled release antibiotic formulation. Further investigations will consist in assessing the antibiotic loading efficiency, the antibiotic release kinetic from the microparticles *in vitro* and antimicrobial activity over period of time relevant for the treatment of infection.

Acknowledgements

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Rev. 2009;38:3484-504.

Chapter 8

Future perspectives

Conclusions and Future perspectives

Millions of fracture fixation devices are implanted annually, and their number is expected to grow further in the future. Due to the fact that implant-associated infection has emerged as one of the most challenging complications after osteosynthesis, research in the field of antibiotic loaded biomaterials (ALBs) aimed at prophylaxis or treatment of these infections has expended tremendously over the last decades.

The local delivery of antibiotics circumvents the issue of systemic toxicity and makes it possible to deliver high concentrations of antibiotics at vascular comprised regions. Furthermore, the short half-life of systemic administered antibiotics can be improved by incorporating the antibiotic into a biomaterial. Despite the recent advances in ALB delivery systems, numerous opportunities for improvement present themselves.

ALBs for infection prophylaxis

Antimicrobials beyond gentamicin-sulfate

ALBs should be able to provide protection under complex circumstances specific for traumatic wounds such as: high bacterial contamination of unknown origin (Gram-positive, Gram-negative, multi-species, (multi-)resistant species), inaccessibility of part of the contaminated areas, poorly defined wound margins and involvement of multiple tissues. One of the requirements therefore is that the incorporated agent, either an antibiotic, or another antimicrobial such as antimicrobial peptides or antibacterial metals should have a broad antimicrobial spectrum. Therefore, it would be important to assess how such diverse antimicrobials agents would be released from stimuli-responsive hydrogels.

Facilitating bone repair

Locally delivered antibiotics from an ALB can have an effect on cell viability of osteoblasts and other eukaryotic cell types if concentrations are high and exposure times are long. In order to counteract any potential deleterious effect, osteopromotive

factors such as bone morphogenetic proteins could be co-introduced in order to improve bone healing in fracture fixation in which an ALB is used.

The in vivo fate of thermo-responsive materials

Stimuli-responsive hydrogels represent an attractive ALB, as they have low viscoelastic moduli before administration, and therefore can easily cover all the contaminated exposed areas of the traumatic anatomical site. Several cues can be used to induce the phase transition from sol to gel, temperature being one of them. Although there is a large amount of polymers or block (co)polymers that display LCST behaviour, only a few have gained particular interest as they gelate at temperatures just below physiological relevant temperature. Amongst them poly(*N*-isopropylacrylamide), poly(*N,N*-diethylacrylamide) and block copolymers of poly(ethylene oxide) and poly(propylene oxide) or poly(ethylene oxide) and poly(lactic-co-glycolic acid). Amongst these poly(*N*-isopropylacrylamide) is the one that shows the sharpest transition between sol and gel state, needed for a fast gelation of the hydrogel.

However, a concern for the use of thermo-responsive polymers based upon (co) polymers of *N*-isopropylacrylamide is their fate in the body, which has not yet been clarified. After degradation, the thermo-responsive grafts stay behind and have to leave the body by renal excretion when they should resolubilize upon shortening of the chain. So far, toxicity testing for *N*-isopropylacrylamide and different molecular weight (co)polymers from the monomer have been performed only *in vivo* for acute or subacute toxicity. However, the exact fate and excretion process of the material still need to be defined within test animals, potentially through specific labelling and quantification *in vivo*. This is crucial for their safe application in to the patients.

ALBs for infection treatment

Delivery systems with a more sustained release are needed for treatment of established infections as clearance takes place over an extended time.

The "Plum pudding" approach

Antibiotic release could potentially be extended through encapsulation in hydrophobic polymer microcarriers dispersed in a stimuli responsive hydrogel. In order to achieve a more predictable release, monodisperse particles can be used. When using hydrophobic polymers which degrade by surface erosion rather than bulk degradation, even zero-order release kinetics can be obtained depending on the dimensions of the implant.

Prodrugs approach

Another approach could be the development of prodrugs, in which the antibiotic attached to a macromolecule through cleavable linkers is released upon exposure to bacterial enzymes or other infection cues.

Furthermore, MICs for bacteria located in a biofilm are multiple times higher than their planktonic counterparts, so also the local concentrations delivered by an ALB should be considerably higher than in the case of infection prophylaxis. Infection with *S. aureus* can even be more complex to treat as the species also tends to invade cells and become intracellular, where many antibiotic are unable to reach them as the cell membrane has low permeability for them. Therefore, either the antibiotics should be modified so they can be internalized easier or targeting ligands which will target the antibiotic loaded vehicle to bone cells could be used in order to effectively clear these pathogens from colonized tissues.

Finally, the efficacy of the emerging ALBs from the ongoing developments have to be assessed in relevant *in vivo* prophylactic and treatment models, in order to be able to successfully move on into clinical trials. This is the only way patients at risk or suffering from implant-associated osteomyelitis can benefit from all these developments.

Summary

This thesis describes the development of gentamicin loaded resorbable polymeric carriers as anti-infective strategy for implant-associated osteomyelitis and their *in vitro* and *in vivo* evaluation. Local delivery of antibiotics has several advantages in the case of trauma to the bone and surrounding tissues. It can deliver high amounts of antibiotics directly to the target site, without inducing toxicity in non-target organs. Furthermore, sufficient antibiotic levels can be reached by local application at sites where the vasculature is disturbed.

A general background with regard to the work in the thesis and antibiotic loaded biomaterials is provided in **Chapter 1**. In **Chapter 2** the problem of implant-associated infection is described and an overview of polymeric carriers for antibiotics delivery is given. These can be passive polymeric carriers, that release their antibiotics by diffusion and/or upon degradation, and active polymeric carriers that release antibiotics upon stimuli provided by bacterial pathogens. Additionally, some polymeric carriers are able to gelate in-situ in response to physiological stimuli to form a depot for antibiotics release. Besides antibiotics, also other anti-infectives such as silver and antimicrobial peptides have been incorporated into biomaterials in the response to emerging antibiotic resistance. **Chapter 3** describes the complexation of gentamicin with negatively charged hyaluronic acid (HA) to form polyelectrolyte complexes (PECs). These complexes have reduced solubility and precipitate out of solution. Several HA conjugates were prepared and screened for their thermo-responsive behaviour and their ability to form polyelectrolyte complexes with gentamicin. The more hydrophilic HA-Jeffamines precipitated out of solution upon complexation. HApN polymers were able to form stable complexes that stayed in solution at RT while precipitating out at 37°C, similar to the more hydrophobic Jeffamine functionalized HA polymers. Hereby the HA derivative based PECs can modulate the availability of gentamicin and potentially increase the half-life and extend the release time of the antibiotic. In **Chapter 4** the preparation and characterization of gentamicin-loaded thermo-responsive HA-poly(*N*-isopropylacrylamide) (HApN) hydrogels is described. At a concentration of 13% w/w the hydrogel had suitable gelation properties, being a moderate viscous fluid at 20°C, whereas at physiological temperatures viscoelastic moduli were in the kPa range. The HApN hydrogel showed an initial burst release of gentamicin *in vitro*, however

the burst was lower than for collagen fleece containing an equal amount of the same antibiotic. In an *in vivo* rabbit humerus model of plating osteosynthesis contaminated with *Staphylococcus aureus* the HApN hydrogel with gentamicin successfully protected the implant site from bacterial colonization at 1 week post-operative. All samples from taken from the implant, bone and surrounding soft tissues from the rabbits to which the HApN hydrogel with gentamicin was administered were culture negative for bacteria. Bone, soft tissue and implant samples deriving from the untreated rabbits on the contrary were colonized by high amounts of Gram-positive bacteria. Histology data showed an absence of bacteria in the humeri of rabbits treated with the HApN hydrogel with gentamicin. Fracture healing upon clearance of a high bacterial load in the presence of the gentamicin-loaded HApN hydrogel is described in **Chapter 5**. Although healing has progressed, bridging of the created osteotomy is not complete 4 weeks post-operatively, as was observed in rabbits that only had surgery. Mechanical testing and histopathological evaluation of the operated humeri showed that the presence of the HApN hydrogel does not significantly influence bone healing in comparison to rabbits that only had surgery. In the histological sections this was observed as the formation of callus both on the cis and trans site of the bone, as well as periost around the osteotomy. Neither did the HApN hydrogel evoke a systemic response as was shown by the absence of differences in haematological values between the rabbits that were operated and the rabbits that were additionally injected with the HApN hydrogel. Humeri of inoculated rabbits that did not receive treatment showed a high degree of necrosis and absence of callus formation in the inoculated area. In the group of rabbits that got inoculated with *S. aureus* and which received the HApN hydrogel with gentamicin bone healing is delayed at the site where the bacteria and the hydrogel had been applied. The presence of dead bacteria might slow down bone healing. However on the contrary to the infected rabbits, callus formation was observed and there was an absence of necrosis. On the long term bone healing in these rabbits is anticipated. In **Chapter 6** the preparation of a lipophilic salt of gentamicin, gentamicin dioctyl sulfosuccinate and its encapsulation in poly(trimethylene carbonate) (PTMC) matrices is described. It was shown that ion-pairing with dioctyl sodium sulfosuccinate does not affect the potency of the antibiotic. The *in vitro* viability was affected negatively at lower molar

concentrations for the gentamicin dioctyl sulfosuccinate as compared to the sulfate salt of gentamicin. However, the lipophilic modified gentamicin was bactericidal towards *S. epidermidis* and *S. aureus* at molar concentrations lower than the concentration for which cell viability was reduced with 50%. The lipophilic modified gentamicin was well solubilized in the PTMC matrix, while gentamicin sulfate was dispersed to the polymer matrix and antibiotic loaded PTMC discs were obtained by compression molding. **Chapter 7** describes the preparation of polymeric microparticles with low dispersity and loaded with gentamicin sulfate or lipophilic modified gentamicin. Membrane emulsification yielded monodisperse gentamicin dioctyl sulfosuccinate loaded PDLLA and PTMC microparticles when polymer and antibiotic were dissolved in the same solvent as dispersed phase in the cross flow membrane emulsification process. By this method monodisperse lipophilic modified gentamicin loaded microparticles with an average diameter of 10 µm and polydispersity index (PDI) < 1.1 were obtained. Finally, in **Chapter 8** final conclusions are drawn and future perspectives of antibiotic loaded biomaterials are discussed. Issues such as bacterial resistance, lowered viability of osteoblasts and other eukaryotic cells in the presence of antibiotics, the *in vivo* fate of thermo-responsive polymers, additional requirements for infection treatment, delivery in response to bacterial presence and targeting of antimicrobials are addressed.

Samenvatting

Dit proefschrift beschrijft de ontwikkeling van degradeerbare polymere afgiftesystemen geladen met gentamicin als infectiewerende strategie tegen implantaat gerelateerde osteomyelitis en hun *in vitro* en *in vivo* evaluatie. Lokale afgifte van antibiotica heeft verschillende voordelen ten opzichte van systemische afgifte in het geval van trauma aan bot en omliggende weefsels. Hoge lokale antibiotica concentraties kunnen worden bewerkstelligd zonder dat andere organen beschadigen door middel van lokale afgifte. Verder kunnen voldoende hoge antibiotica concentraties worden gehaald in weefsels waar de vasculatuur is beschadigd.

De achtergrond met betrekking tot het werk in deze thesis en antibiotica geladen biomaterialen is beschreven in **Hoofdstuk 1**. In **Hoofdstuk 2** wordt het probleem dat implantaat gerelateerde osteomyelitis vormt beschreven, samen met een overzicht van de polymere afgifte systemen voor antibiotica. Een overzicht wordt gegeven van de passieve polymere afgifte sytemen voor antibiotica die hun lading afgeven door middel van diffusie/ of na degradatie, en actieve polymere afgifte systemen die antibiotica afgeven naar aanleiding van stimuli die worden geproduceerd door de aanwezigheid van bacteriën. Verder kunnen sommige polymere afgifte systemen in-situ een gel vormen, als reactie op fysiologische stimuli en als depot voor antibiotica afgifte dienen. Naast antibiotica, zijn er ook andere infectiewerende middelen zoals zilver en antimicrobiële peptiden geladen in biomaterialen als antwoord op een toename in antibiotica resistentie in bacteriën. **Hoofdstuk 3** beschrijft de complexatie van gentamicin met negatief geladen hyaluronan zuur (HA) in polyelectrolyte complexen (PECs). Deze complexen hebben een gereduceerde oplosbaarheid en precipiteren uit de oplossing. Verschillende HA derivaten werden gesynthetiseerd en gescreend op thermoresponsiviteit en de mogelijkheid om PECs te vormen met gentamicin. De meer hydrofiele HA-Jeffamines precipiteerden uit de oplossing na complexatie. HApN polymeren waren in staat complexen te vormen die in oplossing bleven bij kamertemperatuur, terwijl ze precipiteerden bij 37° C, net als de meer hydrophobe Jeffamine gefunctionaliseerde HA polymeren. Op deze manier kunnen PECs op basis van HA derivaten de beschikbaarheid van gentamicin moduleren en potentieel de halfwaardetijd vergroten en de afgiftetijd verlengen. In **Hoofdstuk 4** wordt de synthese en de karakterisatie van gentamicin geladen thermoresponsieve

HA-poly(*N*-isopropylacrylamide) (HApN) hydrogelen beschreven. Goede gelvormende eigenschappen werden bereikt bij een concentratie van 13% w/w. Bij kamertemperatuur (20 °C) was de HApN een matig visceuze oplossing, en bij lichaamstemperatuur waren de viscoelastische moduli in het kPa bereik. De HApN hydrogel gaf initieel een grote hoeveelheid gentamicin af, hoewel de hoeveelheid kleiner was dan voor collageen fleece geladen met een gelijke hoeveelheid antibiotica. De HApN hydrogel met gentamicin beschermde het implantaat en de omliggende weefsel succesvol tegen bacteriële contaminatie 1 week postoperatief in een *in vivo* fractuur model in de humerus van het konijn met osteosynthese hardware en *Staphylococcus Aureus* contaminatie. Alle monsters die werden genomen van het implantaat, het bot en de omringende zachte weefsels van de met de gentamicin geladen HApN hydrogel behandelde konijnen waren vrij van bacteriegroei. Daarentegen, de monsters van het implantaat, het bot en de omringende weefses van onbehandelde konijnen uit de controle groep waren gekoloniseerd door grote hoeveelheden Gram-positieve bacteriën. Histologie ondersteunde deze resultaten en toonde afwezigheid van bacteriën aan in de met de gentamicin geladen HApN hydrogel behandelde konijnen. Het helen van een fractuur na het afdoden van een grote hoeveelheid bacteriën door de gentamicin geladen HApN hydrogel is beschreven in **Hoofdstuk 5**. De gecreëerde osteotomie geneest niet totaal in de 4 weken die deze studie duurde. Mechanische testen en histopathalogisch onderzoek van de geopereerde humeri toonde aan dat de HApN hydrogel geen significant effect heeft op de bot genezing in vergelijking met konijnen die alleen werden geopereerd. Dit kon worden gezien in de vorm van callus formatie aan de cis en trans zijde van het bot, en het vormen van periost rondom de osteotomie. Ook had de toediening van de HApN hydrogel geen systemische reactie tot gevolg, hetgeen kon worden gezien aan de hand van gelijke bloedwaardes voor de groep die alleen geopereerd werd en de groep die de HApN hydrogel toegediend kreeg. De humeri van konijnen die enkel werden geinoculeerd met bacteriën toonden necrose en afwezigheid van nieuw gevormde callus. Tot slot, de heling van de breuk was vertraagd in de groep die werd geinoculeerd met *S. aureus* en die de HApN hydrogel met gentamicin toegediend kreeg. De aanwezigheid van afgedode bacteriën kan het genezingsproces hebben vertraagd. Echter in tegenstelling tot de geinfecteerde konijnen was er nieuwe callus gevormd in deze konijnen en werd er geen necrotisch

weefsel aangetroffen. Op de lange termijn mag verwacht worden dat het bot heelt in deze konijnen. In **Hoofdstuk 6** wordt het bereiden van een lipofiel zout van gentamicin, gentamicin dioctyl sulfosuccinaat en het incapsuleren van dit lipofiele molecuul in poly(trimethyleen carbonaat) (PTMC) matrices beschreven. Er werd aangetoond dat het uitwisselen van het dioctyl sulfosuccinaat voor sulfaat geen effect had op de uiteindelijke antibacteriële capaciteit van het antibioticum. De levensvatbaarheid van cellen wanneer deze aan de antibiotica werden blootgesteld werd negatief beïnvloed *in vitro* bij lagere concentraties voor gentamicin dioctyl sulfosuccinaat dan voor het sulfaat zout van gentamicin. Echter de lipofiel gemodificeerde gentamicin doodde *S. epidermidis* en *S. aureus* bij lagere molaire concentraties dan voor welke de levensvatbaarheid van de cellen met 50% werd gereduceerd. Het lipofiel gemodificeerde gentamicin losde goed op in de PTMC matrix, terwijl het gentamicin sulfaat als dispersie in de polymere matrix werd opgesloten, en antibiotica geladen PTMC schijven konden worden gemaakt met behulp van een pers. **Hoofdstuk 7** beschrijft het vervaardigen van polymere nanodeeltjes met lage dispersiteit en geladen met gentamicin sulfaat of lipofiel gemodificeerd gentamicin. Met behulp van membraan emulsificatie konden monodisperse gentamicin dioctyl sulfosuccinaat geladen PDLLA en PTMC micropartikels worden gemaakt door het antibioticum en het polymer in hetzelfde oplosmiddel als gedispergeerde fase te gebruiken in het kruisstroom membraan emulsificatie proces. Monodisperse lipofiel gentamicin geladen micropartikels konden worden verkregen met een gemiddelde diameter van 10 μm en een polydispersiteit (PDI) < 1.1. Tenslotte werd de balans opgemaakt in **Hoofdstuk 8** en de toekomstperspectieven van antibiotica geladen biomaterialen werden geschetst. Kwesties met betrekking tot resistentie, verlaagde levensvatbaarheid van osteoblasten, het *in vivo* lot van thermoresponsieve polymeren, verdere eisen aan afgifte systemen wanneer het de behandeling van infectie betreft, afgifte in repons op aanwezigheid van bacteriën en het gericht afgeven van antibiotica in bepaalde weefsels werden besproken.

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