Nanostructured Carriers for the Sustained Delivery of Antibacterial Agents

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To my beloved family and Qi
致我的家人和琦
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Chapter 1

General introduction
1 Bone infections

Bacterial infections occur when harmful bacteria invade and reproduce in the human body [1]. Bone tissue, for instance, can be infected when complex bone fractures are exposed to bacteria [2]. Such infections can develop into serious and even life-threatening conditions such as osteomyelitis [2, 3], which is associated with a high morbidity [4]. In addition to bone tissue, artificial bone implants can also be infected. Orthopedic and dental implants are increasingly used for treatment of bone fractures, fixation of non-unions, joint replacement, spinal revision surgery as well as oral and maxillofacial surgery [5]. These implants improve the quality of life by restoring mobility and function and alleviating pain [5-7]. Even though modern implantology has made significant progress during the past decades, infections following invasive surgery remain a major clinical challenge without satisfactory clinical treatment [8, 9]. These infections may lead to implant failure as well as acute and often chronic complications.

The incidence of osteomyelitis varies between individuals and regions, and this type of infection is difficult to treat. The incidence rate of bone implant-associated infections depends on the surgical site and procedure [10]. Typical incidence rates can be as high as 13% for infections related to bone allografts [11], 2% to 5% for spinal infections [5, 10, 12], 2% to 30% for transcutaneous fracture fixation pins [10, 13], and 0.2% to 2.2% for primary total hip replacement [5, 14]. The treatment of these infections places a huge burden on healthcare and cause pain and suffering of the patients [15].

2 Therapeutic strategies for the treatment of bone infections

Traditional modalities for treatment of bone infections involve systemic administration of high amounts of antibiotics for extended periods of time [1, 5]. Systemic delivery of antibiotics is, however, often associated with systemic toxicity [16, 17], antibiotic resistance and recurrence of infections [1, 9, 18]. The surface of bone implants is rapidly coated with serum proteins after implantation in the human body, which promotes cell recruitment and tissue repair by the host [10]. Unfortunately, these serum proteins also facilitate adhesion and invasion of bacteria [19-21]. Subsequently, the
bacteria colonize implant surfaces followed by formation of a biofilm [22, 23], which protects bacteria from antibiotic activity and host immune cell surveillance [10, 23, 24]. These problems impose a major challenge for traditional treatment modalities against infection. A powerful alternative strategy to reduce the complications and side-effects related to the systemic delivery of antibiotics involves local and sustained delivery of high amounts of antibacterial drugs at the site of infection. In addition, the development of new methods to prevent (instead of treat) infections is gaining interest since infection prevention may avoid revision surgery, reduce the suffering of the patients and decrease the medical costs. Therefore, active antibacterial drug release systems should be developed which can deliver antibacterial agents locally in a sustained manner at sufficiently high concentrations to treat or prevent bone infections.

3 Nanocarriers for drug delivery
The design and fabrication of drug delivery systems is crucial to improve the efficacy of antibacterial therapy [3, 25]. These delivery systems should release antibacterial drugs at the local site in a spatiotemporally controlled manner while retaining their bioactivity to optimize their therapeutic efficacy and reduce their side effects [26]. Recently, nanotechnology has provided several tools to develop novel nanosized pharmaceutical carriers (nanocarriers) [27] that facilitate controlled release of a wide range of antibacterial molecules [3, 25, 28, 29].

Nanoparticles (nanospheres) and nanofibers are among the most commonly investigated nanocarriers. Nanoparticles exhibit a considerably higher surface area as compared to traditional microparticles, which can allow higher drug loading efficiencies and more sustained release profiles [30, 31]. The nano-scale dimensions of nanoparticles also provide the possibility for targeted or intracellular delivery of antibiotics [32, 33]. Besides nanoparticles, electrospun nanofibers have also been widely explored for drug delivery purposes due to their high drug loading and encapsulation efficiency, ability to modulate biomolecule release, cost-effectiveness, and ease of processing into e.g. fibrous membranes or sheets [34, 35].

4 Objectives of this study
Gelatin and gelatin-based materials have been widely used in regenerative medicine [36-39] due to their biodegradability, biocompatibility, and
cost-effectiveness. Gelatin nanospheres (GNs) are particularly interesting since they have exceptional physiochemical characteristics for tissue regeneration and biomolecule delivery [31, 40, 41]. The charged nature of GNs renders them suitable for the controlled delivery of oppositely charged bioactive macromolecules such as growth factors [31, 42, 43] and nucleic acids [44, 45]. These macromolecules were shown to bind to oppositely charged GNs through the formation of polycation complexes between macromolecules and GNs [46]. However, it is still not yet known if small biomolecules of low molecular weight (< 1.5 kDa), such as antibiotics, can be released from GNs in a sustained manner. Therefore, detailed studies on the interactions between various antibiotics and gelatin carriers are necessary to understand their mutual interaction and pave the way for development of nanocarrier-based drug delivery systems for local delivery of antibiotics.

In addition to the direct use of GNs as drug delivery vehicles, GNs can also be incorporated into various matrices including coatings and (fibrous) membranes to modify the release characteristics of various biomolecules. Although several local delivery systems have been developed recently including chitosan implant coatings [17, 47, 48] and silk fibroin nanofibrous membranes [49, 50], control over the release of biomolecules from these carriers is still poor due to the lack of interactions between the drugs and carriers. The incorporation of GNs into these delivery systems can be used to modulate their delivery characteristics as well as mechanical and biological performance.

Before the clinical use of GNs can be considered, it is of utmost importance to evaluate the biological properties of GNs thoroughly due to concerns related to the safety of gelatin-derived biomaterials [40, 51]. Consequently, suitable animal models that can provide the requested insight on the biological safety and efficacy of GNs should be developed.

Although local delivery of antibiotics is frequently studied, antibiotics are effective against bacteria only and can induce the development of antibacterial resistance [9, 52]. In fact, the development rate of antimicrobial resistance is exceeding the development rate of new drugs by far [53]. Therefore, local delivery of antiseptics instead of antibiotics can be an effective alternative to kill bacteria without inducing antibacterial resistance.
To address the challenges mentioned above, the current thesis focused on the following scientific questions:

1. What is the current state-of-the-art with respect to the use of gelatin or gelatin-based materials for bone tissue engineering? (Chapter 2)
2. Can gelatin nanospheres be used as carriers for the sustained delivery of small biomolecules such as antibiotics, and what is the mechanism of interaction between antibiotics and gelatin nanospheres? (Chapter 3)
3. Can gelatin nanospheres be deposited inside chitosan coatings applied on the surface of metallic implants to tune the release profile of loaded antibiotics? (Chapter 4)
4. Can gelatin nanospheres be incorporated into electrospun silk fibroin nanofibers to form a novel nano-in-nano fibrous membrane to facilitate sustained delivery of antibiotics and modulate cell adhesion and growth? (Chapter 5)
5. Can zebrafish be used as a preclinical animal model for evaluation of the spatial distribution and immune response of gelatin nanospheres? (Chapter 6)
6. Can antiseptics such as chlorhexidine and silver be loaded onto electrospun chitosan nanofibers to achieve fast release of chlorhexidine and sustained release of silver to induce bactericidal effects without causing antibacterial resistance? (Chapter 7)
References

19. Ahmed, S., et al., Staphylococcus aureus Fibronectin Binding Proteins Are Essential for Internalization by Osteoblasts but Do Not Account for Differences in Intracellular Levels
Sustained delivery of biomolecules from gelatin carriers for applications in bone regeneration

Department of Biomaterials, Radboud University Medical Centre

Therapeutic Delivery, 2014, 5(8):943-58
1 Introduction

Healthy bone tissue is a dynamic tissue that is continuously remodeled by the interdependent processes of bone resorption and bone formation. However, bone defects caused by trauma, tumor resection and congenital abnormalities are common clinical problems. Usually, bone tissue has ability to regenerate itself, but large defects that exceed a certain critical size cannot be healed by themselves [1].

Autologous bone transplantation is the most effective method for bone restoration, because it provides three essential elements: osteoconduction, osteoinduction, and osteogenic cells [2]. Autologous grafts facilitate bone growth along their surface or into their pores (osteocanduction) and stimulate osteoprogenitor cells to differentiate into osteoblasts (osteoinduction) [3]. However, shortage of donors, donor site morbidity, the persistence of pain, risk of infection, risk of hemorrhage, and other disadvantages reduce the attractiveness of this technique [2, 4]. Although these problems can be circumvented by using allografting, clinical risks related to immunological reactions and disease transmission still persist [5]. Consequently, engineered biomaterials that present bioactive cues, such as growth factors (GFs) (osteogenic and angiogenic factors), to the surrounding tissue have recently emerged as a new therapeutic modality for applications in bone regeneration [6]. Such GFs are exposed to cells during bone healing under tight spatial and temporal control. To mimic this highly sophisticated presentation of biomolecules, therapeutic factors such as GFs should be released at optimal doses to target tissue for pre-defined periods of time [7]. To meet this challenge, novel carrier materials are urgently needed to allow for spatiotemporal control over delivery of therapeutic biomolecules [8, 9].

Besides delivery of angio- and osteogenic biomolecules, gelatin carriers can also be used to deliver biomolecules locally for treatment of bone tissue of compromised health due to, for example, ageing, diseases like cancer and osteoporosis, or infection. For instance, osteomyelitis may occur when severe fractures are exposed to environmental bacteria [10]. To combat this phenomenon, systemic administration of antibiotics is the standard treatment modality, but high and frequent doses are needed to achieve the desired antibacterial effect, resulting into, for example, systemic toxicity as well as drug resistance. Moreover, the efficacy of systemic delivery of
antibiotics is often limited by the local low concentrations of antibiotics and the poor blood supply of the bone tissue [11]. Therefore, a local drug delivery system is advantageous because it decreases the systemic toxicity and side effects of systematically delivered antibiotics, while improving the efficacy by delivering higher and sufficient drug concentrations to the infected bone.

Developments in the field of biomaterials research have been applied in the field of pharmaceutical research during the past decades by designing advanced carriers for the sustained release of a wide variety of biomolecules. Since the approval of INFUSE® (a collagen sponge loaded with rhBMP-2) for clinical use in 2002 by the US FDA, biomaterials for local delivery of osteogenic biomolecules have also entered the commercial market [12]. The main strategy for delivering biomolecules during bone regeneration involves encapsulating of biomolecules to a polymer or polymer-based carrier followed by in vivo release mediated through either carrier degradation or biomolecule desorption. To this end, numerous biodegradable and biocompatible synthetic and natural polymers have been used to design carriers for controlled release of biomolecules [13, 14]. Among them, gelatin-based carriers have received specific attention due to their superior biocompatibility, biodegradability, biosafety and cost-effectiveness, even though the animal origin, broad molecular weight distribution and need for additional cross-linking need to be considered when using gelatin as carrier [15, 16].

The current study has reviewed the application of gelatin (in form of bulk hydrogel foams or sheets, microspheres, nanospheres, colloidal gels and composites) as carrier for the controlled delivery of commonly used biomolecules for application in bone regeneration. General properties of gelatin and the preparation methods for gelatin carriers are discussed.

Figure 1. (A) Relative number of papers using gelatin as carrier for biomolecule delivery, (B) Concept for the use of gelatin as carrier for biomolecule delivery (DNA = deoxyribonucleic acid, PRP = platelet rich plasma).
followed by a general overview of the processes relevant for the loading and release of biomolecules. Gelatin has been used as carrier for delivery of a wide variety of biomolecules, but GFs are by far the most extensively studied type of biomolecule. Figure 1A shows the relative number of papers per type of biomolecule corresponding to a PubMed search using keywords “gelatin”, “bone”, “bone regeneration” and “release” which yielded 59 references. Of these, 44, 7, 5 and 3 studies were devoted to the release of GFs, antibiotics, DNA and platelet-rich plasma (PRP), respectively. Therefore, the controlled release of GFs is discussed in detail by focusing on the most commonly studied osteo- and angiogenic GFs: BMPs, bFGF, TGF-β1, VEGF and PRP which contains several GFs. Additionally, the use of gelatin carriers for local and sustained delivery of miscellaneous biomolecules such as nucleic acids (plasmid DNA encoding for BMP-2), and antibiotics is reviewed.

2 Gelatin as a reservoir for biomolecules

2.1 Properties of gelatin

Gelatin is a denatured protein that is prepared through hydrolysis of collagen, which is the main organic component of hard, mineralized tissues. There are two types of commercially available gelatin: cationic gelatin prepared by acid hydrolysis of pig skin type I collagen; and anionic gelatin prepared by alkaline hydrolysis of bovine skin [17]. At neutral pH, cationic gelatin is positively charged (isoelectric point [IEP] of 7-9) while anionic gelatin is negatively charged (IEP of 4.8-5). This charged nature of gelatin renders this biopolymer suitable for controlled delivery of oppositely charged biomolecules.

In addition to its charged nature, gelatin is inexpensive and can be prepared in various shapes and forms using facile and controllable preparation methods. Reversely, the biodegradation of gelatin can be tailored by controlling the cross-linking density. Moreover, gelatin exhibits a beneficial cell compatibility due to the presence of cell recognition peptide sequences, and its pharmaceutical and medical safety is confirmed by long clinical usage [18]. Furthermore, gelatin does not produce harmful byproducts upon enzymatic degradation since it is derived from collagen, which is the most abundant protein in animals [19]. These properties emphasize the strong potential of gelatin as carrier for controlled release of biomolecules.
Chapter 2

for bone regeneration.

2.2 Preparation of gelatin carriers

Gelatin can be processed into hydrogels, porous foams as well as micro- or nanoparticles, and cross-linking can be either physical or chemical to increase the thermal and mechanical stability under physiological conditions [20]. Gelatin hydrogels are generally prepared by cross-linking of gelatin solutions containing glutaraldehyde (GA) or a water-soluble carbodiimide (WSC) as the most commonly used cross-linkers [21, 22]. Gelatin porous foams are usually fabricated by lyophilization of gelatin solutions [23], whereas gelatin microspheres are generally prepared using water-in-oil emulsions [24-27]. Gelatin nanospheres can be prepared using two-step disolvation [17, 27], coacervation-phase separation [28], emulsification-solvent evaporation [29] or nanoprecipitation [30, 31]. A detailed description of the fabrication details of gelatin carriers as well as a description of the cross-linking process of gelatin carriers can be found in reviews of Young et al. [20] and Elzoghby [19].

2.3 Loading and release of biomolecules

Figure 1B shows how gelatin matrices can be used as the carrier for the controlled release of biomolecules. Typically, biomolecules are loaded onto gelatin matrices through polyion complex formation and released upon degradation of gelatin.

2.3.1 Biomolecule loading

The mechanism of loading therapeutic agents onto gelatin carriers to obtain biomolecule immobilization is most frequently based on polyion complexation [20]. Polyion complexation relates to a type of electrostatic interaction formed between positively or negatively charged electrolytes and their oppositely charged counterparts. Herein, polyion complexes formed between biomolecules and gelatin macromers are described. Polyion complexation can be used for controlled release of a wide variety of therapeutic agents such as GFs, polysaccharides, polynucleotides and antibiotics [19, 20]. Many biomolecules are positively charged at neutral pH since their IEPs are higher than physiological pH values of 7 (IEP of bFGF = 9.6, VEGF = 8.6, BMP-2 = 8.5, and TGF-β = 9.5 [18]). Positively charged GFs are electrostatically complexed with negatively charged gelatin macromers. Using this strategy, biomolecules are impregnated into gelatin carriers by
simply dripping aqueous biomolecule solutions onto freeze-dried gelatin carriers, which is defined as diffusional postloading [32]. This procedure effectively facilitates sorption of biomolecules to the carrier matrix and preserves biomolecule bioactivity. Compared to other loading methods, for instance covalent binding through enzymatic cross-linking [33] or loading during carrier preparation, biomolecules are not harmed by harsh processing conditions or organic solvents during diffusional postloading, which is highly beneficial from an applied perspective.

2.3.2 Biomolecule release

Two possible mechanisms of biomolecule release from a biodegradable, charged polymeric carrier have been described by Tabata et al. [34], who indicated that biomolecules can be released from carriers by either environmental changes (such as a change in ionic strength) or degradation of the carrier itself. It is known that gelatin does not degrade by simple hydrolysis but by proteolysis, and the degradation of gelatin depends on its water content and degree of cross-linking [35], where a lower water content or higher cross-linking density results into lower rates of gelatin degradation. In that way, charged biomolecules such as GFs, once complexed with an oppositely charged gelatin carrier, cannot be released from the gelatin matrix unless gelatin degradation occurs. As a consequence, degradation of gelatin matrices that containing GFs with lower water content or higher cross-linking density will be slow, resulting into slow sustained release of GFs over longer periods of time. In that way, the release of biomolecules from gelatin matrices can be regulated by controlling the rate of gelatin degradation. On the other hand, gelatin itself has no active ability to induce bone formation [36, 37]. When the rate of gelatin degradation is slower than the rate of bone regeneration, the gelatin remaining in the defect sites will physically impair bone regeneration even though long-term release of TGF-β1 is achievable [36]. Thus, a gelatin hydrogel with a suitable in vivo degradability functions not only as a matrix for GFs release at the bone defect site, but also as a space provider to prevent ingrowth of soft tissues into the defect. Therefore, an optimal balance between the rates of GF release and new bone formation is pivotal for successful bone regeneration induced by GFs incorporated into gelatin matrices. Based on these mechanisms, gelatin-based materials have been widely studied to obtain controlled and sustained delivery of biomolecules for bone regeneration.
Chapter 2

3 Release of GFs

Recombinant DNA technology has enabled mass production of GFs [38, 39]. Nevertheless, when GFs are applied locally without carriers, their bone-formation capacity was markedly limited because of its short in vivo biological half-life and/or loss of bioactivity [40-42]. One common strategy to circumvent this problem and enhance the in vivo efficacy of GFs is to release the GFs from locally applied carriers over a long period of time [43]. Therefore, several biomaterials have been investigated to provide controlled and sustained delivery of biologically active GFs at the defect site to mimic its temporal profile during natural bone healing in vivo and maximize its bone-inductive ability. As mentioned above, gelatin (in form of bulk hydrogels, microspheres, nanospheres, colloidal gels and composites) is one of the most frequently investigated carrier materials to this end.

3.1 Bone morphogenetic proteins

BMPs are members of the TGF-β superfamily. Generally, their gene expression is upregulated at sites of bone fracture, which shows that BMPs play an important role in bone regeneration [44]. They mainly act as differentiation factors for precursor cells [45] and are present during various stages of bone healing, both in the initial phases of fracture repair [46] and in the later stages involving chondrogenesis and osteogenesis [47]. Among them, BMP-2/4 and BMP-6/7 were confirmed to be osteoinductive although the induction pathways for osteoblastic differentiation of primary human mesenchymal stem cells (hMSCs) are different [48]. Specifically, BMP-2/4 used predominantly BMPR1A and BMPR2 for signaling, whereas ACVR1A and ACVR2A were more sensitive to signaling by BMP-6/7. In addition, BMP-2 can also recruit bone progenitor cells and promote the osteogenic differentiation of these cells [22]. Previous studies showed that these factors elicited new bone formation in animal models [40, 49-51].

3.1.1 BMP-2 release from gelatin bulk hydrogels

Gelatin bulk hydrogels prepared by chemical cross-linking of gelatin solutions followed by freeze-drying have been widely used in bone regeneration since these scaffolds facilitate sustained release of BMP-2, both during ectopic bone formation and orthotopic regeneration of critical-sized bone defects. Table 1 summarizes in vivo studies using gelatin bulk hydrogels (foams/sheets) for the controlled release of BMP-2.
A study conducted by Yamamoto et al. [40] was shown using an ectopic implantation mode that the *in vivo* retention period of BMP-2 prolonged with decreased water content of gelatin bulk hydrogels, resulting into bone formation around the hydrogel incorporating BMP-2. Additionally, Kimura et al. [22] proved that recruitment of bone marrow-derived osteoblast progenitor cells from the blood circulation was determined by the local concentration of BMP-2 upon release from gelatin bulk hydrogel, resulting into controllable volumes of de novo formed bone tissue around the hydrogels. Consequently, it can be concluded that gelatin bulk hydrogels are highly suitable for controlled and sustained delivery of BMPs to obtain bone regeneration.

**Table 1.** *In vivo* studies using gelatin bulk hydrogels for controlled release of BMP-2

<table>
<thead>
<tr>
<th>Animal</th>
<th>Implantation site</th>
<th>Defect size (mm)</th>
<th>Loading amount (μg)</th>
<th>Implantation time (weeks)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>Subcutaneous</td>
<td>—</td>
<td>0.5, 1 and 5</td>
<td>4</td>
<td>[40]</td>
</tr>
<tr>
<td>Mice</td>
<td>Subcutaneous</td>
<td>—</td>
<td>3</td>
<td>7</td>
<td>[22]</td>
</tr>
<tr>
<td>Rabbits</td>
<td>Ulna defect</td>
<td>20</td>
<td>17</td>
<td>6</td>
<td>[52]</td>
</tr>
<tr>
<td>Monkeys</td>
<td>Skull defect</td>
<td>6 in diameter</td>
<td>5, 50 and 200</td>
<td>12</td>
<td>[49]</td>
</tr>
<tr>
<td>Rabbits</td>
<td>Alveolar clefts defect</td>
<td>6 × 6</td>
<td>17</td>
<td>4</td>
<td>[50]</td>
</tr>
<tr>
<td>Dogs</td>
<td>Orbital floor fracture</td>
<td>10 × 10</td>
<td>10</td>
<td>5</td>
<td>[44]</td>
</tr>
<tr>
<td>Rabbits</td>
<td>Ulna defect</td>
<td>20</td>
<td>17</td>
<td>12</td>
<td>[51]</td>
</tr>
</tbody>
</table>

Besides the above mentioned ectopic bone formation using gelatin bulk hydrogels containing BMP-2, several successful critical bone regeneration studies have been listed in Table 1. Although complete bone healing was obtained for each of these studies, direct comparison between these studies is complicated by the differences in animal type, defect location and size, dose of BMP-2 and implantation period. Therefore, systematic studies should be performed under identical experimental conditions with respect to the type of animal, defect site and water content of gelatin hydrogels before unambiguous conclusions can be drawn on the optimal conditions for bone regeneration.

### 3.1.2 BMP release from gelatin microspheres

The association between BMP-2 and gelatin is largely based on electrostatic interactions [53]. When BMP-2 (with IEP of 8.5) was loaded through diffusional postloading onto negatively or positively charged gelatin microspheres (exhibiting an IEP of 5 and 9, respectively and cross-linked by
40 mM glutaraldehyde), the burst release was minimal followed by linear in vitro release kinetics, while slower release was observed from the negatively charged gelatin microspheres compared to the positively charged gelatin microspheres [54]. However, when the gelatin microspheres were cross-linked by 10 mM glutaraldehyde, the release of BMP-2 was controlled by gelatin degradation and did not depend on the type of gelatin [54]. This suggested that controlled release of BMP-2 can be achieved using gelatin microspheres as carrier. In addition, when gelatin microspheres were dispersed through oligo (poly(ethylene glycol) fumarate) (OPF) matrices, the burst release of BMP-2 from the resulting composite hydrogel was reduced and the retention of BMP-2 was improved upon loading BMP-2 onto gelatin microspheres instead of the OPF matrix phase [55]. However, this formulation did not induce bone augmentation in a rat model [56]. In contrast, a study conducted by Li et al. [57] showed that bone defect healing in osteoporotic goats was accelerated due to the controlled release of rhBMP-2 from composites consisting of gelatin microspheres and calcium phosphate cements. This difference may be due to the dense geometry of the hydrogel and insufficient degradation of the hydrogel composites [56], which inhibited the formation of an interconnected pore network structure that would allow for bone ingrowth.

### 3.1.3 BMP-2 release from gelatin-based composites

Gelatin-based composites containing BMP-2 were also used for bone regeneration. Weinand et al. [58] observed the formation of bone tissue after seeding human bone marrow-derived mesenchymal stem cells (hBMSCs) and adipose-derived stem cells (ASCs) onto gelatin/β-tricalcium phosphate (β-TCP) composite scaffolds containing BMP-2-loaded gelatin microspheres. Besides, successful bone regeneration using gelatin/β-TCP scaffolds loaded with BMPs has also been reported both in vitro and in vivo. Yang et al. [59] prepared gelatin/β-TCP scaffolds and then soaked these scaffolds in BMP-4 solution to form a BMP-containing 3D porous scaffold, showed improved cell attachment and osteoblast differentiation compared to scaffolds without loading of BMP-4. Takahashi et al. [60] investigated the in vivo release profile of BMP-2 from gelatin sponges containing 5 μg BMP-2 and various contents of β-TCP followed by evaluation of the in vivo ectopic osteoinduction activity of these composite sponges. In vivo release of BMP-2 was shown to be independent of the β-TCP content, but the extent of
ectopic bone formation increased with decreasing β-TCP content. A study conducted by Matsumoto et al. [61], however, showed that bone defects of 5 mm diameter in rat mandibles were almost entirely regenerated after 8 weeks of implantation of gelatin sponges containing 50 wt% of β-TCP and 5 μg BMP-2. Similarly, Tsuzuki et al. [42] obtained significantly higher amounts of bone regeneration after 16 weeks of implantation of gelatin sponges containing 50 wt% of β-TCP and 3 μg BMP-2 into the third metacarpal bones of horses. These results seem to indicate that the incorporation of degradable β-TCP bioceramics only stimulated bone formation in orthotic sites as studied by Matsumoto et al. and Tsuzuki et al. [42, 61], but not in ectopic implantation sites as investigated by Takahashi et al. [60]. No solid explanation was given to account for these differences between ectopic and orthotopic implantations.

3.1.4 Factors influencing BMP-2-induced bone formation

From the above-mentioned in vivo studies, it can be clearly concluded that gelatin is a suitable carrier for the controlled delivery of BMPs during bone regeneration. Nevertheless, the results from several in vivo studies are often controversial irrespective of the form of the gelatin carrier, in other words, bulk hydrogel, micro- or nanosphere or composite. It should be realized that bone regeneration is a complex and multifactorial process regulated by BMP dose, carrier properties, animal type and implantation site.

BMP dosing is crucial for successful bone regeneration since low doses are ineffective, whereas high doses of BMP in commercially available products (typically in the milligram range [62]) were shown to cause complications such as excessive bone growth, back/leg pain, ectopic bone formation, risk of cancer, as well as osteolysis [12]. Although it is obvious that pre-clinical studies cannot be correlated to human clinical studies, side effects of high dose of BMP-2 were also observed in animal studies. For instance, a BMP-2 dose of more than 150 µg caused detrimental side effects such as cyst-like bone formation and significant inflammation in a rat femoral segmental defect model [63]. These complications stress the need for carrier materials that allow for delivery of BMPs at lower effective doses. The BMP-2 doses in the studies listed in Table 1 were much lower and typically in the microgram range. Although bone regeneration was successfully achieved in most of the above-mentioned in vivo studies, the BMP dosing was different among
these studies and no consensus has been reached yet about the optimal BMP dose. Additionally, several studies even reported controversial results for the same type of gelatin carrier, in other words, for gelatin microspheres [56, 57]. Comparing all the in vivo studies, it can be concluded that the BMP dose is not the only key factor regulating the regeneration of bone defects. Instead, it has become evident that the release profile of BMPs determines the bone healing process. For instance, in the study conducted by Takahashi et al. [49], bone regeneration in monkey skull defects was significantly enhanced even at a low BMP-2 dose of 5 μg per site with BMP-2 release for 12 weeks. Yamamoto et al. and Takahashi et al. found that gelatin bulk hydrogels with a water content of 97.8% exhibited optimal BMP-2 release profiles for effective induction of bone formation [49, 52], which was mainly controlled by the degradability of the gelatin carrier. Consequently, optimization of the release profile of BMP-2 by the material design is pivotal to promote bone regeneration and to reduce the BMP-2 dose, so as to shorten the period and lower the amount of BMP required for bone regeneration.

3.2 Basic fibroblast growth factor

In bone tissues, bFGF is produced by cells of the osteoblastic lineage and stored in the bone matrix, and it acts as an autocrine/paracrine factor [64, 65]. bFGF links to acidic polysaccharides such as heparan sulfate and heparin located in the extracellular matrix, where bFGF is protected from in vivo deactivation and can be released through enzymatic degradation of the polysaccharides [66]. Although the mechanism of bFGF on bone formation has not been completely elucidated, bFGF is reported to exhibit anabolic effects in the early stages of fracture healing through its potent mitogenic action on immature mesenchymal cells [67]. Moreover, this GF stimulates proliferation of osteoblasts in the late differentiation stages and enriches the population of cells competent for osteogenic differentiation and maturation [68]. Besides, it stimulates local production of other factors such as TGF-β [69] and BMPs [68]. Furthermore, bFGF accelerates callus remodeling resulting from both osteoblastic callus formation and osteoclastic callus resorption [70, 71].

3.2.1 bFGF release from gelatin in vitro

Anionic gelatin (IEP = 5) was selected as the carrier for bFGF (IEP = 9.6)
because of the formation of polyion complexes with cationic bFGF, which was beneficial for the controlled release of bFGF. On the contrary, almost 100% of bFGF released from cationic bulk gelatin initially by simple desorption since complexes between bFGF and cationic gelatin could not be formed [66]. Another strategy to influence the release rate of bFGF from anionic gelatin carriers was pursued by Leeuwenburgh et al. who reduced the release rate of bFGF from gelatin microspheres by adding calcium phosphate nanocrystals [18], thereby slowing down the degradation rate of gelatin microspheres.

3.2.2 bFGF release from gelatin bulk hydrogel in vivo

Gelatin bulk hydrogels have frequently been used to obtain the sustained release of bioactive bFGF during bone regeneration. Yamada et al. [72] implanted anionic gelatin hydrogel impregnated with 100 μg bFGF into 6 mm defects in rabbit skulls, resulting into complete closure of the defects within 12 weeks. This accelerating effect of bFGF on bone formation at the skull defect was also observed at the same dose of bFGF (i.e. 100 μg) in rabbits [43] and monkeys [73] preclinical models. Gelatin hydrogel sheets loaded with 100 μg bFGF accelerated devascularized sternum healing after bilateral removal of the internal thoracic artery in normal [74] and diabetic [75] rats as well as in dogs [70], due to the angiogenic and osteogenic effects of bFGF. The same amount of bFGF also induced faster recovery and lower demineralization of transverse fractures in proximal sesamoid bone in horse upon incorporation into gelatin hydrogel sheets as compared to screw fixation only [76].

3.2.3 bFGF release from gelatin microspheres in vivo

For applications in bone regeneration, anionic gelatin microspheres were used as carrier for release of bFGF to treat femoral head osteonecrosis in rabbits [26] and alveolar bone defect in dogs [77]. Both studies showed positive effects on bone formation. Moreover, gelatin microspheres loaded with bFGF were used in combination with bone marrow mononuclear cells to treat the medial femoral condyle bone defects in rabbits. This therapy enhanced neovascularization and new bone formation [78]. Furthermore, Ichinohe et al. [79] combined bFGF-loaded gelatin microspheres with hydroxyapatite (HA)-coated titanium nonwoven fabrics for the treatment of skull defects in rabbits. Compared to bFGF-free titanium nonwoven fabrics, titanium fabrics coated with HA with/without free bFGF, this combination
significantly enhanced bone regeneration by enhancing the local bFGF concentration at the bone site for prolonged time periods.

3.2.4 Dosing of b-FGF

From the above-mentioned studies on the use of bFGF for bone regeneration, it is obvious that controlled and sustained release of biologically active bFGF from gelatin microspheres and hydrogels accelerated bone regeneration. The amount of bFGF used, however, was different among these studies. Kawaguchi et al. [80] suggested that a threshold amount of bFGF (ranging between 100-1000 μg per defect site) was needed to effectively stimulate bone healing. Consequently, an amount of 200 μg of bFGF per implantation site was used for the treatment of ulna fractures in monkeys [80]. The same amount of bFGF was used in treating mandibular ridges defects in dogs [81]. However, the most frequent dose of bFGF used for bone regeneration at different fracture sites in different animal models was 100 μg. Kodama et al. [68] proved that even lower doses of 20 μg bFGF per site were already sufficient to stimulate new bone formation in maxillae of mice, and the amount of bFGF used for effective bone regeneration around fenestrated implants in dogs was even as low as 10 μg [82]. These results confirm that the optimal dose of bFGF used is strongly dependent on the fracture site and type of animal.

3.3 Transforming growth factor

TGF-β1 is the most abundant isoform of the three TGF-β isoforms that are detected in bone [83]. It is secreted by bone cells in a latent form and is stored in the ECM. Although the mechanism of action of TGF-β1 on the process of bone regeneration is still not fully elucidated, the most commonly accepted model involves recruitment of osteoblast progenitors and stimulation of their proliferation, which results into the expansion of the pool of committed osteoblasts and enhanced differentiation of the progenitor cells at the early stages [84]. Nevertheless, this growth factor blocks later phases of differentiation and mineralization, which means that the osteogenic ability of TGF-β1 is only effective in the early stage of fracture healing. Similar to bFGF, TGF-β1 can form polyion complexes with anionic gelatin and can be released in vivo upon gelatin degradation. As a consequence, several studies aimed to sustain the delivery of TGF-β1 from gelatin-based matrices after implantation in rabbit bone defects [36, 37, 85, 86].
Similar to the release of BMPs and bFGF, the release of TGF-β1 from gelatin hydrogel was shown to correlate strongly to the degradation rate of gelatin [37]. When gelatin hydrogels containing 0.1 μg TGF-β1 were implanted into rabbit calvarial defects, gelatin hydrogels with water contents of 90 and 95 wt% induced abundant bone regeneration [37]. In addition, Hong et al. investigated the osteogenic ability of gelatin hydrogels containing TGF-β1 using a rabbit skull defect model [36]. After 6 weeks of implantation of a gelatin hydrogel (water content 95%) loaded with at least 0.1 μg TGF-β1, significant bone regeneration was observed. The same authors also implanted gelatin microspheres containing 0.5 μg TGF-β1 at the rabbit skull bone gap between the autologous bone fragment and the surrounding intact bone, resulting into significant bone formation after 3 weeks implantation [85]. Moreover, gelatin microspheres loaded with 0.25 μg TGF-β1 were added to calcium phosphate cement at 5 wt% to allow for the formation of porosity and enhance the bone response [86]. However, bone formation was not improved upon release of TGF-β1 from gelatin microspheres. On the other hand, release of TGF-β1 from these cements accelerated the degradation of these composites after 12 weeks of implantation, which was most likely caused by a stimulatory effect on osteoclasts.

3.4 Vascular endothelial growth factor

VEGF is a potent angiogenic GF and it induces proliferation and migration of endothelial cells. Besides, VEGF promotes new bone formation by increasing mesenchymal stem cell chemotaxis and stimulating osteoblast differentiation and proliferation due to an indirect effect on osteoprogenitor cells [87]. In vitro release of VEGF from gelatin microspheres displayed a characteristic burst release on short term followed by sustained release on long term based on degradation of gelatin [24]. Control over the delivery of VEGF could be achieved by altering the extent of microsphere cross-linking [24]. Ozturk et al. fabricated a gelatin/hydroxyapatite cryogel scaffolds containing 5 μg VEGF and implanted them into critical-sized defects in the proximal tibiae of rabbits [88]. The bone healing rate was better for the scaffold containing VEGF compared to the VEGF-free control scaffolds after 6 weeks, but there was no difference at 12 weeks, which suggested that VEGF plays an important role particularly in the early phases of fracture healing [88].
3.5 Multiple release of growth factors

Bone regeneration using tissue engineering strategies is a highly complex process regulated by many factors including the structural characteristics of the ECM analogs, the presence and activity of angiogenic and osteogenic GFs, and the osteogenic potential of cells [89]. Generally, GFs are presented to cells in vivo in a spatially and temporally controlled manner. Since bone is a highly vascularized tissue and angiogenesis is pivotal for bone regeneration, neovascularization facilitates recruitment of mesenchymal stem cells and osteoblasts necessary for bone repair [87]. Thus, besides osteogenesis also angiogenesis should be addressed to obtain successful bone regeneration. Moreover, recruitment of host osteoblast progenitor cells in the circulating blood to the implant site is also beneficial for bone formation [22]. Therefore, several in vivo studies were performed to investigate potential synergistic effects of delivery of multiple signaling factors, for example, BMP-2, VEGF, bFGF and TGF-β from gelatin microspheres [25], gelatin bulk hydrogels [89] and colloidal gelatin gels [27, 32, 90] were performed to mimic the natural bone healing process.

3.5.1 Dual release of growth factors from gelatin microspheres

Patel et al. [25] investigated the effects of dual delivery of VEGF and BMP-2 for bone regeneration in a rat cranial critical size defect. They loaded 12 μg VEGF and/or 2 μg BMP-2 onto gelatin microparticles and incorporated these microparticles into porous polymer scaffold made from poly(propylene fumarate). Significantly higher bone volumes were observed following dual release of VEGF and BMP-2 compared to single growth factor release after 4 weeks of implantation, pointing to synergistic effects, in other words, VEGF induced promotion of chemotaxis and differentiation of osteoblasts. However, differences between single and dual release were not observed anymore after 12 weeks, suggesting that dual growth factor release was most effective at early stages of bone healing and synergistic effects of dual release result in faster healing times.

3.5.2 Dual release of growth factors from gelatin hydrogels

Stromal cell-derived factor-1 (SDF-1, also known as C-X-C motif chemokine 12, CXCL12), is critical to induce migration and homing of stem cells to a target site [91] and recruits endothelial progenitor cells that are necessary for angiogenesis [92], Additionally, it is reported that the SDF-1 plays a
crucial role in the migration of bone marrow stromal cells (BMSCs) to the fracture site, where recruited cells actively participated in endochondral bone repair in a segmental bone defect model [93]. Therefore, it was hypothesized that co-delivery of SDF-1 and BMP-2 would stimulate stem cell recruitment, and angiogenesis (by SDF-1) as well as osteogenesis (by BMP-2) [89]. To prove this hypothesis, gelatin hydrogel were loaded with SDF-1 and BMP-2 to treat critical-sized ulna defects and induce the ectopic bone formation in rats [89]. The initial release of SDF-1 was accelerated by the combination with BMP-2, whereas the release of BMP-2 was not influenced by the co-delivery of SDF-1. Dual release of 5 µg SDF-1 and 3µg BMP-2 from these gelatin hydrogels induced a significantly greater extent of bone formation in both the critical-sized bone defect and the subcutaneous implantation site compared to the single release of either SDF-1 or BMP-2, which confirms the synergistic effects of both factors.

3.5.3 Dual release of growth factors from gelatin nanospheres colloidal gel

Recently, our laboratory developed a colloidal gel system made of oppositely charged gelatin nanospheres. Due to the reversible non-covalent (i.e. electrostatic and/or hydrophobic) interactions between these nanoparticles, cohesive and self-healing colloidal gels were obtained [17]. These nanostructured colloidal gelatin gels exhibited superior viscoelastic properties over microsphere-based gels with respect to elasticity, injectability, structural integrity, and self-healing behavior upon severe network destruction [27]. The high specific surface area of the nanospheres facilitated stronger polyion complexation of GFs to oppositely charged gelatin nanospheres, while the GFs were more effectively entrapped in the nanopores between tightly packed nanospheres, which resulted into zero-order release kinetics of BMP-2 without any initial burst release [27]. Additionally, sustained delivery of GFs from these colloidal gelatin gels was controlled by fine-tuning the cross-linking densities of gelatin nanospheres [27, 32].

These colloidal gelatin gels were also shown to be suitable for dual release of angiogenic and osteogenic GFs. Wang et al. [32] monitored the in vitro dual release kinetics of osteogenic BMP-2 and angiogenic bFGF from colloidal gelatin gels, and compared the effect of single or dual delivery of BMP-2 and bFGF on bone regeneration using a rat femoral condyle defect
model. Fast release of bFGF and slow release of BMP-2 was achieved by loading bFGF onto rapidly degrading cationic gelatin nanospheres of low cross-linking density and by loading BMP-2 onto slowly degrading anionic gelatin nanospheres of high cross-linking, respectively. The amount of new bone formation was highest for BMP-2 loaded gels while lowest for gels loaded with both BMP-2 and bFGF. The authors suggested that the lack of synergistic effects was caused by overdosing of bFGF relative to BMP-2 (bFGF:BMP-2 ratio of 1:1), thereby impeding bone formation. Therefore, identical colloidal gelatin gels were loaded with lower doses of bFGF relative to BMP-2 (bFGF:BMP-2 ratio of 1:5) and inserted into porous titanium scaffolds to treat critical-sized femoral bone defect model in rats [90]. Significantly more bone was formed outside the porous titanium scaffolds for gels containing two GFs, which points to a synergistic effect of dual delivery of bFGF and BMP-2.

3.5.4 Release of platelet-rich plasma from gelatin hydrogels

PRP is blood plasma that is enriched with platelets, which contains and releases several types of GFs including a.o. platelet-derived growth factors and TGF-β [94, 95]. Local application of PRP from bone grafts has been proposed to enhance bone regeneration since 1998 [95] as PRP is easily purified from the patient's own peripheral blood, thereby avoiding transmission of diseases. Gelatin hydrogels have also been considered as carrier for the local application of PRP during bone regeneration. Hokugo et al. [96] prepared gelatin hydrogel containing PRP for application in bone defects in rabbit ulna, resulting into successful healing after 4 weeks of implantation. In this study, PRP platelets were activated by exposure to gelatin molecules upon impregnation into the hydrogel, resulting into the secretion of platelet-derived growth factors and TGF-β1. The released PRP GFs were then immobilized onto the carrier through electrostatic interaction with gelatin macromers. As described above, the immobilized GFs were released upon enzymatic degradation of gelatin and subsequently stimulated bone formation. PRP induced bone regeneration was also observed in rabbit calvarial defects treated with PRP impregnated gelatin hydrogel [97].

4 Release of nucleic acids

GFs are by far the most popular class of biomolecules for use in bone
regeneration. Nevertheless, the fragile nature of GFs is still a major concern for the long-term efficacy of growth factor-based therapy [98]. To circumvent this problem, gene transfection strategies have been proposed, which involve the introduction of exogenous genes into cells for therapeutic purposes [99]. Specifically, gene delivery therapies in bone regeneration aim at effective delivery of plasmid DNA encoding a therapeutic protein which stimulates bone formation after secretion by cells.

To this end, cationized gelatin has been used for the controlled and sustained delivery of DNA. DNA, a polyanionic macromolecule, can electrostatically bind to cationized gelatin to form electrostatic complexes [100]. Thus, the release of plasmid DNA from gelatin carrier is driven by the enzymatic degradation of the cationized gelatin, and the release kinetics can be controlled through adjusting the cross-linking density of the gelatin. Moreover, it was suggested that DNA can remain complexed with degradation fragments of the cationized gelatin carriers [101]. In that way, degradation of the DNA by nucleases can be reduced, which facilitates cellular uptake through interaction of the positively charged complexes with negatively charged cell membranes [100].

Several studies were performed aiming at delivery of plasmid DNA encoding BMP-2 from cationized gelatin microspheres-containing scaffolds \textit{in vitro} [102] and \textit{in vivo} [103, 104]. Herein, amino groups were introduced onto cationic gelatin (IEP-9) through chemical conversion of carboxyl groups [100] followed by preparation of cationized gelatin microspheres and incorporation into OPF hydrogels. \textit{In vitro} release of plasmid DNA from hydrogels with or without gelatin microspheres was dominated by the degradation of the OPF network, and loading of DNA onto the cationized gelatin microspheres prolonged the duration of DNA release [102]. When plasmid DNA was loaded onto either gelatin microspheres or onto OPF hydrogel matrix, the \textit{in vivo} release kinetics of DNA was similar, and the release of DNA from the composites was in accordance with the degradation of the microspheres within the OPF [103].

When the hydrogel composites loaded with plasmid DNA were implanted into a critical-sized rat cranial defect no enhancement of bone formation was observed after 30 days of implantation [104]. In another study, poly(propylene fumarate) scaffolds were filled with gelatin microspheres loaded with plasmid DNA complexed with a biodegradable branched triacrylate/amine polycationic polymer to enable controlled release of DNA
Although the release of plasmid DNA could be varied by controlling the degradation rates of the TAPPs, release of DNA did not result in enhanced bone formation [105]. However, a study conducted by Hosseinkhani et al. showed that a scaffold seeded with complexes of cationized gelatin and plasmid DNA encoding BMP-2 effectively enhanced in vivo ectopic bone formation [99]. In this study, the cationized gelatin-plasmid DNA complexes were impregnated into the lyophilized collagen sponges containing poly(glycolic acid) (PGA) fibers. The lack of bone formation resulting from plasmid DNA delivery as observed by Kasper et al. [104] and Chew et al. [105] was related to poor cellular uptake of DNA, which resulted into a low transfection efficiency. Generally, it can be concluded that strategies using gelatin carriers for in vivo delivery of DNA have not yet been successful so far. Nevertheless, recent data indicate that gelatin nanospheres have the potential to enter the cell through endocytosis, thereby enhancing the transfection efficiency of DNA. In addition, it was recently shown that gelatin nanospheres can be used successfully for intracellular delivery of small interfering RNA [106]. Based on these preliminary data, intracellular delivery of DNA from gelatin nanospheres instead of microspheres will be a viable strategy to achieve stimulation of new bone formation.

5 Release of antibiotics

Bone defects caused by severe trauma are highly susceptible to infection. This type of infection easily develops into osteomyelitis, which is a severe bone infection and associated with a high morbidity [107]. Besides, osteomyelitis typically lead to complications such as bone resorption, bone dysfunction, and progressive inflammatory destruction of bone [108]. Traditional treatment of osteomyelitis involves systemic administration of antibiotics to eradicate the residual bacteria after the removal of the affected tissue [109]. This treatment, however, may lead to systemic toxicity and side effects. To circumvent these clinical complications, local delivery of antibiotics is a potential alternative treatment option that would increase local concentrations and decrease required dosing and corresponding side effects. To this end, the beneficial properties of gelatin for local delivery have been exploited to facilitate controlled release of antibiotics in vitro and in vivo to treat bone infection or osteomyelitis caused by bone reconstruction.
5.1 Release of antibiotics from gelatin microspheres

Shi et al. [110] loaded colistin sulfate onto gelatin microparticles by diffusional postloading, and incorporated the swollen gelatin microparticles into polymethylmethacrylate to form antibiotic-releasing polymethylmethacrylate/gelatin/antibiotic constructs. In these constructs, gelatin microparticles served as both biomolecule carrier and porogen. Colistin released continuously during 10 or 14 days with an average release rate above 10 µg/ml per day in vitro. After degradation of the gelatin microparticles a porous structure was formed, which was suggested to favor ingrowth of fibro-vascular and other tissue types.

5.2 Release of antibiotics from gelatin hydrogels

Wu et al. [111] developed a β-TCP bactericidal scaffold reinforced with a gentamicin-doped gelatin/genipin hydrogel. The initial burst release of gentamicin from this scaffold was followed by zero-order release kinetics which effectively inhibited the growth of Staphylococcus aureus both in vitro and in vivo. Using a rat osteomyelitis model, it was shown that osteomyelitis could be fully cured within 3 weeks of implantation. Commonly used antibiotics (gentamicin or vancomycin) have also been loaded into interpenetrating hydrogel networks based on poly(acrylic acid) and gelatin by simple diffusional post-loading to treat osteomyelitis in rabbits [112]. This scaffold degraded completely and healed the infection within 6 weeks after implantation. No differences in healing rate were observed between the release of gentamicin or vancomycin.

5.3 Release of antibiotics from gelatin/bioceramics composites

Antibiotics have also been loaded to osteoconductive and osteoinductive bioceramics like β-TCP [113, 114] or hydroxyapatite (HA) [115] to prevent bone infections during bone repair or implant surgery. Yaylaoğlu et al. [116] mixed gelatin, gentamicin and calcium phosphate by means of solution-casting followed by glutaraldehyde cross-linking of gelatin to prepare membrane-like composites. Gentamicin released from the composite in a burst manner both in vitro and in vivo, but details on the release kinetics were not provided in that article. In another study, Sezer et al. [113] prepared β-TCP/gelatin composite microspheres cross-linked with different amounts of glutaraldehyde using water-in-oil emulsification, where gentamicin was
loaded onto these microspheres by diffusional post-loading. The same authors also added these gentamicin-loaded microspheres into poly(ε-caprolactone) matrices to prepare biodegradable antibiotic-releasing composite films for bone regeneration [114]. A minimal initial burst (20-30%) followed by sustained release of gentamicin was observed until 5 days of soaking when 78% of the antibiotic was released upon degradation of gelatin microspheres and poly(ε-caprolactone) matrices. Kim et al. [115] fabricated porous gelatin-HA nanocomposites loaded with tetracycline during cross-linking of the lyophilized scaffold in an acetone-water solvent mixture using carbodiimide chemistry. Tetracycline release kinetics were studied as a function of HA content and cross-linking density. The majority of the tetracycline was released during an initial burst (up to 10 h) followed by a more sustained release until 3 days. In this study, however, the amount of biomolecule loading was diffusion-limited by the large dimensions of the porous gelatin-HA scaffolds. Nevertheless, biomolecule loading by simple swelling of gelatin or gelatin composite in drug solution [110, 112-114] can increase the loading efficiency up to around 100%.

### 5.4 Future improvements of gelatin-based carriers for release of antibiotics

Generally, it should be emphasized that an objective comparison and evaluation of the above-mentioned studies and materials is not straightforward by any means due to several complicating factors in the study design. Firstly, the majority of the release studies were performed in vitro which cannot be correlated to the in vivo conditions. Release periods and dosing of antibiotics largely varied among the various studies, stressing that consensus on the desired release kinetics and duration is still lacking although it is known that the local concentration of antibiotics should exceed the minimal inhibitory concentration (MIC) of the respective antibiotic. Therefore, studies comparing the loading and release mechanism of different types of antibiotics from different forms of gelatin carriers (i.e. bulk hydrogels and spheres) are needed to optimize the antibacterial effect of local delivery of antibiotics from gelatin-based carriers.

### 6 Conclusion

Gelatin has gained considerable interest in the field of bone regeneration as carrier for local delivery of biomolecules. These biomolecules bind to
Delivery of Biomolecules from Gelatin Carriers for Bone Regeneration

gelatin matrices through formation of polyion complexes with gelatin macromolecules upon diffusional postloading, thereby retaining their biological activity. Upon enzymatic degradation of gelatin carriers, the loaded biomolecules are released at rates which can be precisely tuned by adjusting the cross-linking density of gelatin. This mechanism has been proved for large biomolecules such as the GFs and DNA, whereas the loading and release mechanism of small molecules such as antibiotics remain to be elucidated. Current challenges for clinical translation and commercialization of gelatin-based carriers for bone regeneration and infection include upscaling of carrier manufacturing under the strict regulations of good manufacturing practice, and selection of proper preclinical animal models to correlate in vivo release kinetics with detailed characterization of the response of bone tissue to local delivery of biomolecules.

7 Future perspective

Despite two decades of research on the use of gelatin carriers for local biomolecule delivery, gelatin carriers have not yet been used for clinical treatment of bone defects/infections and/or commercially available medical devices. Proof-of-concept for the suitability of gelatin carriers for local delivery of biomolecules to bone tissue has been obtained in a plethora of pre-clinical animal studies. These studies, however, were performed in a wide range of animal models and types of bone, which complicates comparison of the obtained data in terms of properties of the gelatin carriers (e.g. gelatin type, form and cross-linking) and characteristics of the respective biomolecules (e.g. biomolecule type, dosing and loading).

In order to meet these challenges, development of cost-effective upscaling methods to prepare gelatin carriers (as bulk hydrogels, micro- and nanoparticles, etc.) under the strict regulations of good GMP will become a critical issue. Specific attention should be paid to the physicochemical characteristics of the commercially available gelatins in terms of molecular weight, isoelectric point and purity to guarantee reproducible industrial production of gelatin-based carriers. In addition, clinicians and experts in chemistry, materials science, bone biology and pharmaceutical research should join forces to optimize biomolecule release from gelatin carriers according to clear and pre-defined criteria in terms of dosing, loading and in vivo release kinetics. After these optimization studies, the efficacy of biomolecule delivery from gelatin carrier should be directly compared to
conventional carriers for local delivery of biomolecules such as collagen fleeces and sponges. Finally, advanced pre-clinical imaging tools such as micro single-photon emission computed tomography (microSPECT) [117] should be used to monitor the in vivo release of labeled biomolecules in bone tissue both spatially and temporally, and correlate these findings to the actual effects of biomolecule release on bone tissue using histology and histomorphometry.
References

39. Yamamoto, M., Y. Takahashi, and Y. Tabata, Controlled release by biodegradable


92. Honczarenko, M., et al., Human bone marrow stromal cells express a distinct set of
112. Changez, M., V. Koul, and A.K. Dinda, Efficacy of antibiotics-loaded interpenetrating


Chapter 3

Influences of the molecular weight and charge of antibiotics on their release kinetics from gelatin nanospheres

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1 Introduction

Bacterial infections occur when harmful bacteria invade and reproduce in the host. This infectious disease can develop into serious, even life-threatening conditions such as osteomyelitis [1]. This type of infection is associated with a high morbidity [2] and occurs when a complicated bone fracture is exposed to bacteria [3]. Traditional treatment modalities for osteomyelitis involve systemic administration of antibiotics for several weeks to months [4], which is often associated with toxicity, antibiotic resistance and recurrence of infections [5, 6]. In addition, bone infections are frequently caused by more than one type of bacteria [7, 8], which requires the delivery of different types of antibiotics from a single carrier to avoid ineffective treatment and antibiotic resistance. A powerful strategy to reduce the complications and side-effects related to the systemic use of antibiotics involves local and sustained delivery of multiple antibiotics at the site of infection. In order to facilitate this strategy, a suitable carrier system should be developed that is biocompatible, safe, and most importantly, biodegradable in a controllable manner [9]. Gelatin, a denatured protein prepared by hydrolysis of collagen, is particularly attractive as a drug carrier for controlled release due to its non-toxicity, bioactivity, cost-effectiveness, tunable charge and degradability [10-13].

Gelatin nanospheres (GNs) have been extensively studied as carriers for controlled delivery of therapeutic biomolecules of high molecule weight such as growth factors [14-20] and nucleic acids [21-26]. These macromolecules were shown to bind to oppositely charged gelatin carriers through the formation of polyelectrolyte complexes [12] between macromolecules and gelatin matrices, thereby facilitating sustained delivery of bioactive macromolecules. However, it is still unknown if small biomolecules of low molecular weight (< 1.5 kDa) such as antibiotics can be released from GNs in a sustained manner. Therefore, detailed studies on the interactions between various antibiotics and gelatin carriers are necessary to understand their mutual interaction upon diffusional post-loading. These studies will be useful to become able to adjust the release kinetics of antibiotics from GNs and exploit the full potential of gelatin as carrier for local delivery of antibiotics.

Recently, it has been shown that the physicochemical characteristics of antibiotics have a strong influence on the release kinetics of antibiotics.

Properties of Antibiotics Influence Their Release from Gelatin Nanospheres
from synthetic polymers [27]. Shah et al. [27] found that the charge and molecular weight of antibiotics influenced the incorporation into and release of antibiotics from poly (DL-lactic-co-glycolic acid) microparticles. Negatively charged antibiotics were incorporated less efficiently than positively charged antibiotics, while antibiotics of higher molecular weight revealed a more sustained release. Nevertheless, the mechanisms of interaction between antibiotics and polymeric carriers have not been studied before, thereby hindering full understanding of the loading and release of antibiotics from polymeric carrier materials. Therefore, the aim of our study was to investigate the fundamental relationship between the physicochemical characteristics of antibiotics and the kinetics of their release from GNs, aiming at the development of a simple and practical strategy towards local and sustained delivery of antibiotics. We hypothesized that sustained release of antibiotics from GNs could only be achieved provided that sufficiently strong electrostatic and hydrophobic interactions could be established between the antibiotics and GNs, which were hypothesized to depend strongly on the molecular weight and charge of the antibiotics.

To this end, we selected four commonly used water-soluble antibiotics of different molecular weight and charge (i.e. gentamicin, moxifloxacin, vancomycin and colistin, see Table 1 and Figure S1 for more detail). Firstly, we compared the long-term kinetics of the release of these antibiotics from GNs of low and high cross-linking densities in the absence or presence of collagenase. Additionally, we studied the short-term release of these antibiotics from GNs as a function of pH, ionic strength and detergent concentrations without any collagenase present to investigate the fundamental interactions between the antibiotics and gelatin carriers. Finally, we correlated the release kinetics of the antibiotics with the degradation rate of gelatin carriers to unravel the mechanisms of antibiotic release from gelatin carriers.
2 Materials and Methods

2.1 Materials
Gelatin type B (from bovine skin, 225 bloom, isoelectric point (IEP) ~ 5), Sigma-Aldrich, The Netherlands); gentamicin sulfate (potency ≥ 590 µg/mg), moxifloxacin hydrochloride (VETRANAL™, analytical standard), vancomycin hydrochloride hydrate (potency ≥ 900 µg/mg) and colistin sulfate (potency ≥ 15000 U/mg) were purchased from Sigma-Aldrich, all of the antibiotics salts are soluble in water. Relevant physicochemical properties of these antibiotics are listed in Table 1. Glutaraldehyde (25 wt% solution in water) was purchased from Acros Organics while all other chemicals were purchased from Sigma-Aldrich.

2.2 Preparation of gelatin nanospheres
Gelatin nanospheres of low and high cross-linking densities (GNLs and GNHs) were prepared using a two-step desolvation method [28]. In brief, 1.25 g gelatin type B was dissolved in 25 ml deionized water to form a 5 w/v% solution at 50 °C. 25 ml acetone was then added to precipitate the high molecular weight gelatin. Gelatin type B was selected to allow for complexation with oppositely charged antibiotics. After precipitation, the supernatant was discarded and the gelatin was re-dissolved in water at 50 °C, followed by adjusting the pH of the gelatin solution to 2.5 using hydrochloric acid solution. Thereafter, 80 ml acetone was added dropwise (~2 ml/min) into the gelatin solution under vigorous stirring to form GNs. Subsequently, the GNs were stabilized by adding different amounts of glutaraldehyde into the suspension yielding molar ratios of glutaraldehyde relative to the amine content of gelatin of 1:2 (GNL, 74 µl of glutaraldehyde per gram of gelatin) and 4:1 (GNH, 592 µl of glutaraldehyde per gram of gelatin), respectively. After cross-linking for 16 h, 100 mM glycine solution was added to block unreacted aldehyde groups. The suspension was then subjected to three cycles of centrifugation (5000 rpm for 60 min) and re-suspension in deionized water by vortexing, after which the pH of the nanosphere suspension was adjusted to pH 7.0 and lyophilized for 24 h. The lyophilized GNs were stored at 4 °C for further use.

2.3 Characterization of gelatin nanospheres
The morphology of the GNs was characterized by scanning electron
microscopy (SEM, JEOL 6301). Images of the GNs were taken at an accelerating voltage of 5 kV. The size and size distribution of GNs were measured using dynamic light scattering (DLS, Zetasize Nano-S, Malvern Instruments Ltd., Worcestershire, United Kingdom) by dispersing the GNs in 5 mM HEPES buffer, at pH 7.0. ζ-potential values of GNs were measured by laser doppler electrophoresis with a Zetasizer Nano-Z (Malvern Instruments Ltd., Worcestershire, United Kingdom) using a folded capillary cell (DTS 1060) in HEPES buffer (5 mM, pH 7.0). The water content of GNs was detected according to a previously published method [17] by comparing the weight of GNs in dry and fully swollen state in PBS. The amount of residual free amine groups as present in the cross-linked gelatin spheres was quantified using the 2,4,6-trinitrobenzene sulfonic acid (TNBS) assay. Specifically, approximately 2 ~ 4 mg GNs were dispensed in 2 ml NaHCO3 solution (pH 8.5, 4 w/v%) and reacted with 0.01M 2,4,6-trinitrobenzenesulfonic acid (TNBS) for 2 h at 40 °C, followed by hydrolyzing with 3 ml HCl (1 M) for 1.5 h at 60 °C. After hydrolysis, the solution was cooled to room temperature and 5 ml sterile deionized water was added. Finally, the absorbance of the solution was measured at λ = 420 nm with an enzyme-linked immunosorbent assay (ELISA) plate reader (EL800, Bio-Tek Instruments. Inc.). The weight percentage of free amine groups per milligram of gelatin was calculated according to the standard curve prepared by using glycine as the standard reagent.

2.4 Long-term release of antibiotics from gelatin nanospheres

A diffusional post-loading method was used to load the antibiotics onto GNs, which involved direct mixing of the solution containing antibiotics with lyophilized GNs. The volume of antibiotic-containing solution was lower than required for complete swelling of GNs yielding a colloidal gel with a solid content of 20 w/v%, while the amount of antibiotic was fixed at 5 μg per gram of GNs. Briefly, 25 μl of 1 mg/ml antibiotic in PBS solution was dripped onto 5 mg of GNs in an 1.5 ml Eppendorf® (n = 5). After loading, the mixtures were stored at 4 °C overnight to allow for complete swelling of gelatin carriers and sorption of antibiotics, followed by centrifugation at 13000 rpm for 5 min. 1 ml PBS containing 400 ng collagenase IA and 0.001 w/v% sodium azide was added into the tubes to start the in vitro release study. The tube was then incubated at 37 °C on a rotating plate at a speed of 100 rpm. At each time point, the tubes were centrifuged at 13000
rpm for 5 min to sediment the gelatin spheres, after which 900 μl of the supernatant was obtained for further analysis and refreshed with the same amount of medium.

### 2.5 Influence of pH, ionic strength and detergent concentration on the short-term release of antibiotics

GNHs were used as carriers for an additional release study on the kinetics of antibiotic release in media with different i) pH (PBS at pH 7.4 at an ionic strength of 154 mM as well as citrate buffers at pH 5 and 3 at a similar ionic strength of 154 mM), ii) ionic strength (10 mM, 100 mM and 1000 mM NaCl solutions in water) and iii) detergent concentration (0.002 v/v%, 0.01 v/v% and 0.1 v/v% Tween 20). As described previously, 25 μl of solutions containing 1 mg/ml antibiotic in PBS were mixed with 5 mg GNs in 1.5 ml Eppendorf® tubes and stored at 4 °C overnight to form antibiotic-loaded colloidal gels. To start the short-term release studies, 1 ml of the above-mentioned media were added into each tube separately (n = 3). At each time point, the tubes were centrifuged at 13000 rpm for 5 min to sediment the gelatin spheres, after which 900 μl of the supernatant was obtained for further analysis and refreshed with the same amount of medium.

### 2.6 Detection of antibiotics in the supernatants

The concentration of gentamicin was monitored by an indirect ELISA method. A gentamicin-bovine serum albumin conjugate was coated onto the ELISA-plate as a competitor for the soluble gentamicin in the sample. The concentration of gentamicin was determined in relation to a gentamicin calibration curve (range: 1 - 1000 ng/ml). The detection was performed by a specific anti-gentamicin primary antibody (Abcam, USA) and a horseradish peroxidase (HRP) labeled secondary antibody (Dako, Denmark) subsequently visualized by 3,3′,5,5′-tetramethylbenzidine (TMB, Sigma-Aldrich, USA) and measured at 450 nm in an ELISA reader (MultiSkan FC, Thermo Scientific).

Moxifloxacin [29] and vancomycin [30] concentrations were detected by reverse phase high performance liquid chromatography (RP-HPLC) using a Hitachi HPLC machine. This system consisted of a Hitachi L-2130 pump, a Hitachi L-2400 UV detector and a Hitachi L-2200 auto samper, as well as a LiChrospher® RP-18 endcapped HPLC column (125 mm × 4 mm, particle size 5 μm). For moxifloxacin, the mobile phase consisted of 50
mM phosphate buffer (pH 2.6) and acetonitrile (75:25, v/v). For analysis of moxifloxacin concentrations, 50 μl of moxifloxacin-containing samples were injected and the effluent was monitored at 296 nm with a flow rate of 1 ml/min. Regarding the detection of vancomycin, a mixture of 50 mM ammonia phosphate buffer (pH 3, adjusted with H3PO4) and acetonitrile (90:10, v/v) was used as the mobile phase, at a flow rate of 1 ml/min and an injection volume of 50 μl. The UV detector was set at 240 nm to quantify the vancomycin concentrations without any interference from gelatin degradation products. Standard calibration curves in the range of 0.1 to 25 μg/ml and 0.2 to 25 μg/ml were prepared for the quantification of moxifloxacin and vancomycin, respectively. Release of colistin was monitored using liquid chromatography-mass spectrometry (LC/MS) [31]. The liquid chromatography was performed using a Shimadzu system with two LC20AD pumps, a SIL20AC autosampler, a CTO20AC oven at 35 °C and an RP-C18 column (Phenomenex Gemini NX, 50 mm × 2.0 mm, 3 μm, 110A), while the mass spectrometry was conducted using a Thermo® LCQ Fleet® electrospray ion-trap mass spectrometer. The mobile phase consisted of acetonitrile and water (both containing 0.1% formic acid), with a gradient of acetonitrile from 5% to 100% in 10 min at a flow rate of 0.2 ml/min. 100 μl of sample was injected and the doubly charged ions ([colistin A + 2H]2+ at m/z 585.9 and [colistin B + 2H]2+ at m/z 578.8) were analyzed in the mass spectra collected in the range of 100 to 2000 in order to determine the amount of colistin present in the samples. The amount of colistin was quantified with a standard calibration curve in the range of 0.79 to 25 μg/ml.

### 2.7 In vitro degradation of gelatin nanospheres

The degradation behavior of GNs was monitored under the same conditions as the release of antibiotics, except for the fact that the GNs were loaded with 25 μl deionized water instead of antibiotic-containing PBS. The amount of gelatin degradation at various time points was quantified by measuring the total protein released in the supernatant using the bicinchoninic acid (BCA) assay. Briefly, 25 μl deionized water was loaded onto 5 mg GNs in 1.5 ml Eppendorf® tubes and stored at 4 °C overnight to allow for the complete swelling of GNs (n = 5). 1 ml PBS containing 400 ng/ml collagenase IA and 0.001 w/v% sodium azide was then added into the tube, which was then incubated at 37 °C on a rotating plate. At each time point, 900 μl of
supernatant was collected for the BCA assay after centrifugation at 13000 rpm for 5 min to sediment the GNs, and the tubes were refreshed with the same amount of fresh medium.

2.8 Statistics
All of the measurements were depicted as average ± standard deviation and the statistical analyses were conducted using GraphPad Prism. Differences among groups were analyzed by Analysis of Variance (ANOVA) followed by a Tukey post-hoc test, and a value of P < 0.05 was considered as a statistically significant difference.

3 Results
3.1 Long-term release of antibiotics from gelatin nanospheres
In the presence of collagenase, vancomycin and colistin were released from GNLs and GNHs in a sustained manner for more than 14 days (Figure 1A and 1B). Comparable amounts of colistin and vancomycin were released from GNLs (Figure 1A), whereas almost two times more vancomycin was released from GNHs as compared to colistin. In the absence of collagenase, however, sustained release was only observed for vancomycin, whereas the release of colistin stopped after two days (Figure 1C and 1D).

![Figure 1](image_url). Release kinetics of antibiotics from gelatin nanospheres of low (A, C) and high (B, D) cross-linking densities in the presence (A, B) or absence of collagenase (C, D).
contrast, gentamicin and moxifloxacin were released in a burst-wise manner irrespective of the gelatin cross-linking densities or the presence of collagenase (Figure 1A-D).

### 3.2 Influence of pH, ionic strength and detergent concentration on the short-term release of antibiotics

To investigate the interactions between antibiotics and gelatin carriers in more detail, antibiotics were released from GNs in media of different pH, ionic strength and detergent concentration. The corresponding release profiles of the various antibiotics are shown in Figure 2 - 5.

#### 3.2.1 Colistin release

Release of colistin from both GNLs and GNHs revealed two distinct phases (Figure 2A). In the presence of collagenase, an initial burst release after 1 day was followed by a phase of sustained release. Without collagenase, on the contrary, no phase of sustained colistin release was observed after the initial burst phase. More than two times more colistin was released from
GNLs compared to GNHs in the burst release stage, irrespective of the presence of collagenase.

The amount of colistin release after 3 days increased strongly with decreasing pH from about 20% at neutral pH (7.4) to almost 80% at acidic conditions (pH of 3.0) (Figure 2B). Although the release of colistin also increased with increasing ionic strength, the effect of ionic strength on colistin release was much less pronounced than the pH (Figure 2C). Similarly, the effect of the addition of detergent (Tween 20) was also minor compared to the effect of pH (Figure 2D) since colistin was not released at low concentrations while less than 15% of colistin was released when the concentration of Tween 20 was increased to 0.1 v/v%.

### 3.2.2 Vancomycin release

Release of vancomycin from GNHs did not depend on the presence or absence of collagenase. More vancomycin was released from GNLs, whereas the presence of collagenase resulted into more vancomycin

![Figure 3](image_url)

**Figure 3.** Release of vancomycin from gelatin nanospheres of low (GNL) and high cross-linking densities (GNH) in the presence (GNL-CA and GNH-CA) or absence of collagenase (GNL and GNH) (A); release of vancomycin from gelatin nanospheres of high cross-linking density in media of different pH (B), ionic strength (C) and detergent concentrations (D) in the absence of collagenase.
release from day 7 onward (Figure 3A). The addition of Tween 20 had the most pronounced effect on the rate and amount of vancomycin release since vancomycin release was negligible at low detergent concentrations but increased dramatically to more than 90% at the highest detergent concentration of 0.1 v/v% (Figure 3D). In contrast, the effects of pH and ionic strength on the release of vancomycin were less pronounced (Figure 3B and 3C) although it can be concluded that the release of vancomycin increased with increasing ionic strength and decreasing pH.

### 3.2.3 Gentamicin and moxifloxacin release

Gentamicin and moxifloxacin displayed a comparable burst-type release behavior characterized by more than 70% of cumulative release within less than 1 day irrespective of the degree of cross-linking (GNLs vs. GNHs) or the presence of collagenase (Figure 4A and Figure 5A). Gentamicin release increased considerably with increasing ionic strength (Figure 4C), while the addition of detergent stimulated release of gentamicin at the

![Figure 4](image_url)

**Figure 4.** Release of gentamicin from gelatin nanospheres of low (GNL) and high cross-linking densities (GNH) in the presence (GNL-CA and GNH-CA) or absence of collagenase (GNL and GNH) (A); release of gentamicin from gelatin nanospheres of high cross-linking density in media of different pH (B), ionic strength (C) and detergent concentrations (D) in the absence of collagenase.
highest detergent concentration only (i.e. 0.1 v/v%, Figure 4D). The effect of pH was negligible since differences between the various pH values were statistically insignificant (Figure 4B). Moxifloxacin release was not affected by differences in pH (Figure 5B), while moxifloxacin release increased only moderately with increasing ionic strength and detergent concentration.

**Figure 5.** Release of moxifloxacin from gelatin nanospheres of low (GNL) and high cross-linking densities (GNH) in the presence (GNL-CA and GNH-CA) or absence of collagenase (GNL and GNH) (A); release of moxifloxacin from gelatin nanospheres of high cross-linking density in media of different pH (B), ionic strength (C) and detergent concentrations (D) in the absence of collagenase.

**4 Discussion**

The main goal of the current study was to investigate the main interaction forces between antibiotics and gelatin matrices in order to unravel the different mechanisms of antibiotic release from GNs. To this end, we studied the release kinetics of four commonly used antibiotics of different molecular weight and charge (Table 1 and Figure S1) from GNLs and GNHs (Figure S2). We observed that colistin and vancomycin could be released in a sustained manner for more than 14 days in the presence of collagenase, whereas gentamicin and moxifloxacin were released in a burst-wise
manner within less than two days (Figure 1A and 1B). Gelatin is a denatured protein which is prepared through the hydrolysis of collagen. At neutral pH, gelatin A (IEP of 7-9) is positively charged while gelatin B (IEP of 4.8-5) is negatively charged [32]. This charged nature of gelatin renders this biopolymer suitable for controlled delivery of oppositely charged biomolecules. The most widely accepted mechanism to explain release kinetics of bioactive macromolecules (e.g. growth factors) from gelatin carriers relies on the formation of polyion complexes between charged biomolecules and oppositely charged gelatin carriers, where sustained release of biomolecules is enabled by enzymatic degradation of gelatin [12]. However, our results showed that the most positively charged antibiotic (i.e. gentamicin [33], see Table 1) was not released in a sustained manner from oppositely charged GNs. In contrast, vancomycin had a higher molecular weight and was released in a sustained manner although this antibiotic contains a much lower amount of positively charged amine groups than gentamicin. Finally, most sustained release profiles were observed for colistin of high molecular weight and intermediate charge. These results indicate that the release of colistin was most likely caused by the formation of polyion complexes, whereas the formation of such complexes between gelatin carriers and gentamicin or vancomycin was unlikely. Therefore, we have explored the interactions between antibiotics and gelatin carriers in more detail in order to obtain fundamental insight into the release kinetics of the selected antibiotics.

Generally, gelatin macromolecules consist of both positively (~ 13% lysine and arginine), negatively (~ 12% glutamic and aspartic acid) and hydrophobic amino acids (~ 11% leucine, isoleucine, methionine and valine) [10]. This composition facilitates the establishment of electrostatic and hydrophobic interactions between antibiotics and gelatin carriers. To investigate the formation of electrostatic interactions between antibiotics and gelatin nanospheres, the charged nature of gelatin was affected by i) decreasing the pH of the release medium (to switch the charge of GNs from negative to positive), and ii) increasing the ionic strength of the medium (to screen the charge of GNs). Moreover, to examine the formation of hydrophobic interactions between antibiotics and gelatin nanospheres, we added detergent Tween 20 into the release media since amphiphilic Tween 20 can disrupt such hydrophobic interactions and accelerate the release of antibiotics that are bound to gelatin carriers by hydrophobic forces [34].
The strong increase in colistin release with decreasing pH was a clear indication that electrostatic interactions were formed between colistin and GNs. At neutral pH, gelatin carriers and colistin were oppositely charged, whereas gelatin carriers were uncharged at a pH value of 5.0 which was similar to the isoelectric point of gelatin type B [13]. At a lower pH of 3, gelatin carriers and colistin were similarly charged due to protonation of gelatin’s carboxylate and amine groups [13]. Apparently, electrostatic interactions could be formed between gelatin and colistin provided that their respective charges were opposite. Subsequent release of colistin from GNHs was shown to be pH-responsive (Figure 2B) since the extent of gelatin degradation under similarly acidic conditions was negligible (Figure S3B). The formation of electrostatic interactions between cationic colistin molecules and anionic gelatin macromers was further confirmed by the fact that colistin release increased with increasing the ionic strength (Figure 2C). Obviously, screening of the charges of carriers resulted into a decreased mutual affinity between colistin and gelatin and strongly enhanced colistin release. With increasing ionic strength, the electric double layer of the gelatin nanospheres became more compressed [28]. As a consequence, more positively charged sodium ions entered the Stern layer [35, 36] and competed with positively charged colistin molecules to establish stable complexes with the negatively charged groups of gelatin. Since only a limited amount of colistin (27%) was released after 3 days into solutions of highest ionic strength (1000 mM, Figure 2C), it can be concluded that the electrostatic interactions between colistin and gelatin carriers were strong and not easily disrupted by charge screening. These results are in line with the observation that colistin was only released upon enzymatic degradation of gelatin after the initial burst (Figure 2A), which stresses that diffusional post-loading of colistin onto gelatin carriers resulted into a tight interaction between the carriers and antibiotics. In addition, it was shown that only 13% of colistin was released into media of very high detergent concentration (0.1 v/v% Tween 20) (Figure 2D), which confirms that hydrophobic interactions hardly contributed to the formation of bonds between colistin and gelatin. Summarizing, our findings provide evidence that the interactions between colistin and gelatin nanospheres were strong and mainly of electrostatic nature. Owing to the high strength of these interactions, the release of colistin from GNs was strongly dependent on the enzymatic degradation of GNs (Figure
2A, Figure S3A) since colistin was not released anymore after the initial burst from both GNLs and GNHs when collagenase was not present. The initial burst release of colistin that preceded the linear, sustained release phase corresponded weakly bound colistin since gelatin hardly degraded during the first 2 days (Figure S3A). Highly cross-linked GNs displayed a lower burst release (~ 20%) than GNs of lower cross-linking density (~ 50%) (Figure 2A). This observation was attributed to the lower amount of free amine groups for GNHs that resulted from cross-linking using glutaraldehyde, which rendered these carriers more negatively charged than GNLs (Table S1).

Electrostatic interactions were also formed between vancomycin and gelatin carriers, as the release of vancomycin from GNHs increased moderately with increasing pH (Figure 3B) and ionic strength (Figure 3C). These electrostatic forces were, however, not the only type of forces that contributed to the binding of vancomycin to gelatin. Vancomycin was released almost completely in the medium with the highest concentration (0.1 v/v%) of Tween 20 (Figure 3D), thereby confirming that both electrostatic and hydrophobic forces contributed to the strong affinity between vancomycin and gelatin. Moreover, in the presence of collagenase, the degradation rate of GNLs was much faster than that of GNHs from day 5 onwards (Figure S3A). Apparently, the release rate of vancomycin was affected by the degradation rate of gelatin nanospheres due to the formation of strong hydrophobic and electrostatic interactions between vancomycin and gelatin. Consequently, the release of vancomycin was slightly accelerated after 5 days by the presence of collagenase for GNLs only, whereas the presence of collagenase did not affect the kinetics of vancomycin release for GNHs of higher cross-linking density (Figure 3A).

Apparently, vancomycin release was controlled by weak interactions up to the fifth day of soaking, while enzymatic degradation of GNLs became active after 5 days of soaking. Both electrostatic and hydrophobic interactions were formed between gentamicin and GNs, since the release of gentamicin increased dramatically with increasing ionic strength and concentration of Tween 20 (Figure 4C and 4D). However, the combined action of both forces was still too weak to bind gentamicin sufficiently strong to gelatin carriers, resulting into a lack of bonding strength between gentamicin and gelatin and correspondingly a burst-wise release profile. Moxifloxacin, on the other hand, was hardly affected by changes in pH or ionic strength, while its release was only
slightly affected by increasing the concentration of Tween 20. From our findings, it can be concluded that the interactions that are formed between antibiotics and gelatin carriers are strongly dependent on the inherent physicochemical properties of these antibiotics, thereby directly determining antibiotics release kinetics. Specifically, molecular weight and charge are two major parameters that contribute to differences in release kinetics. In the current study, molecular weight had a stronger effect on the release kinetics of antibiotics than antibiotic charge density. Antibiotics of higher molecular weight (i.e. colistin and gentamicin) were released in a sustained manner irrespective of their charge density, whereas antibiotics of low molecular weight were released in a burst-type manner even though antibiotics of highly positive charge such as gentamicin were investigated. Apparently, the number of non-covalent interactions between gelatin carriers and antibiotics is a more important criterion for the formation of a tight bonding between antibiotics and gelatin than their charge difference. Finally, we investigated the relationship between the release of antibiotics and the degradation of gelatin carriers by plotting the cumulative release of antibiotics vs. the cumulative degradation of GNs (Figure 6). After the initial burst release, linear correlations between antibiotic release and GNs degradation were observed for both high molecular weight colistin and vancomycin (Figure 6), whereas low molecular weight gentamicin and moxifloxacin were released from gelatin carriers in a burst-type manner without any correlation to the amount of GNs degradation. Summarizing, our data suggests that antibiotics of relatively high molecular weight can be released in a sustained manner from GNs. These results confirmed that the release of antibiotics from gelatin carriers strongly depends on the physicochemical characteristics of antibiotics. As a consequence, the data

![Figure 6. Correlations between antibiotics release and degradation of gelatin nanospheres of low (A) and high (B) cross-linking densities.](image-url)
presented herein are instrumental for the design of novel gelatin-based carrier systems for local delivery of antibiotics. Although this study provides useful information on the interactions between antibiotics and GNs, several stumbling blocks towards clinical translation and commercialization of GNs for local delivery of antibiotics need to be overcome. Besides the complexity of gelatin-antibiotic interactions, the variable physicochemical characteristics of commercially available gelatin in terms of molecular weight, isoelectric point and purity remain a challenge. This holds particularly true for GNs, since their synthesis is sensitive to small variations in e.g. the molecular weight distribution and/or charge of gelatin. Therefore, it is of utmost importance to reproducible industrial production of GNs by securing access to gelatin of controllable physicochemical properties. Subsequently, the antibacterial effects of gelatin carriers loaded with antibiotics should be investigated in extensive in vitro and in vivo studies. In doing so, the efficacy of antibiotics delivery from gelatin carrier should be directly compared to conventional carriers for local delivery of antibiotics such as collagen fleeces and sponges.

5 Conclusions

The release of antibiotics loaded onto GNs by simple diffusional post-loading was shown to depend strongly on the physicochemical properties of the antibiotics. Antibiotics of high molecular weight (colistin and vancomycin) were released in a sustained manner for more than 14 days, whereas antibiotics of low molecular weight (gentamicin and moxifloxacin) were released in a burst-wise manner. The interactions between antibiotics and gelatin carriers were mainly dominated by i) strong electrostatic forces for colistin, ii) strong hydrophobic and electrostatic forces for vancomycin, iii) weak electrostatic and hydrophobic forces for gentamicin, and iv) weak hydrophobic forces for moxifloxacin. The release kinetics of these four selected antibiotics strongly correlated to the binding affinity of antibiotics to gelatin carriers which decreased in the order colistin (high molecular weight and charge) > vancomycin (high molecular weight and neutral) > gentamicin (low molecular weight and charge) > moxifloxacin (low molecular weight and neutral).

Acknowledgement

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Properties of Antibiotics Influence Their Release from Gelatin Nanospheres

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References

19. Kuroda, Y., et al., Treatment of experimental osteonecrosis of the hip in adult rabbits with
Properties of Antibiotics Influence Their Release from Gelatin Nanospheres


36. Travesset, A. and S. Vangaveti, Electrostatic correlations at the Stern layer: Physics or
**Supplementary information**

**Table S1.** Parameters of gelatin nanospheres

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Nanospheres (swollen state)</th>
<th>Low CL</th>
<th>High CL</th>
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</thead>
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<tr>
<td>Molar ratio of GA/NH₂</td>
<td></td>
<td>1/2</td>
<td>4/1</td>
</tr>
<tr>
<td>Particle size (nm)</td>
<td></td>
<td>590.5 ± 8.7</td>
<td>397.6 ± 5.0</td>
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<td>Water content (%)</td>
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<td>Amount of free amine groups (μg/mg)</td>
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<td>32.7 ± 1.3</td>
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<td>ζ-potential (mV)</td>
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<td>-10.2 ± 0.7</td>
<td>-21 ± 1.2</td>
</tr>
</tbody>
</table>

*Note: CL = cross-linking density; GA = glutaraldehyde.*

**Figure S1.** Chemical structures of gentamicin (A), moxifloxacin (B), colistin (C) and vancomycin (D).

**Figure S2.** Scanning electron micrographs of gelatin nanospheres of low (A) and high (B) cross-linking densities.
Figure S3. Degradation of gelatin nanospheres (GNs) of low and high cross-linking densities in medium containing 400 ng/ml collagenase (A); degradation of gelatin nanospheres of high cross-linking density in medium of different pH without collagenase (B)
Electrophoretic deposition of chitosan coatings modified with gelatin nanospheres to tune the release of antibiotics

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1 Introduction
Orthopedic and dental implants are increasingly used for applications such as bone fractures and fixation of nonunions, joint replacement, spinal revision surgery, and oral and maxillofacial surgery [1]. These implants improve the quality of life by restoring mobility and function and alleviating pain [2]. Although modern implantology has made significant progress over the past few decades, implant-associated infections following invasive surgeries remain a major clinical challenge without satisfactory clinical treatment, which may lead to implant failure and acute and often chronic complications [3-5]. Therefore, orthopedic implants that have the capacity to kill bacteria and facilitate host-tissue integration to prevent implant-associated infections must be developed [1, 6, 7].
To achieve such functional performance, the application of a biologically active coating as a local antibacterial drug delivery system to implant surfaces is advocated as an effective strategy. Compared to traditional systemic delivery by oral or intravenous administration, this local delivery can provide a site-specific release of antibacterial drugs at high concentrations for a prolonged time period without causing systemic toxicity and antibacterial resistance [3, 4, 8]. Consequently, various coating techniques, including electrophoretic deposition (EPD) [3, 9, 10], solvent casting [11], dip coating [12], and sol-gel coating [13] have been used to deposit such biologically active coatings onto implants. These techniques enable incorporation of antibacterial drugs into the coatings.
Among the various coating techniques, EPD is a rapid, cost-effective, site-selective, and versatile technique that can produce uniform, stable, and mechanically resistant coatings with tunable thickness at room temperature [9, 10]. Additionally, the EPD technique allows incorporation of drug delivery vehicles into the coating matrix to enhance the control over drug delivery [3]. As a coating matrix, chitosan has been widely coated on implant surfaces using the EPD technique [3, 14] due to its beneficial properties for drug delivery [15-18] and intrinsic antibacterial properties [19]. Control over release of the drugs, however, is generally limited due to a poor affinity between the drugs and chitosan. Therefore, suitable drug delivery carrier vehicles should be incorporated into the EPD coatings.
More recently, gelatin nanospheres (GNs) have been widely used as drug delivery vehicles because of their excellent bioactivity, nontoxicity, cost-
effectiveness and tunable charge and degradation rate [20]. Previously, it was shown that both macromolecules (e.g., growth factors [21, 22] and nucleic acids [23]) and small molecules (e.g., vancomycin and colistin [24]) could be released from GNs in a sustained manner due to molecular interactions between the drugs and GNs, such as hydrophobic and electrostatic interactions [24], which offers improved control over drug delivery compared to that of traditional diffusional release. Therefore, the objective of the current study was to prepare biologically active coatings to facilitate sustained release of antibacterial drugs from the surface of the implants to prevent implant-associated infections. To this end, we deposited chitosan coatings containing GNs loaded with antibiotics onto 316L stainless steel plates by means of the EPD technique (Figure 1). We hypothesized that sustained release of vancomycin from the EPD coatings would be achieved by the strong interactions between vancomycin and the GNs [24], whereas moxifloxacin would be released in a burst-type manner due to its weak affinity with GNs [24]. We studied the release kinetics of vancomycin and moxifloxacin from EPD coatings in vitro and examined the antibacterial effect of the EPD coatings using the zone of inhibition test against *Staphylococcus aureus*.

### 2 Materials and Methods

#### 2.1 Materials

Gelatin type B (from bovine skin, 225 bloom, isoelectric point (IEP) ~ 5) and chitosan (molecular weight in the range of 190-310 kDa with a deacetylation degree of 75-80%) were purchased from Sigma-Aldrich. Vancomycin hydrochloride hydrate (potency ≥ 900 µg/mg) and moxifloxacin hydrochloride (analytical standard) were purchased from Sigma-Aldrich. Glutaraldehyde (25 wt% solution in water) was purchased from Acros.
Organics, while all other chemicals were purchased from Sigma-Aldrich.

2.2 Preparation and characterization of gelatin B nanospheres

GNs were prepared by a two-step desolvation method [25]. Briefly, 25 ml of a 5% (w/v) solution of gelatin B in deionized water was prepared at 50 °C, and 25 ml acetone was then added to precipitate the gelatin chains of high molecular weight. The supernatant was discarded after precipitation, and the gelatin was redissolved in water at 50 °C followed by adjustment of the pH of the gelatin solution to 2.5. Thereafter, 80 ml of acetone was added to the gelatin solution at a constant rate of 4 ml/min under vigorous stirring to form GNs. Subsequently, the GNs were stabilized by the addition of 740 μl of 25 wt% glutaraldehyde. After 16 h of cross-linking, 105 ml of a 100 mM glycine solution was added to block the unreacted aldehyde groups. The suspension was then centrifuged at 5000 rpm for 60 min and resuspended in deionized water by vortexing. After three cycles of washing, the pH of the GNs suspension was adjusted to pH 7.0 and lyophilized for 24 h. The lyophilized GNs were stored at 4 °C for further use.

The morphology of the GNs was characterized by scanning electron microscopy (SEM, LEO 435 VP, Zeiss, Germany). The size and size distribution of GNs in the swollen state were measured in HEPES buffer (5 mM, pH 7.0) using dynamic light scattering (DLS, Zetasizer Nano-S, Malvern Instruments Ltd., Worcestershire, U.K.). The ζ-potential of the GNs was examined by laser doppler electrophoresis with a Zetasizer Nano-Z instrument (Malvern Instruments Ltd.) in the same HEPES buffer.

To investigate the interaction between the GNs and chitosan coatings, the ζ-potential of the GNs was also measured after they were thoroughly mixed with chitosan solutions. To this end, a suspension containing 2 mg/ml GNs and 0.5 mg/ml chitosan was prepared (see Table 1) and magnetically stirred overnight to ensure a strong interaction between the GNs and chitosan coatings.

Table 1. Composition of antibiotic-free suspensions used for EPD

<table>
<thead>
<tr>
<th>Group</th>
<th>GNs (mg/ml)</th>
<th>Chitosan (mg/ml)</th>
<th>GNs/chitosan ratio (wt/wt)</th>
<th>Solvent ratio (v/v, ethonal/water)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>—</td>
<td>0.5</td>
<td>—</td>
<td>1:1</td>
<td>4.8</td>
</tr>
<tr>
<td>2</td>
<td>0.25</td>
<td>0.5</td>
<td>1:2</td>
<td>1:1</td>
<td>4.8</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>0.5</td>
<td>1:1</td>
<td>1:1</td>
<td>4.8</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>0.5</td>
<td>4:1</td>
<td>1:1</td>
<td>4.8</td>
</tr>
</tbody>
</table>

Note: GNs are gelatin nanospheres; EPD is electrophoretic deposition.
chitosan. The GNs were then collected by centrifugation and redispersed in a citrate buffer (5 mM, pH 4.8, similar to the pH of the suspensions used for EPD (see Table 1) by sonication. Subsequently, the size distribution and $\zeta$-potential of the chitosan-modified GNs were characterized in the citrate buffer as described above.

### 2.3 Electrophoretic deposition process

A chitosan solution (1 mg/ml) was prepared when chitosan was dissolved into 2% (v/v) acetic acid and magnetically stirred at 200 rpm. Pure ethanol of the same volume was added dropwise into the chitosan solution under magnetic stirring to minimize electrolysis of the water during the EPD process. Different amounts of GNs were then added to the chitosan solution. The GNs were dispersed homogeneously inside the chitosan solution by sonication for 30 s (cycles of 0.9 s of working time and 0.1 s pauses) at 40% amplitude with a Branson digital sonifier (Emerson industrial Automation, Branson Europe, Dietzenbach, Germany). The compositions of the GNs/chitosan suspensions used for coating deposition using EPD are listed in Table 1.

Two parallel 316L stainless steel plates 12 mm diameters were placed in the EPD cell and used as the deposition and counter electrodes. The distance between the electrodes was fixed at 10 mm, and the deposition area was 1 cm². After optimization of the applied voltage (10-50 V) and deposition time (10-300 s), the deposition process was carried out at 15 V over 60 s, yielding a homogeneous and crack-free coating with maximal deposit mass. Oxidation of the stainless steel substrate during the anodic EPD process was negligible due to the excellent corrosion resistance of the stainless steel and the gentle conditions employed in the current experiment [26].

<table>
<thead>
<tr>
<th>Group</th>
<th>GNs (mg/ml)</th>
<th>Chitosan (mg/ml)</th>
<th>GNs/chitosan ratio (wt/wt)</th>
<th>Antibiotic</th>
<th>Antibiotic concentration in suspension (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V-GNs High</td>
<td>2</td>
<td>0.5</td>
<td>4:1</td>
<td>vancomycin</td>
<td>120</td>
</tr>
<tr>
<td>V-GNs Low</td>
<td>2</td>
<td>0.5</td>
<td>4:1</td>
<td>vancomycin</td>
<td>40</td>
</tr>
<tr>
<td>V-Control</td>
<td>—</td>
<td>0.5</td>
<td>—</td>
<td>vancomycin</td>
<td>40</td>
</tr>
<tr>
<td>M-GNs</td>
<td>2</td>
<td>0.5</td>
<td>4:1</td>
<td>moxifloxacin</td>
<td>40</td>
</tr>
<tr>
<td>M-Control</td>
<td>—</td>
<td>0.5</td>
<td>—</td>
<td>moxifloxacin</td>
<td>40</td>
</tr>
<tr>
<td>GNs-Control</td>
<td>2</td>
<td>0.5</td>
<td>4:1</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

**Note:** GNs are gelatin nanospheres, EPD is electrophoretic deposition; V is vancomycin; and M is moxifloxacin.
It should be noted that the proposed coating can be obtained not only on 316L stainless steel plates but also on other conductive substrates such as titanium and titanium alloys using similar EPD parameters. After deposition, the deposited cathodes were gently removed from the EPD cell and kept in air at room temperature for 20 min followed by drying at 37 °C for 24 h. To incorporate antibiotics into the EPD coatings, 500 μl of PBS containing vancomycin (12 and 4 mg/ml) or moxifloxacin (4 mg/ml) was dropped onto 100 mg of GNs in a 50 ml centrifuge bottle (Falcon). The GNs were then stored at 4 °C overnight to allow for total absorption of the antibiotics followed by addition of 50 ml of the chitosan solution. Subsequently, the suspension was sonicated as described previously. The compositions of the suspensions used for deposition of drug-loaded coatings are listed in Table 2. The EPD coatings loaded with antibiotics were then prepared under the same conditions as described before.

2.4 Characterization of the electrophoretic deposition coating

The morphology of the EPD coatings was characterized by SEM (Model Auriga, Zeiss, Germany). The roughness of the surfaces of the EPD coatings was measured using a laser profilometer (UBM, ISC-2). The wettability of the EPD coatings was determined by detecting the static water contact angles of the surfaces with a DSA30 instrument (Kruess GmbH, Germany). The adhesion strength of the composite coatings (GNs/chitosan weight ratio of 4:1) to the substrates was measured before and after incubation in phosphate buffered saline (PBS) for 3 weeks by means of a scratch tester (CSM instruments, Revetest, Switzerland) (n = 7). In this test, a diamond indenter with a tip radius of 22 μm was drawn across the composite-coated surfaces with a linearly increasing load. A scratch length of 5 mm, a load range of 0.09-20 N, and a scratch speed of 10 mm/min were used for the measurement. Because the acoustic emission response was too weak, the load force which corresponded to the first failure by detachment (observed using an optical microscope) was interpreted as the critical load.

2.5 Release of antibiotics

The coated circular stainless steel plates were placed into glass bottles (n = 5), followed by addition of 1 ml of PBS. The glass bottles were then placed on a shaking plate with a shaking rate of 90 rpm at 37 °C to perform the release study. At t = 6 h and 1, 2, 3, 5, 7, 10, and 14 days, 900 μl of the
supernatant was collected and refreshed with the same amount of PBS. The collected supernatant was stored at 4 °C until the concentrations of the antibiotics were determined using reverse phase high-performance liquid chromatography (RP-HPLC).

2.6 Detection of vancomycin and moxifloxacin

Concentrations of vancomycin and moxifloxacin in the supernatants of the release study were detected by RP-HPLC using a Hitachi HPLC machine which consisted of a Hitachi L-2130 pump, a Hitachi L-2400 UV detector, a Hitachi L-2200 auto sampler, and a LiChrospher RP-18 end-capped HPLC column (125 mm × 4 mm, particle size 5 μm). Standard calibration curves in the ranges of 0.1 to 25 μg/ml and 0.2 to 25 μg/ml were prepared for the quantification of moxifloxacin and vancomycin, respectively.

The mobile phase consisted of 50 mM ammonia phosphate buffer (pH 3, adjusted with H3PO4) and acetonitrile (90/10 v/v) was used for detection of vancomycin, and 50 mM phosphate buffer (pH 2.6) and acetonitrile (75/25 v/v) was used for detection of moxifloxacin. Thirty microliters of supernatant containing vancomycin or moxifloxacin was injected with a flow rate of 1 ml/min. The concentrations of the antibiotics were quantified at 196 and 296 nm for vancomycin and moxifloxacin, respectively, using standard calibration curves in the concentration range between 0.2 and 200 μg/ml.

2.7 Cytotoxicity

The lactate dehydrogenase (LDH) activity assay was used to evaluate the cytotoxicity of the EPD coatings by following the manufacturer’s standard protocol (Thermo Fisher Scientific). Briefly, human foreskin fibroblasts (HFFs, isolated from foreskin specimens of a healthy donor by following national guidelines for working with human materials) were cultured in an αMEM medium (Gibco, Invitrogen Corp., Paisley, Scotland). The cells were trypsinized and reseeded into a 24-well plate containing the EPD-coated substrates (n = 5) listed in Table 2 at a cell density of 2 × 104 cells/cm2. Thermonox coverslides (Thermo Fisher Scientific) and 5% DMSO were used as positive and negative controls, respectively. In addition, wells containing 2% (v/v) Triton - X100 and only culture medium were used as the high and low controls, respectively. After 24 h of incubation, 100 μl aliquots of metabolized medium from each well were transferred to a 96-well plate
in duplicate, and 100 µl of freshly prepared reaction medium was added followed by incubation at room temperature for 30 min. The activity of the LDH released from the cytosol of damaged cells was determined by measurement the absorbance of the suspension at a wavelength of 590 nm in a Bio-Tek FL600 microplate fluorescence reader (Biotek, Winooski, VT). The cytotoxicity was calculated with the following equation:

\[
\text{Cytotoxicity} = \left( \frac{\text{experimental value} - \text{low control}}{\text{high control} - \text{low control}} \right) \times 100\%
\]

The final results are listed as cytocompatibility.

### 2.8 Antibacterial effects

A zone of inhibition test was used to examine the antibacterial effect of the EPD coatings against *S. aureus* (ATCC 25923TM, Manassas, VA) according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) disc diffusion method [27]. Briefly, the isolated bacterial colonies were dispersed in sterile saline (0.85% w/v NaCl in water) to reach a density of the McFarland 0.5 standard. The bacterial suspension was then diluted to a concentration of approximately 1.5 × 10⁷ colony-forming units (CFU) per milliliter. After dilution, the bacterial suspension was inoculated on the entire surface of a Mueller-Hinton agar plate (Thermo Fisher Scientific). The coated discs were then placed on the bacterium-inoculated agar plate. The inhibition zone was measured after incubation at 36 °C for 20 h. Moreover, the morphology of the bacteria on the surface of the EPD coating discs was examined by SEM (Zeiss Sigma-300, Germany). In brief, bacterial cells were washed with PBS and then fixed with 2 wt% glutaraldehyde in a 0.1 M sodium cacodylate solution for 5 min followed by washing with the 0.1 M sodium cacodylate solution. Subsequently, the bacterial cells were dehydrated in a graded series of ethanol (70, 80, 90, 96 and 100 % ethanol and 100 % water-free ethanol) for 5 min and then dried in air in tetramethylsilane. The samples were sputter-coated with 10 nm chromium and then examined using SEM.

### 2.9 Statistics

All measurements were depicted as average ± standard deviation, and the statistical analyses were conducted using GraphPad Prism. Differences among groups were analyzed by analysis of variance (ANOVA) followed by a Tukey post-hoc test, and a value of p < 0.05 was considered as a statistically
significant difference.

3 Results

3.1 Electrophoretic deposition coating

GNs (Figure 2A) with a diameter of approximately 593 nm in the swollen state (Table 3, HEPES buffer result) and a \( \zeta \)-potential of -28.9 mV were successfully deposited on the surface of 316L stainless steel plates using the EPD technique (Figure 2). The bare chitosan coating (Figure 2B) was smooth, while randomly distributed GNs were observed when they were added (Figure 2C). With an increase in the amount of GNs in suspension, the surface of the coating was highly packed with homogeneously distributed GNs (Figures 2D and 2F). The GNs were mostly visible only at the surface of

![Figure 2. Scanning electronic micrographs of the gelatin nanospheres (A) and electrophoretically deposited gelatin nanosphere/chitosan coatings at ratios of 0:1 (B), 1:2 (C), 1:1 (D and E) and 4:1 (F and G); (E) and (G) are the cross-sections of (D) and (F), respectively. Arrows in E and G indicate the gelatin nanospheres distributed in the coating.](image)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>HEPES buffer (pH 7)</th>
<th>Citrate buffer (pH 4.8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without chitosan</td>
<td>Without chitosan</td>
</tr>
<tr>
<td>Particle size (nm)</td>
<td>593 ± 5</td>
<td>1291 ± 67</td>
</tr>
<tr>
<td>( \zeta )-potential (mV)</td>
<td>-28.90 ± 0.67</td>
<td>-5.48 ± 0.32</td>
</tr>
</tbody>
</table>
the EPD coatings at GNs/chitosan ratios lower than or equal to 1 (Figures 2C and 2E), while a homogeneous distribution of GNs throughout the coating was observed when the weight ratio between the GNs and chitosan was increased to 4:1 (Figure 2G).

The topography and wettability of the deposited coatings with different compositions were determined by roughness and contact angle measurements, respectively. The roughness of the EPD coatings was correlated to the weight ratio between the GNs and chitosan. Coatings with a GNs:chitosan ratio of 1:2 displayed a roughness higher than those of experimental groups (Figure 3A). The contact angles of the coated samples decreased when the amount of GNs increased (Figure 3B).

The results of the scratch test used to evaluate the adhesion strength of the GNs/chitosan coatings to the substrate are shown in Figure 4. The cross

| Figure 3. Roughness (A) and contact angles (B) of electrophoretically deposited coatings with different ratios between the gelatin nanospheres and chitosan. p < 0.05 (*). |
| Figure 4. Typical scratch track on a GNs and chitosan coating (4:1 weight ratio) before (A) and after (B) incubation in PBS for 3 weeks. The arrows show the scratch direction, and the crosses show the first coating detachment to determine the critical load. |
on the scratch line, which stands for the position of the first detachment under the linearly increasing load, was used to determine the critical load in the present study. The critical load force of the raw GNs/chitosan coating was measured to be $8.1 \pm 0.3$ N (Figure 4A). An appreciable increase in the critical load force to $10 \pm 0.3$ N was observed after degradation in PBS for 3 weeks (Figure 4B).

### 3.2 Release of antibiotics from EPD coatings

Sustained release of vancomycin was observed only from EPD coatings containing GNs loaded with high amounts of vancomycin (Figure 5A). An amount of vancomycin ($p < 0.0001$) was released from the experimental group containing a high amount of vancomycin (V-GNs High groups) was significantly higher compared to those of the other two vancomycin-loaded groups. In contrast, moxifloxacin was released in a burst-type manner regardless of the presence of GNs inside the coatings (Figure 5B). Moreover, burst release of vancomycin from coatings containing only vancomycin without GNs was observed (Figure 5A).

### 3.3 Cytocompatibility

Figure 6 shows the cytocompatibility of the EPD coatings measured using the LDH activity assay. None of the EPD coatings were cytotoxic. Further, no significant difference was observed among the groups with various compositions.

### 3.4 Antibacterial efficacy

An inhibition zone diameter for S. aureus was measured to examine the antibacterial efficacy of the EPD coatings (Figures 7A-C). The samples that
contained moxifloxacin with or without GNs displayed an obvious inhibition zone (Figures 7A and 7B), whereas none of the groups with or without vancomycin induced the formation of an inhibition zone (Figure 7C and Figure S1). SEM was used to observe the morphology of S. aureus on the surface of the coatings with and without vancomycin. Bacteria were clearly observed on all of these coatings (Figure 7D-F) except for those that contained GNs loaded with a high amount of vancomycin (Figures 7G and 7H).

4 Discussion

The aim of the present study was to develop a method to apply an antibacterial coating to the surface of metallic implants to prevent implant-associated infections. To this end, we used the EPD technique to deposit positively charged chitosan containing negatively charged GNs onto the surface of 316L stainless steel plates. In these EPD coatings, GNs served as delivery vehicles which facilitated improved control over the antibacterial
efficacy of the coatings. The GNs were successfully incorporated into the EPD coatings as indicated by a homogeneous distribution of the spheres throughout the coatings (Figures 2F and 2G). During the EPD process, water around the cathode was electrolyzed, resulting in a locally increased pH [14]. Meanwhile, the protonated chitosan macromolecules migrated to the cathode under the influence of the electric field, neutralized their charge by electron uptake, and adhered to the electrode at an alkaline pH [14]. To explore the possible interaction between GNs and chitosan during the EPD process, we measured the size and ζ-potential of the GNs with and without chitosan in a citrate buffer, which had a pH similar to that of the suspension used for EPD. The pure GNs in the acidic citrate buffer were less negatively charged and larger in size than those in the neutral HEPES buffer, which may be caused by moderate degrees of aggregation at reduced ζ-potential values. After being thoroughly mixed with chitosan macromolecules, the negatively charged GNs became positively charged, as indicated by a shift in ζ-potential from -5.48 to 11.1 mV (Table 3). This change, in addition to an increase in the diameters of the GNs from 1291 to 1389 nm, indicated that the GNs were covered by a thin chitosan layer with a thickness of approximately 50 nm (Table 3). Consequently, the formation of homogeneous and intimately mixed GNs/chitosan coatings at the cathode was realized by a two-step process: (i) electrostatic adsorption of positively charged chitosan molecules onto negatively charged GNs and (ii) co-deposition of free chitosan molecules and chitosan-coated GNs onto the working electrode driven by the applied electric field [9, 10, 28]. Thus, we assume that the amount of deposited GNs was directly controlled by the original weight ratio between the GNs and chitosan in the EPD suspension (Figures 2B-G), as confirmed by our previous investigation of EPD-deposited composite coatings [3, 26].

The weight ratio between GNs and chitosan not only influenced the amount of GNs inside the EPD coatings but also affected the topography and hydrophobicity of the EPD coatings (Figure 3). The highest roughness values were observed for the EPD coatings at a GNs and chitosan weight ratio of 1:2 (Figure 3A) due to the presence of discontinuous GNs (Figure 2C). At GNs and chitosan ratios higher than 1:1, the surfaces of the EPD coatings were highly packed with a confluent layer of GNs (Figure 2D) which lowered the roughness value. Generally, we showed that the surface roughness and
wettability of the coatings could be tuned by a simple adjustment of the weight ratio between GNs and chitosan. In addition to the surface roughness, the wettability of metallic implant surfaces is crucial for processes such as protein adsorption and cell attachment [29-31], which ultimately control the extent of osseointegration [3]. Recently, it was reported that chitosan-coated surfaces with a contact angle of $76 \pm 5^\circ$ induced higher amounts of albumin and fibronectin adsorption as well as enhanced osteoblast cell attachment compared to more hydrophilic Ti surfaces (contact angle $32 \pm 6^\circ$) [29]. These results suggest that the high contact angles as observed for our composite coatings can be considered beneficial for the process of osseointegration (Figure 3B). Our composite coatings became more hydrophilic with increasing amounts of GNs, which can be explained by the hydrophilicity of water-swollen GNs [22, 24]. Consequently, the wettability of the chitosan/gelatin coatings can be fine-tuned by a simple adjustment of the amount of GNs inside the coatings.

Furthermore, coatings applied to orthopedic and dental implants should display sufficient adhesion strength to survive implant installation. In the present study, the critical load force (as a measure of the adhesion strength of the EPD coatings to the stainless steel plates) increased from $8.1 \pm 0.3$ N for as-prepared coatings to $10 \pm 0.3$ N for coatings after incubation in PBS for 3 weeks (Figure 4). This increase suggests that the composite coatings remain stable during the incubation process. The slight increase in adhesion strength could be attributed to the detachment of weakly bonded components while strongly bonded components remained tightly adhered to the stainless steel plate. The adhesion strength as observed in the current study was similar to or higher than the adhesion strength in the range from 6 to 13 N there were reported previously for electrophoretically deposited cellulose nanocrystal/bioactive glass [32], electrodeposited calcium phosphate/chitosan [33], plasma sprayed hydroxyapatite [34] and biomimetic-assembled hydroxyapatite [35]. Consequently, we assume that the adhesion strength of the deposited GNs/chitosan coatings was sufficient to justify their application as a biodegradable antibacterial coating on orthopedic and dental implants. After implantation, bodily fluids that contain enzymes such as lysozyme and collagenase may penetrate the coatings and thereafter degrade chitosan and the GNs [21, 36], respectively, thereby facilitating the osseointegration of the implant.
To render the electrophoretically deposited chitosan/gelatin coatings biologically active, we loaded two types of antibiotics (i.e., vancomycin and moxifloxacin) onto the GNs and then deposited them together with chitosan onto the stainless steel plates. As such, the release of antibiotics from these novel coatings would be proceeded by desorption from the GNs carriers and subsequent diffusion through the chitosan barrier matrix. To test our hypothesis that antibiotics can be released from these EPD coatings in a sustained manner provided that strong interactions exist between the antibiotics and GNs, we compared the release kinetics of vancomycin and moxifloxacin from the EPD coatings. We observed that vancomycin was released in a sustained manner for up to 2 weeks (Figure 5A), which is consistent with the fact that vancomycin can establish relatively strong hydrophobic and electrostatic interactions with GNs [24]. In contrast, moxifloxacin was released in a burst-type manner (Figure 5B), which is in line with the weak interaction between moxifloxacin and GNs, as reported previously [24]. In addition, we observed that sustained release of vancomycin from electrophoretically deposited coatings could be obtained only when GNs were incorporated into the coatings (Figure 5A), which confirmed the added value of the incorporation of GNs into the chitosan coatings. These results are an improvement compared to a recent report where chitosan/gelatin hybrid nanospheres were formed through a one-step EPD process [37]. In this study, the antibiotic was released within 2 h, which does not yet meet clinical requirements.

The cytotoxicity test confirmed the excellent cytocompatibility of the coatings. Although chitosan has been recognized as an antibacterial material [15, 19], the coatings without antibiotics did not form an inhibition zone (Figure S1), and growth of bacteria on the surface of these coatings was observed (Figure 7D). These results indicate that chitosan was not effective against S. aureus in the present study. Therefore, it was necessary to incorporate antibiotics inside the coatings to render them antibacterial. The antibacterial efficacy of the coatings loaded with moxifloxacin was confirmed because the burst release of moxifloxacin resulted in an obvious inhibition zone (Figures 7A and 7B). Although coatings that contained GNs loaded with a high amount of vancomycin did not induce the formation of an inhibition zone (Figure 7C), it was found that bacteria did not grow on the surface of these coatings (Figures 7G and 7H), which indicates that these coatings had the ability to kill the contact bacteria. These results
suggeste that the released vancomycin did not diffuse on the agar plate due to its high affinity with the GNs. Therefore, the antibacterial effects of these novel EPD-processed coatings might be improved by increasing the amount of vancomycin in the EPD coatings. These results suggest that the antibacterial efficacy of electrophoretically deposited gelatin/chitosan coatings can be further improved by simultaneous incorporation of GNs loaded with either moxifloxain (to achieve an initial burst release, killing invaded bacteria) or vancomycin (to guarantee a long-term antibacterial effect, killing latent bacteria).

5 Conclusion
A novel antibacterial implant coating was developed by electrophoretic deposition of chitosan coatings containing gelatin nanospheres to improve control over the delivery of antibiotics and prevent implant-associated infections. The coatings adhered tightly to the stainless steel substrates, and gelatin nanospheres were distributed homogeneously throughout the chitosan matrix of the coatings. The surface roughness and hydrophobicity of the coatings can be fine tuned by a simple adjustment of the weight ratio between the gelatin nanospheres and chitosan in the suspensions. Vancomycin and moxifloxacin were released in a sustained and burst-type manner, respectively, while the coatings were highly cytocompatible and exhibited sufficient antibacterial effects. These results suggest that the antibacterial capacity of metallic implants can be tuned by orthogonal control over the release of (multiple) antibiotics from electrophoretically deposited chitosan/gelatin nanosphere coatings, which offers a new strategy to prevent implant-associated infections.

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References

19. Song, J., et al., Antibacterial effects of electrospun chitosan/poly(ethylene oxide)
Gelatin Nanospheres Facilitate Antibiotic Release from EPD Coatings

37. Cai, X., et al., Surface functionalization of titanium with tetracycline loaded chitosan-
Supplementary information

Figure S1. Optical image of the inhibition zone of coatings containing GNs without antibiotics.
Chapter 5

Electrospun nanofibrous silk fibroin membranes containing gelatin nanospheres for controlled delivery of biomolecules

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1 Jinlong Shao
1 Yang Zhang
2 Eva Kolwijck
1 John A. Jansen
1 Sander C.G. Leeuwenburgh
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2 Department of Medical Microbiology, Radboud University Medical Centre
1 Introduction

The design and fabrication of an effective and efficient drug delivery system is of vital importance in the fields of tissue regeneration \([1-3]\) and antibacterial therapy \([4, 5]\). An ideal delivery systems should release drug molecules at the required local site in a spatiotemporally controlled manner while retaining their bioactivity, thereby optimizing their efficacy and reducing their side effects \([6, 7]\). To this purpose, numerous nanostructured carriers including liposomes \([8-10]\), dendrimers \([10-12]\), micelles \([10, 13]\), nanoparticles \([14, 15]\) and electrospun nanofibers \([16, 17]\) have been developed.

Among these nanocarriers, electrospun nanofibers have been explored for drug delivery purposes due to their high drug loading and encapsulation efficiency, ability to modulate the release of biomolecules, cost-effectiveness, and ease of processing \([17, 18]\). More recently, silk fibroin (SF) has gained interest as a biomaterial due to several beneficial properties such as its biocompatibility, biodegradability, and its processability into multiple shapes including nanofibers \([19-23]\). Such nanofibers can be further cross-linked by water annealing to improve their chemical and mechanical stability. Consequently, SF nanofibers have been widely used as drug carriers \([24, 25]\) and membranes for guided tissue regeneration/guided bone regeneration (GTR/GBR) \([26, 27]\).

The release of biomolecules from SF nanofibers is based on diffusion. Consequently, control over the rate of drug delivery is poor due to a lack of interaction between biomolecules and SF \([25]\). Therefore, we hypothesized that enrichment of electrospun SF membranes with additional drug carriers will improve the suitability of SF nanofibers for drug delivery.

Charged gelatin nanospheres (GNs) are promising candidates in view of their excellent bioactivity, tunable biodegradability and charge, non-toxicity and cost-effectiveness \([28-30]\). Positively charged gelatin type A nanospheres (GANs) and negatively charged gelatin type B nanospheres (GBNs) have been developed previously \([28, 29]\). These nanospheres were shown to facilitate sustained delivery of macromolecules such as growth factors \([31]\) and nucleic acid \([32]\). Additionally, the GNs were capable of delivering small molecules including vancomycin and colistin in a controlled manner \([30, 33]\). These beneficial features of GNs are attributed to strong molecular interactions between biomolecules and GNs, i.e., polyion complexation,
hydrophobic and electrostatic interactions [29, 30]. Consequently, GNs are superior drug carriers compared to other nanostructured delivery vehicles that rely on release by diffusion. Therefore, we aimed to incorporate oppositely charged GNs into the SF nanofibers as an effective strategy to provide additional controlled drug delivery performance of electrospun SF nanofibers.

To this end, we dispersed vancomycin-loaded GNs into an aqueous SF solution and fabricated SF nanofibers embedded with GNs by using a colloid-electrospinning technique [34]. To monitor the distribution of GANs and GBNs inside the nanofibers, we labeled GANs and GBNs with blue and far-red fluorescent dyes, respectively. Thereafter, we used single or co-axial nozzle electrospinning to adjust the amount of oppositely charged GANs and GBNs inside the SF nanofibers and to organize the spatial distribution of GNs. Subsequently, we studied the in vitro release kinetics of vancomycin from the nanofibrous SF/GN membranes and examined the antibacterial effect of these membranes against *Staphylococcus aureus* (*S. aureus*) by using a zone of inhibition test. Finally, we evaluated the cytotoxicity of the membranes using lactate dehydrogenase (LDH) activity assay, and cultured periodontal ligament (PDL) cells on the membranes containing different amounts of GNs to evaluate the cytocompatibility of these membranes.

2 Materials and Methods

2.1 Materials

Silk cocoons were provided by Dr Aichun Zhao (State Key Laboratory of Silkworm Genome Biology, Southwest University, Chongqing, P.R. China). Poly(ethylene oxide) (PEO) (molecular weight = 900 kDa), gelatin type A (from porcine skin, 300 Bloom, isoelectric point (IEP) ≈9) and gelatin type B (from bovine skin, 225 bloom, isoelectric point (IEP) = 5) were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). Glutaraldehyde (25 wt% in water) was purchased from Acros Organics (Geel, Belgium). DyLight™ 405 and 650 NHS Esters were purchased from ThermoFisher Scientific (Bleiswijk, the Netherlands). All other chemicals were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands).

2.2 Extraction of silk fibroin from silk cocoons

SF was extracted from silk cocoons according to a previously reported protocol [20]. Briefly, 5 g of silk cocoons was boiled in a 0.02 N Na₂CO₃
solution for 30 min to remove sericin proteins. After boiling, the silk material was rinsed in cold deionized water for 20 min with three repetitions and then dried at ambient temperature for 24 h. The dried silk materials was then dissolved in a 9.3 M LiBr (Sigma-Aldrich, Zwijndrecht, the Netherlands) solution at 60 °C for 4 h. Thereafter, the SF solution was dialyzed in deionized water with a Slide-A-Lyzer™ dialysis cassette (3.5 K, Thermo Fisher Scientific, Rockford, USA) for 48 h with six times changes of water, followed by centrifugation twice at 5,000 rpm for 20 min to remove the impurities. The SF solution was stored at 4 °C for further experiments.

2.3 Preparation of gelatin nanospheres
GANs and GBNs were prepared using a two-step desolvation method [31]. Briefly, 1.25 g gelatin was dissolved in 25 ml deionized water at 50 °C to form a 5 w/v% solution. The high molecular weight gelatin in the solution was then precipitated by adding 25 ml acetone. The supernatant was discarded afterwards, and 25 ml deionized water was added to re-dissolve the precipitated gelatin at 50 °C. The pH of the gelatin solution was then adjusted to 2.5 using hydrochloric acid solution. After that, 75 ml acetone for gelatin A or 80 ml for gelatin B was added dropwise to the gelatin solution (~ 4 ml/min) under vigorous stirring to form GNs. Subsequently, the GNs were stabilized by adding glutaraldehyde into the suspension yielding molar ratios between glutaraldehyde and amines (as present in gelatin) of 1:1 for GANs (i.e., 132 μl of glutaraldehyde per gram of gelatin A) and 4:1 for GBNs (i.e., 592 μl of glutaraldehyde per gram of gelatin B). After 16 h of cross-linking, 100 ml of 100 mM guanidine and 105 ml of 100 mM glycine solution for gelatin A and B, respectively, was added to block the unreacted aldehyde groups. The suspension was then subjected to three cycles of centrifugation at 5000 rpm for 60 min and re-suspension in deionized water by vortexing, followed by adjusting the pH of the nanosphere suspension to 7.0. The suspension was stored at 4 °C for further use.

2.4 Fluorescent labeling of gelatin nanospheres
GANs and GBNs were fluorescently labeled with DyLight™ 405 ester in blue and 650 NHS Esters in far-red, respectively, according to a standard protocol (ThermoFisher Scientific, Bleiswijk, the Netherlands). In brief, 200 mg of GNs were dispersed in 20 ml of phosphate buffered saline (PBS). The pH of the GNs suspension was then adjusted to 7.2 by using 1M sodium
hydroxide solution. Thereafter, 25 μg of NHS esters were dissolved in 200 μl of N,N dimethylformamide (Serva Electrophoresis GmbH, Heidelberg, Germany), and the solution was then added dropwise into the GNs suspensions under magnetic stirring at 400 rpm. After 1 h of reaction, the suspension was subjected to two cycles of centrifugation (5000 rpm for 60 min) and re-suspension in deionized water by vortexing. The suspension was stored at 4 °C until further use.

2.5 Characterization of gelatin nanospheres

The size and size distribution of GNs (either with or without conjugated fluorescent dyes) were measured in 5 mM HEPES buffer at pH 7.0 in swollen state by using dynamic light scattering (DLS, Zetasizer Nano-S, Malvern Instruments Ltd., Worcestershire, United Kingdom). The ζ-potential of the GNs was examined by laser Doppler electrophoresis using a Zetasizer Nano-Z (Malvern Instruments Ltd., Worcestershire, United Kingdom) in the same HEPES buffer.

2.6 Electrospinning nanofibers containing gelatin nanospheres

2.6.1 Single nozzle electrospinning

To assist the water phase electrospinning of SF, 75 mg of PEO was dissolved in 3 ml of SF solution (7.5 wt%) under magnetic stirring at 120 rpm to form a standard batch of SF/PEO (75/25 w/w) solution. Different amounts of GBNs were then added into the SF/PEO solution under magnetic stirring to form a colloid suspension. The composition of SF/PEO/GBN colloid suspensions used for electrospinning is listed in Table 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>SF solution (mg/ml)</th>
<th>SF solution (ml)</th>
<th>PEO (mg)</th>
<th>SF/PEO/GBN (wt/wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>75</td>
<td>3</td>
<td>75</td>
<td>9/3/0</td>
</tr>
<tr>
<td>2</td>
<td>75</td>
<td>3</td>
<td>75</td>
<td>9/3/0.9</td>
</tr>
<tr>
<td>3</td>
<td>75</td>
<td>3</td>
<td>75</td>
<td>9/3/3</td>
</tr>
<tr>
<td>4</td>
<td>75</td>
<td>3</td>
<td>75</td>
<td>9/3/6</td>
</tr>
</tbody>
</table>

For visualization of the distribution of GNs inside the nanofibers, NHS-Ester labeled GNs were added into the SF/PEO solution (Group 1 in Table 1) under magnetic stirring at 120 rpm (Figure 1, a). The weight ratios of GANS/GBNs were varied from 75/25, 50/50 to 25/75. The weight ratio of SF/PEO/GN was fixed at 9/3/0.45 for all the experimental groups. This ratio allowed
**Figure 1.** Schematic diagrams of the preparation of silk fibroin (SF)/poly(ethylene oxide) (PEO)/gelatin nanosphere (GN) nanofibers by using colloid electrospinning with single (a) or co-axial (b) nozzles.

**Table 2.** Composition of vancomycin-containing suspensions for facile monitoring of the spatial distribution of GNs inside the nanofibers using confocal microscopy.

<table>
<thead>
<tr>
<th>Group</th>
<th>SF/PEO/GBN (wt/wt)</th>
<th>GBN (mg)</th>
<th>Vancomycin (mg)</th>
<th>Vancomycin/GBN (wt %)</th>
<th>Drug/polymer (wt %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V-Control</td>
<td>9/3/0</td>
<td>—</td>
<td>1.5</td>
<td>—</td>
<td>0.50</td>
</tr>
<tr>
<td>20 wt% GBNs</td>
<td>9/3/3</td>
<td>75</td>
<td>1.5</td>
<td>2</td>
<td>0.40</td>
</tr>
<tr>
<td>33 wt% GBNs</td>
<td>9/3/6</td>
<td>150</td>
<td>1.5</td>
<td>1</td>
<td>0.33</td>
</tr>
</tbody>
</table>

For the vancomycin-loaded groups (Table 2), vancomycin was first loaded
onto GBNs by mixing aqueous vancomycin solutions with GBNs suspensions at different vancomycin/GBN weight ratios. The vancomycin/GBN mixture was kept at 4 °C overnight to allow for complete absorption of vancomycin into GBNs. Thereafter, this mixture was added to the silk/PEO solution to form the colloidal suspensions for electrospinning.

The electrospinning process was performed using a commercially available electrospinning set-up (Esprayer ES-2000S, Fuence Co., Ltd, Tokyo, Japan) with optimized conditions (Figure 1). Briefly, the prepared colloid suspensions were ejected at a flow rate of 20 μl/min at a voltage of 22 - 28 kV. The distance between the spinneret and collector was fixed at 20 cm. The obtained electrospun nanofibers were collected on an aluminum foil. After electrospinning, the fibrous membranes of approximately 200 μm thick were cross-linked by water annealing overnight to induce β-sheet formation followed by drying at room temperature. Thereafter, the electrospun membranes were punched into discs with a diameter of 11.5 mm. The samples were stored in a desiccator at room temperature until further use.

### 2.6.2 Core-shell electrospinning

The suspension containing fluorescently labeled GANs or GBNs was added into the standard batch of SF/PEO solution (Group 1, Table 1) under magnetic stirring at 120 rpm to form a colloidal suspension. The weight ratio of SF/PEO/GN was kept at 9/3/0.45. Co-axial electrospinning was conducted in an electrospinning set-up as described previously [35] with minor modifications. The core-shell spinneret contained a Teflon chamber for the shell solution and metal chamber for the core solution. The inner metal chamber was connected to a 21 G needle (with inner and outer diameters of 0.514 and 0.820 mm, respectively) while the inner diameter of the shell outlet was 2 mm. A high voltage (15-18 kV) was applied onto the inner metal chamber. Colloidal suspensions containing GANs or GBNs were used as core or shell feeds, respectively (Figure 1b). These core and shell feeds were ejected at different flow rates controlled by syringe pumps (BD Scientific Inc., USA). The distance between the spinneret and collector was fixed at 20 cm. The obtained electrospun nanofibers were collected on an aluminum foil or a glass slide for further characterization. Detailed information on the core-shell electrospinning parameters are listed in Table 3.
2.7 Characterization of the nanofibers

2.7.1 Microscopy
The morphology of the electrospun nanofibers was observed by scanning electron microscopy (SEM, JEOL 6301) at an acceleration voltage of 10 kV with gold sputtered on the sample surfaces. The distribution of the GNs inside the nanofibers was examined using confocal laser scanning microscopy (CLSM, Olympus FV1000, Zoeterwoude, the Netherlands).

2.7.2 FT-IR analysis
Electrospun membranes (Group 1 in Table 1) before and after cross-linking were used for FT-IR analysis. The contribution of the different SF conformations to the amide I region was determined by Fourier self-deconvolution and subsequent curve fitting according to a previously reported method [36]. In brief, the absorbance of each membrane was measured with 400 scans at a resolution of 4 cm⁻¹ at wavenumbers ranging from 400 to 4000 cm⁻¹ on an attenuated total reflectance infrared spectroscopy (ATR-IR, UATR two, PerkinElmer, the Netherlands). Thereafter, the second derivative was applied to the original spectra in the amide I region to check the peak positions in cm⁻¹. The Fourier self-deconvolution was then performed using the PerkinElmer software. The parameters of deconvolution were selected based on the resolution and peak positions corresponding to the positions of the minima in the second derivative of the initial spectra. Subsequently, curve fitting was applied to the deconvoluted spectra with Gaussian line shape profiles to measure the relative areas of the amide I components.

<table>
<thead>
<tr>
<th>Group</th>
<th>GAN suspension</th>
<th>GBN suspension</th>
<th>SF/PEO/GBN (wt/wt)</th>
<th>Core feeding rate (ml/h)</th>
<th>Shell feeding rate (ml/h)</th>
<th>Core/shell ratio (wt/wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Core</td>
<td>Shell</td>
<td>9/3/0.45</td>
<td>0.4</td>
<td>2</td>
<td>1/5</td>
</tr>
<tr>
<td>2</td>
<td>Core</td>
<td>Shell</td>
<td>9/3/0.45</td>
<td>0.6</td>
<td>1.8</td>
<td>1/3</td>
</tr>
<tr>
<td>3</td>
<td>Core</td>
<td>Shell</td>
<td>9/3/0.45</td>
<td>1.2</td>
<td>1.2</td>
<td>1/1</td>
</tr>
<tr>
<td>4</td>
<td>Core</td>
<td>Shell</td>
<td>9/3/0.45</td>
<td>1.6</td>
<td>0.8</td>
<td>1/0.5</td>
</tr>
<tr>
<td>5</td>
<td>Shell</td>
<td>Core</td>
<td>9/3/0.45</td>
<td>2.0</td>
<td>0.4</td>
<td>5/1</td>
</tr>
<tr>
<td>6</td>
<td>Shell</td>
<td>Core</td>
<td>9/3/0.45</td>
<td>1.8</td>
<td>0.6</td>
<td>3/1</td>
</tr>
<tr>
<td>7</td>
<td>Shell</td>
<td>Core</td>
<td>9/3/0.45</td>
<td>1.2</td>
<td>1.2</td>
<td>1/1</td>
</tr>
<tr>
<td>8</td>
<td>Shell</td>
<td>Core</td>
<td>9/3/0.45</td>
<td>0.8</td>
<td>1.6</td>
<td>0.5/1</td>
</tr>
</tbody>
</table>

Table 3. Composition of suspensions containing fluorescently labeled GNs for core-shell electrospinning.
2.8 Vancomycin release and detection

Vancomycin loaded membranes (Table 2) were used for the release study. The nanofibrous membrane discs weighed 10 mg and were placed into a 1.5 ml Eppendorf® tube (n = 5) containing 1 ml PBS. The tubes were then incubated at 37 °C and gently shaken at 90 rpm. At each predetermined time point, 900 μl supernatant was collected and refreshed with PBS. The collected supernatant was stored at 4 °C for further analysis. The concentration of vancomycin was detected by reverse-phase high performance liquid chromatography (RP-HPLC) according to a previously reported method [30]. A Hitachi HPLC machine was used in the present study. This HPLC system consisted of a Hitachi L-2130 pump, a Hitachi L-2400 UV detector, a Hitachi L-2200 autosampler and a LiChrospher® RP-18 endcapped HPLC column (125 mm × 4 mm, particle size 5 μm). The mobile phase consisted of ammonia phosphate buffer (50 mM, pH 3 adjusted with H3PO4) and acetonitrile at a volume ratio of 90/10. The injection volume was 30 μl with a flow rate of 1 ml/min. The concentration of vancomycin was quantified at 196 nm using a standard calibration curve in the concentration range between 0.2 and 25 μg/ml.

2.9 Cytotoxicity

The electrospun membranes were sterilized under UV light for one hour and the cytotoxicity of the sterilized membranes was evaluated by a LDH activity assay following a standard protocol (ThermoFisher Scientific, Bleiswijk, the Netherlands). Briefly, human foreskin fibroblasts (HFFs, isolated from the foreskin specimens of a healthy donor by following the national guidelines for working with human materials) were cultured in αMEM medium (Gibco, Invitrogen Corp., Paisley, Scotland). The cells were trypsinized and reseeded onto a 48-well plate containing electrospun membrane discs at a seeding density of 2 × 104 cells/cm2 (Table 2, n = 5). Wells containing only culture medium and wells containing 2 v/v% Triton - X100 were used as low and high controls, respectively. Thermanox™ coverslips (ThermoFisher Scientific, Bleiswijk, the Netherlands) and 10% DMSO were used as positive and negative controls, respectively. After 24 h of incubation, a 100 μl aliquot of metabolized medium was transferred into a 96-well plate in duplicate, followed by addition of 100 μl of the freshly prepared reaction medium. The mixture was incubated at room temperature for 30 min. The cytotoxicity of the electrospun membranes was determined by measuring the activity of
the LDH released from the cytosol of damaged cells. The absorbance of
the suspension was measured at a wavelength of 590 nm in a Bio-Tek®
FL600 microplate fluorescence reader (Biotek, VT, USA). The cytotoxicity
was calculated according to the following equation:
\[
\text{Cytotoxicity} = \left( \frac{\text{experimental value} - \text{low control}}{\text{high control} - \text{low control}} \right) \times 100\%
\]

2.10 Antibacterial effect
The antibacterial effect of the electrospun membranes (Table 2) was
determined by a zone of inhibition test against *S. aureus* (ATCC® 25923TM,
Manassas, USA) [18]. In brief, the isolated bacterial colonies were
dispersed in sterile saline (0.85% NaCl w/v in water) to reach the density
of a McFarland 0.5 standard. After dispersion, the bacterial suspension
was diluted to a concentration of approximately 3 × 10^7 colony-forming
units (CFU) per ml. Thereafter, the bacteria were inoculated by spreading
the bacteria suspension evenly over the entire surface of an agar plate
with a sterile cotton swab, followed by placing the electrospun membrane
discs (n = 4) on the Muller Hinton agar plate. The radius of the zone of
inhibition was measured from the centre point of the disc to the border
of the inhibition zone after incubation at 36 °C for 20 h. The results were
listed as the diameter of the inhibition zone.

2.11 Periodontal ligament cells growth on the electrospun silk
fibroin membranes
For the evaluation of the cytocompatibility of the SF membranes for later
use in GTR/GBR applications, PDL cells were cultured on the electrospun
SF membranes (Table 2). PDL cells were isolated from healthy donors with
informed consent and cultured in Dulbecco's modified eagle medium
(DMEM, Gibco, Invitrogen Corp., Paisley, Scotland) supplemented with
10% fetal bovine serine and 1% penicillin/ streptomycin. The cells were
trypsinized and reseeded onto a 48-well plate containing electrospun
membrane discs at 2 × 10^4 cells/cm^2 (Table 2, n = 5). The Thermanox™
coverslips (ThermoFisher Scientific, Bleiswijk, the Netherlands) was used as
positive control.

2.11.1 Live/Dead assay
The viability of PDL cells growing on the electrospun SF membranes at
24 h was determined by using the LIVE/DEAD® Viability/Cytotoxicity Kit (ThermoFisher Scientific, Bleiswijk, the Netherlands) following the manufacturer’s standard protocol. Briefly, the LIVE/DEAD® reagent stock solutions were removed from the freezer and warmed to room temperature. Thereafter, calcein AM and ethidium homodimer-1 (EthD-1) stock solutions were diluted in the DMEM culture medium at a final concentration of 2 μM and 4 μM for calcein AM and EthD-1, respectively. Subsequently, 300 μl of the staining solution was directly added onto the membranes. After 30 min of incubation at 20-25°C, the membranes were washed with PBS and then placed on the microscope slides. Image acquisition of the PDL cells was performed by using CLSM (Olympus FV1000, Olympus FV1000, Zoeterwoude, the Netherland) with a 20× objective.

2.11.2 Cell morphology
The morphology of the PDL cells cultured on the SF membranes was examined with SEM (Zeiss Sigma-300, Germany). In brief, PDL cells were washed with PBS and then fixed with 2 wt% glutaraldehyde in 0.1 M sodium-cacodylate solution for 20 min. After fixation, the PDL cells were washed with 0.1 M sodium-cacodylate solution for 5 min. Subsequently, the PDL cells were dehydrated in a graded series of ethanol (70, 80, 90 and 96% ethanol for 5 min each, and then 100 % ethanol and 100 % water-free ethanol for 10 min each), followed by adding enough tetramethylsilane on top of samples and dried in fume hood. The samples were sputter-coated with 10 nm chromium and then examined under SEM.

2.12 Statistics
Statistical analyses were conducted using GraphPad Prism and all results were reported as mean ± standard deviation. Differences among groups were analyzed by one-way analysis of variance (ANOVA) followed by a Tukey post hoc test, and a p-value of < 0.05 was considered as a statistically significant difference.

3 Results
3.1 Characterization of the electrospun fibrous membranes
Fluorescently labeled GANs and GBNs with size of approximately 400 and 600 nm in swollen state, respectively, were prepared (Table S1). No significant difference in size was observed for the nanospheres before and
after conjugation of fluorescent dye (Table S1). Uniform and defect-free SF nanofibers containing negatively charged GBNs with a size of approximately 600 nm (Table S1) in swollen state were prepared successfully (Figure 2, a-c; Figure 3, c and d). Although the nanofibers became less uniform in size with increasing amount of GBNs (Figure 2), defect-free fibers were obtained up to a GBNs content of 33 wt% at an SF/PEO/GBN weight ratio of 9/3/6 (Figure 2, d). Besides GBNs, GANs were also incorporated into the nanofibers (Figure 3, a-b) to form a nano-in-nano fibrous composite. The distribution of the GANs and GBNs inside the SF nanofibers was homogeneous (Figure 3). At an SF/PEO/GBN weight ratio of 9/3/0.45, individual GBNs were uniformly arranged along the nanofibers (Figure 3, c-d).

To check the efficiency of water annealing on the SF electrospun fibrous membranes, we used FT-IR analysis to examine these membranes (Group 1, Table 1) before and after water annealing. After water annealing, extensive strong β-sheet formation was observed as reflected by a stretch vibration at 1625 cm⁻¹ [36] (Figure 4, a). Weak β-sheet formation was observed as indicated by a stretch vibration at 1697 cm⁻¹ [36] (Figure 4, a). In the spectra of uncross-linked membranes, however, we only observed a stretch vibration at 1646 cm⁻¹ attributing to the random coil conformation [36] (Figure 4, a and d). To investigate the conformational changes of SF in more detail, Fourier self-deconvolution to the original spectra were
performed, followed by curve fitting using Gaussian line shape profiles (Figure 4, b-c). The conformation of approximately 50.01% of the silk fibroin was strong intermolecular β-sheet, which was induced from random coils and intramolecular β-sheet by cross-linking (Figure 4, b-d). In addition, around 3.39% of silk fibroin was induced to weak β-sheet (Figure 4, b-d).

Figure 4. FT-IR analysis of SF/PEO nanofibers before and after water annealing. Original FT-IR absorbance spectra of the amide I region between 1720 and 1580 cm⁻¹ (a). Fractions distribution (blue dashed lines) of different SF conformations in the amide I region before (b) and after (c) cross-linking, red dashed lines are the fitted curve. Contribution of different conformations of silk fibroin (d).

### 3.2 Spatial distribution of gelatin nanospheres: single-nozzle electrospinning

SF nanofibers containing oppositely charged GANs and GBNs (Table S1) were fabricated by a simple single-nozzle electrospinning technique (Figure 5, g-i). To explore the possibility to control the distribution of oppositely charged GANs and GBNs inside electrospun SF membranes, GANs and GBNs were added into the SF/PEO solution at different weight ratios. The distribution of GANs (Figure 5, a-c) and GBNs (Figure 5, d-f) inside the nanofibers was clearly homogeneous, while the amount of GANs or GBNs inside the nanofibers correlated with their initial amount in the SF/PEO/GN colloidal suspensions (Figure 5, a-f). At a GAN/GBN weight ratio of 50/50, an equal distribution of GANs and GBNs was observed without overlap of the nanospheres (Figure 5, h).

This excellent control over the spatial distribution of GANs and GBNs was, however, only possible at low amounts of GANs and GBNs in the SF/PEO/GN colloidal suspensions. By increasing the amount of GNs to a SF/PEO/GAN/GBN weight ratio of 9/3/3/3, flocculation was observed with obvious phase separation (arrow in Figure S1) which hindered electrospinning.
3.3 Spatial organization of gelatin nanospheres: core-shell electrospinning

**Figure 5.** Confocal laser scanning micrographs of single-nozzle electrospinning of SF nanofibers containing both gelatin A and B nanospheres (GANs and GBNs) at an SF/PEO/GN weight ratio of 9/3/0.45. The weight ratios of GAN/GBN are 25/75 (a, d and g), 50/50 (b, e and h) and 75/25 (c, f and i). GANs are labeled blue (a-c) and GBNs are labeled red (d-f). Scale bars represent 10 µm.

**Figure 6.** Confocal laser scanning micrographs of core-shell electrospinning of SF nanofibers containing gelatin A and B nanospheres (GANs and GBNs) at an SF/PEO/GN weight ratio of 9/3/0.45. The shell (GBNs solution)/core (GANs solution) feeding rate was 5/1 (a, e and i), 3/1 (b, f and j), 1/1 (c, g and k) and 0.5/1 (d, h and l), respectively. GANs are labeled blue (a-d) and GBNs are labeled red (e-h). Scale bars represent 10 µm.
To prepare the SF nanofibers with high amounts of GANs and GBNs, we used a core-shell electrospinning technique. The electrospinning process was stable up to an SF/PEO/GN weight ratio of 9/3/3 (20 wt% of GNs to polymer). The distribution of these high amounts of GANs and GBNs in the nanofibers could, however, not be monitored using CLSM. Therefore, we fixed the SF/PEO/GN weight ratio at 9/3/0.45 when electrospinning colloidal suspensions containing GANs or GBNs as core or shell solutions to allow for CLSM observation. SF nanofibers containing both GANs and GBNs were obtained using core-shell electrospinning with GANs and GBNs as core and shell suspensions, respectively (Figure 6). The amount of GANs and GBNs inside the nanofibers was correlated to the feeding ratio between core and shell suspensions (Figure 6, a-h). Identical results were obtained when using colloid suspensions containing GANs and GBNs as shell and core suspensions, respectively (Figure S2).

3.4 Release of vancomycin from electrospun silk fibroin nanofibrous membranes

To test our hypothesis that the incorporation of GNs into the nanofibers would improve the release kinetics of biomolecules, vancomycin-loaded GBNs were electrospun into the SF nanofibers and monitored the release kinetics of vancomycin by means of HPLC. Burst release of vancomycin was observed on the first day for both samples with and without the presence of GBNs (Figure 7, a). In contrast, sustained release of vancomycin was observed for more than 14 days for membranes containing GBNs (Figure 7, b). The released amounts of vancomycin from membranes containing 20 wt% vs. 33 wt% of GBNs were statistically insignificant (Figure 7, b).

3.5 Cytotoxicity

The cytotoxicity of the electrospun SF nanofibrous membranes was
determined by a LDH activity assay as shown in Figure 8. These results indicated that none of the electrospun SF membranes was cytotoxic. No significant differences were observed among the various electrospun membranes.

### 3.6 Antibacterial effect

A zone of inhibition test was used to determine the antibacterial effects of the various SF nanofibrous membranes. An obvious inhibition zone was observed for all the membranes loaded with vancomycin (Figure 9, b-d). The zones of inhibition were significantly different for membrane samples with or without the presence of GBNs (Figure 9, e). The diameter of the inhibition zone was correlated to the weight percentage of vancomycin loaded inside the membranes (Table 2; Figure 9, e).

![Figure 8. Cytotoxicity of silk fibroin nanofibrous membranes containing different amounts of gelatin B nanospheres (GBNs) loaded with vancomycin. * p < 0.05, *** p < 0.001.](image)

![Figure 9. Zone of inhibition test against Staphylococcus aureus. Optical images of the inhibition zones surrounding electrospun silk fibroin membranes containing neither gelatin B nanospheres (GBNs) nor vancomycin (a, control), membranes containing only vancomycin (b, V-control), membranes containing 20 (c) and 33 wt% (d) of GBNs loaded with vancomycin.](image)

### 3.7 Viability of periodontal ligament cells on silk fibroin nanofibrous membranes

To explore the potential of the SF nanofibrous membranes for GBR/GTR applications, PDL cells were cultured on the membranes containing different amounts of GBNs. A live/dead assay was performed to evaluate the viabilities of the PDL cells, which revealed that the majority of the cells were alive on all membranes (Figure 10, a-c, live cells were stained green while dead cells were stained red). Spreading of the PDL cells on the membranes containing GBNs was clearly observed using electron
microscopy (Figure 10, b-c), while spherical cell clusters were observed on membranes without GBNs (Figure 10, a). Additionally, the mechanical integrity and fibrous structure of the membranes were retained after 48 h of cell culture (Figure 10, d-f). SEM characterization of the cell attachment also confirmed that PDL cells adhered and spread only on membranes containing GBNs after 48 h of cell culture (Figure 10, e-f).

**Figure 10.** Confocal laser scanning micrographs (a-c) and scanning electron micrographs (d-f) of periodontal ligament cells cultured on membranes without GBNs (Control, a, d), with 20 (b, e) and 33 wt% (c, f) of GBNs. Viabilities of PDL cells were evaluated by live/dead staining after 24 h culture (a-c) where live and dead cells were stained green and red, respectively. The morphology of the PDL cells was examined after 48 h culture (d-f). Scale bars represent 100 µm in (a-c) and 20 µm in (d-f).

**Discussion**

The aim of present study was to develop an electrospun nanofibrous SF-based drug delivery system for the controlled release of biomolecules. To this end, GNs were added into SF/PEO solutions to form colloidal suspensions for electrospinning. Defect-free nanofibers containing GNs were successfully prepared (Figure 2 and 3) with a GNs content of up to 33 wt% (Figure 2, d). This result demonstrated that electrospinning is a powerful method to prepare nano-in-nano structures [34, 37].

Prior to colloidal electrospinning, GNs were dispersed into aqueous SF solutions as vehicles for drug delivery while the SF solution was used as fiber-forming template [34]. Inflammable and toxic organic solvents were not used, which is in line with the concept of green electrospinning [38,
At low amounts of GNs in the colloidal suspension, the GNs could be dispersed without substantial aggregation (Figure 3). Uniform and defect-free fibers were obtained with SF/PEO/GNs weight ratios equal to or lower than 9/3/3 (Figure 2, b-c). With increasing the amount of GNs to SF/PEO/GNs weight ratio of 9/3/6, fibers became less uniform (Figure 2, d) which may be caused by the aggregation of GNs.

After electrospinning, water annealing was used to induce cross-linking by β-sheet formation [36] inside the nanofibers to ensure both mechanical and chemical stability of the nanofibrous membranes. This process effectively induced the conformation change of the silk fibroin with 30.31% of strong β-sheets formation (Figure 4, b-d) from random coils and weak β-sheets. The mechanical integrity of the nanofibrous membranes was conserved after water annealing (Figure S3), and this integrity was also maintained after the membranes were placed into the PBS medium for 14 days of release of loaded drugs (Figure S4).

Using the electrospinning technique as presented herein, oppositely charged GANs and GBNs were incorporated into SF nanofibers by means of single-nozzle without any substantial aggregation (Figure 5). In addition, this technique allowed control over the spatial distribution of the GNs in a straightforward manner. The distribution of GANs and GBNs inside the nanofibers was fine-tuned by adjusting the weight ratio between GANs and GBNs (Figure 5). Single-nozzle electrospinning was, however, not suitable for solutions containing high amounts of oppositely charged GANs and GBNs due to agglomeration of GANs and GBNs (Figure S1). This problem was overcome by using a co-axial electrospinning method, which guaranteed that agglomeration of GNs could be avoided since suspensions containing GANs and GBNs were only mixed at the tip of the nozzle. By adjusting the feeding ratios between core and shell suspensions, the distribution of GANs and GBNs could be easily manipulated in an efficient way (Figure 6). These results indicated that the colloidal electrospinning technique was a straightforward and effective strategy to confine GNs drug carriers at the nanoscale in nanofibrous membranes.

Although SF nanofibers have been widely used as drug delivery vehicles for e.g., GTR/GBR [26, 27], the release of biomolecules from these carriers/membranes is generally based on diffusion and consequently difficult to control due to a lack of interaction between the biomolecules and SF [25]. Herein, we hypothesized that this problem could be solved by incorporating...
GNs as drug delivery vehicles into SF nanofibers. To test our hypothesis, we electrospun SF nanofibrous membranes containing vancomycin-loaded GBNs by means of colloidal electrospinning (Table 2). Release of vancomycin from nanofibrous membranes containing GBNs was more sustained after the initial burst release compared to GBNs-free membranes (Figure 7). These results indicated that the GBNs were capable of prolonging the release of vancomycin due to the formation of interactions between vancomycin and GBNs as observed previously [30]. Nevertheless, only limited amounts of vancomycin can be bound onto GBNs to achieve a sustained release without any burst [30]. The amount of vancomycin loading in the present study (Table 2) surpassed the vancomycin-binding capability of GBNs, which in turn induced the burst release of vancomycin in the first day. To investigate the influence of the amount of GBNs on the release of vancomycin, we incorporated the same amount of vancomycin into the membranes with different amount of GBNs (Table 2). The release of vancomycin from membranes containing different amounts of GBNs was, however, statistically insignificant. This may be caused by the dissolution of PEO from the fibers. With more GBNs inside the nanofibers, the PEO was dissolved faster (Figure S4, d-f) since the SF/PEO layer was thinner as compared to nanofibers containing less GBNs. The disappearance of PEO in turn accelerated the exposure of GBNs to the medium, which resulted in the acceleration of vancomycin release. Consequently, the capability of the GBNs for the sustained release of vancomycin was comprised by the fast dissolution of PEO from the nanofibers.

The cytotoxicity test confirmed the excellent cytocompatibility of the vancomycin-loaded SF nanofibrous membranes (Figure 8). Additionally, the antibacterial efficacy of these membranes was confirmed since the release of vancomycin led to distinct inhibition zones (Figure 9, b-e). The diameter of the inhibition zone was correlated to the amount of the vancomycin released from the nanofibrous membranes (Figure 7, a; Figure 9, e). The amount of burst release of vancomycin in the first day decreased in the same order as the original weight percentage of vancomycin relative to the polymer content, i.e., V-control > 20 wt% GBNs > 33 wt% GBNs was in the same decreasing order (Figure 7, a; Table 2).

The cytocompatibility of the SF nanofibrous membranes was confirmed by direct culture of PDL cells onto the membranes. Most PDL cells were alive
after 24 h of cell culture on these membranes (Figure 10, a-c). In addition, the PDL cells attached and spread only on the membranes containing GBNs (Figure 10, b-c and e-f), which confirmed the added value of the incorporation of GBNs into the nanofibers. The GBNs contain cell recognition sites such as Arg-Gly-Asp (RGD) peptide sequences which modulate cell adhesion [28]. Upon dissolution of the PEO from the nanofibers, the GBNs became exposed and presented cell binding sites, thereby facilitating the attachment and spreading of the PDL cells.

**Conclusion**

Novel silk fibroin nanofibrous membranes containing gelatin nanospheres with a nano-in-nano structure were successfully fabricated. Both oppositely charged gelatin A and B nanospheres were incorporated into the nanofibers. The distribution of gelatin A and B nanospheres could be fine-tuned at the nanoscale by adjustment of either the weight ratio between the nanospheres by using single nozzle electrospinning, or the relative feeding rate of core and shell solutions by using co-axial nozzle electrospinning. A more sustained release of vancomycin was achieved from membranes containing gelatin B nanospheres inside the nanofibrous membranes as compared to the nanosphere-free membranes. These membranes were highly cytocompatible and exhibited antibacterial effects against *Staphylococcus aureus*. Additionally, these membranes supported the attachment and spreading of periodontal ligament cells. These results indicate that the nano-in-nano fibrous silk fibroin membranes containing gelatin nanospheres are promising candidates to control the release of (multiple) bioactive molecules.

**Acknowledgement**

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References

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33. Song, J., et al., Electrophoretic deposition of chitosan coatings modified with gelatin nanospheres to tune the release of antibiotics. ACS Appl Mater Interfaces, 2016.
### Supplementary information

**Table S1.** Parameters of gelatin nanospheres in the swollen state

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Gelatin A</th>
<th>Gelatin B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No dye NHS-405 labeled</td>
<td>No dye NHS-650 labeled</td>
</tr>
<tr>
<td>Particle size (nm)</td>
<td>400 ± 5</td>
<td>390 ± 2</td>
</tr>
<tr>
<td>ζ-potential (mV)</td>
<td>11.80 ± 0.49</td>
<td>-1.67 ± 0.08</td>
</tr>
</tbody>
</table>

**Figure S1.** Optical images of silk fibroin (SF)/gelatin nanospheres (GANs and GBNs) colloid suspensions at SF/PEO/GBNs of 9/3/6 (a) and SF/PEO/GANs/GBNs of 9/3/3/3. Arrow indicates the phase separation between silk fibroin and gelatin nanospheres.

**Figure S2.** Confocal laser scanning micrographs of core-shell electrospinning of silk fibroin nanofibers containing gelatin A and B nanospheres (GANs and GBNs, in shell and core, respectively) at silk fibroin:PEO:gelatin nanospheres weight ratio of 9:3:0.45 with core:shell feeding rate of 1:5 (a, e and i), 1:3 (b, f and j), 1:1 (c, g and k) and 0.5:1 (d, h and i), respectively. GANs are labeled blue (a, b, c and d) and GBNs are labeled red (e, f, g and h). Scale bars represent 10 µm.
Figure S3. Scanning electronic micrographs of electrospun silk fibroin (SF) nanofibers without gelatin B nanospheres after water annealing.

Figure S4. Scanning electronic micrographs of cross-linked electrospun silk fibroin (SF) nanofibers without GBNs (a, d), with 20 (b, e) and 33 wt% (c, f) of GBNs after 14 days of vancomycin release in PBS. Scale bars represent 4 μm in (a-c) and 800 nm in (d-f).
Chapter 6

Injection of gelatin nanospheres into zebrafish: a pilot study

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1 Introduction

Gelatin and gelatin-based materials have been widely used in the field of regenerative medicine due to their biodegradability, biocompatibility, and cost-effectiveness [1-4]. Gelatin nanospheres (GNs) are particularly interesting in view of their advantageous features for regenerative medicine and drug delivery [5-7].

First of all, the charged nature of GNs renders them suitable for controlled delivery of oppositely charged bioactive macromolecules such as growth factors [5, 8, 9] and nucleic acids [10, 11]. Beside macromolecules, GNs were shown to facilitate delivery of small biomolecules such as vancomycin and colistin in a sustained manner resulting from the formation of electrostatic and hydrophobic bonds between GNs and the specific drugs [12]. Moreover, the degradation profile of GNs can be easily fine-tuned by simple adjustment of the cross-linking density during their preparation [5, 9, 12, 13]. Furthermore, GNs can be chemically modified using various functional groups such as targeting-ligands owing to their proteinaceous nature. This modification provides the possibility for targeted delivery of biomolecules [6, 14].

The clinical applicability of GNs is, however, debated due to the potentially harmful effects of GNs related to the animal origin of gelatin [6, 15]. Although gelatin has been clinically applied as a plasma expander for several decades, retrospective analyses from observational studies found that the use of gelatin was associated with an increased renal impairment for patients with severe sepsis [16]. Therefore, preclinical evaluation of GNs in animal models is crucial before the clinical translation of GNs can be considered.

Although in vivo studies regarding the use of GNs for bone tissue regeneration have been reported previously [8, 9], real-time tracking of the GNs inside animals and monitoring of the host response to GNs is not yet possible. A clear understanding of the spatial distribution of GNs upon injection into various types of tissues is of critical importance since this distribution determines the final clinical applicability of the GNs. Currently, no suitable animal model which would allow combined real-time tracking of GNs and monitoring of the host response to GNs is available.

Because of their tractable genetic and embryologic characteristics [17], zebrafish (Danio rerio) have been used since the 1970s as a powerful
model in biomedical research for drug screening [18-20], translational neuroscience [21, 22], physiological analysis [23, 24], infection biology [25] as well as evaluation of nanocarriers for drug delivery [26, 27]. The zebrafish embryo has fully functional organs and a strong physiological and genetic homology to mammals [17, 22] e.g. with respective to their innate immune system which is mainly comprised of neutrophils and macrophages at 3 d post fertilization [28]. These unique characteristics enable the evaluation of GNs with zebrafish embryo alternatives instead of mammalian models, which is in line with ethical requirements related to reducing, replacing or refining animal experiments [18]. In addition, the high fecundity, external fertilization and rapid development of zebrafish embryos render them cost- and space-effective for in vivo research [22, 29], and even allows medium to high throughput analyses [25, 30]. Moreover, transgenic zebrafish lines that express green fluorescent proteins (GFP) under control of a macrophage-specific promoter (e.g., the macrophage expressed gene 1 (mpeg1) promoter [31]) allow the in vivo real-time visualization and detailed analysis of macrophage responses to GNs in transparent zebrafish embryos until 7 d post fertilization [32]. Consequently, the zebrafish embryo holds strong promise as animal model for the in vivo evaluation of GNs.

The aim of this study was to perform a preclinical real-time evaluation of GNs in a transgenic zebrafish model with GFP-expressing macrophages. To this end, we labeled GNs fluorescently with a far-red dye to allow for their visualization within the zebrafish embryo. Subsequently, we injected the labeled GNs suspension into either the blood stream or muscle tissue of 3-day-old zebrafish embryos by micro-injection. We hypothesized that this model would allow for real-time visualization of the distribution of GNs inside the zebrafish and monitoring of the interactions between macrophages and GNs by using confocal laser scanning microscopy (CLSM).

2 Materials and Methods

2.1 Materials

Gelatin type B (from bovine skin, 225 bloom, isoelectric point (IEP) ~ 5) was purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). Glutaraldehyde (25 wt% solution in water) was purchased from Acros Organics (Geel, Belgium). DyLight™ 650 NHS Esters were purchased from ThermoFisher Scientific (Bleiswijk, the Netherlands). All other chemicals
were purchased from Sigma-Aldrich. The zebrafish embryo medium (E3 medium) was home made.

2.2 Preparation of gelatin nanospheres

GNs were prepared by a two-step desolvation method [12]. Briefly, 1.25 g of gelatin was added to 25 ml deionized water to form a 5 w/v% solution at 50 °C under magnetic stirring. Twenty-five ml of acetone was subsequently added to precipitate the high molecular weight gelatin chains. After precipitation, the supernatant was discarded and the gelatin was re-dissolved in water at 50 °C. Thereafter, the pH value of the gelatin solution was adjusted to 2.5, followed by addition of 80 ml of acetone at a constant rate of 4 ml/min under a stirring speed of 1200 rpm to form GNs. Subsequently, the GNs were cross-linked by addition of 740 μl of 25 wt% glutaraldehyde. After 16 h of reaction, the cross-linking process was stopped by adding 105 ml of 100 mM glycine solution. The suspension was centrifuged subsequently at 5000 rpm for 60 min and re-suspended in deionized water by vortexing. After 3 cycles of washing, the GNs suspension was stored at 4 °C until further use.

2.3 Fluorescent labeling of gelatin nanospheres

To trace the GNs inside the zebrafish, the GNs were fluorescently labeled with a far-red dye by DyLight™ 650 NHS esters according to a standard protocol (ThermoFisher Scientific, Bleiswijk, the Netherlands). Briefly, 200 mg of GNs were dispersed in 20 ml of phosphate buffered saline (PBS), followed by adjustment of the pH of the GNs suspension to 7.2 using 1M sodium hydroxide solution. Thereafter, 50 μg of NHS ester were dissolved in 200 μl of N,N-dimethylformamide (Serva Electrophoresis GmbH, Heidelberg, Germany). The NHS ester solution was then added dropwise to the GNs suspension under magnetic stirring at 400 rpm. After one hour of reaction, the suspension was centrifuged at 5000 rpm for 60 min and re-suspended in deionized water by vortexing. The suspension was stored at 4 °C until further use.

2.4 Characterizations of gelatin nanospheres

The morphology of the GNs before and after fluorescent labeling was characterized by scanning electron microscopy (SEM, LEO 435 VP Zeiss, Germany). The size and size distribution of GNs in swollen state before and after fluorescent labeling were measured in HEPES (5 mM, pH 7.0) and PBS (154 mM, pH 7.4) by means of dynamic light scattering (DLS, Zetasizer
Nano-S, Malvern Instruments Ltd., Worcestershire, United Kingdom). The ζ-potential of GNs was examined by laser Doppler electrophoresis with a Zetasizer Nano-Z (Malvern Instruments Ltd., Worcestershire, United Kingdom) in the same HEPES and PBS buffer.

2.5 Zebrafish husbandry

The zebrafish embryos used in this study were produced by the transgenic line Mpeg1:Kaede. Macrophages of these embryos are fluorescent due to the expression of GFP. The embryos were maintained in E3 medium and kept in an incubator (VWR Incu-line, Amsterdam, the Netherlands) at 28.5 °C. E3 medium was refreshed and dead embryos were removed every day. All experiments with zebrafish embryos were conducted in agreement with the local animal welfare regulations (European Union Council Guidelines 86/609/EU).

2.6 Micro-injection of the gelatin nanospheres

Fluorescently labeled GNs were suspended in deionized water at a concentration of 18 mg/ml for the micro-injection into zebrafish embryos. The GNs suspension was vortexed for 1 min before administration to zebrafish embryos. The zebrafish embryos with fluorescent macrophages were first selected using a fluorescent stereo microscope (Leica M205 FA, Eindhoven, the Netherlands). These embryos were subsequently anesthetized with 0.03% tricaine (buffered 3 aminobenzoic acid ethyl ester, Sigma-Aldrich, Zwijndrecht, the Netherlands) and aligned in the U-shape grooves in an agarose plate casted with a specific mold. GNs were injected into either the blood stream via the duct of curvier or the tail muscle tissue of mpeg 1:Kaede zebrafish embryos at 3 d post fertilization using a glass capillary (Harvard Apparatus, Holliston, MA). The outer diameter of the openings of all glass capillaries was adjusted to a size of approximately 15 μm under the light microscope (Leica M50, Eindhoven, the Netherlands), leading to an injection volume of approximately 3 nl of the GNs suspension. The same volume of nanosphere-free PBS was injected into muscle tissue as control. Injections were performed with an Eppendorf FemtoJet. A light microscope was used to monitor the injection procedure.

2.7 Confocal imaging of the gelatin nanospheres and macrophages

To observe the distribution of GNs and the response of macrophages to
the GNs in the zebrafish embryos, we used CLSM (Leica DMI6000 inverted microscope, mounted with the Leica TCS SP8 SMD, Eindhoven, the Netherlands) to monitor the GNs and fluorescently labeled macrophages after 3 h and 24 h post injection. For imaging, the zebrafish embryos were first anesthetized with 0.03% tricaine (Sigma-Aldrich, Zwijndrecht, the Netherlands). Subsequently, they were placed in a glass-bottom culture dish (35 mm, MatTek Corporation, Ashland, USA) filled with low melting point agarose. After hardening of the agarose, E3 medium was added to cover the agarose with the embryo entirely and a water lens was used for visualization.

Since the fluorescent Kaede protein expressed in macrophages of this zebrafish line was reported to undergo an irreversible photoconversion from green to red fluorescence upon UV illumination (350 - 400 nm) [33, 34]. We used a filter at wavelength of 504-555nm to observe macrophages and a filter at wavelength of 659-764 nm to observe GNs. By using these two filters, the color conversion of the Kaede protein did not impede visualization of the macrophages in the zebrafish since no far-red signals was observed at the injection site (Figure S1, d and f).

2.8 In vitro macrophage culture studies

THP-1 derived macrophages were cultured in the presence of GNs to monitor the in vitro cellular internalization of GNs. Briefly, THP-1 human monocytic cells were cultured in RPMI 1640 medium (Gibco™, Fisher Scientific, Paisley, Scotland) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/mL streptomycin at 37 °C in a 5% CO2 and 95% air environment. The cells were seeded on Thermanox coverslips (ThermoFisher Scientific, Bleiswijk, the Netherlands) at a cell density of 2.5 × 105 cells/cm2. Phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich, Saint Louis, USA) was then added at a final concentration of 50 ng/ml to generate macrophages. After 48 h of culture, fluorescently labeled GNs were added to the medium (at a concentration of 180 μg/ml). Macrophages were further incubated with the GNs for 5 h and 24 h. After incubation, Cell-Tracker™ CMFDA green (ThermoFisher Scientific, Bleiswijk, the Netherlands), staining the cell cytoplasm, was added at a concentration of 10 μM. After 30 min of incubation, the cells were washed 3 times with PBS and fixed with 4% paraformaldehyde. Thereafter, DNA/nuclei of these cells were stained with 4’,6-diamidin-2-phenylindol (DAPI, Roche Applied Science, Mannheim,
Germany) and the cells were mounted with ProLong® Diamond Antifade Mountant (ThermoFisher Scientific, Bleiswijk, the Netherlands) for imaging.

3 Results

3.1 Characterization of fluorescently labeled gelatin nanospheres

Fluorescent labeling of GNs did not affect the morphology of the nanospheres (Figure 1), while their size in swollen state in PBS buffer increased slightly from 329 to 387 nm (Table 1). The average size of the fluorescently labeled GNs in swollen state in HEPES and PBS buffers was approximately 566 and 387 nm, respectively. The GNs became less negatively charged in PBS buffer of high ionic strength buffer compared to HEPES buffer of much lower ionic strength. The lower nanosphere size in PBS buffer was attributed to screening of the repulsive negative charges between the nanospheres.

3.2 In vivo distribution of and macrophage response to gelatin nanospheres after intravenous injection

Around 3 nl of the aqueous suspension containing fluorescently labeled GNs (Figure 1 and Table 1) were successfully injected intravenously (Figure 2, a-d) into zebrafish embryos at 3 d post fertilization. Three hours after intravenous injection, the GNs were spread throughout the large blood vessels (Figure 2, a and d, yellow arrow in d) and small blood vessels of the embryos (Figure 2, d, white arrows). These GNs were observed as separate aggregated clusters (Figure 2, c), whereas individual GNs could not be observed due to the limited resolution of the CLSM.

Three hours after intravenous injection, the macrophages were mainly

| Table 1. Parameters of gelatin nanospheres in the swollen state |
|-----------------|-----------------|-----------------|
| Parameters      | No dye          | NHS-650 labeled |
|                 | PBS (154 mM, pH 7.4) | HEPES buffer (5 mM, pH 7) | PBS (154 mM, pH 7.4) |
| Particle size (nm) | 329 ± 5         | 566 ± 6         | 387 ± 7         |
| ζ-potential (mV)   | -6.63 ± 0.86    | -25.00 ± 0.50   | -6.55 ± 0.20    |
present in the blood vessels (Figure 2, c and f), whereas macrophages were hardly observed in muscle tissue of embryos (Figure 2, e and f). Co-localization of GNs and macrophages was clearly observed within the blood circulation 24 h after injection (Figure 3, a-c and f, white arrows in b-c), when the majority of the macrophages was observed in close proximity to the GNs (Figure 3, d-f).
3.3 *In vivo* distribution of and macrophage response to gelatin nanospheres after intramuscular injection

Approximately 3 nl of fluorescently labeled GNs (Figure 1 and Table 1) were injected intramuscularly (Figure 4, a-c). In contrast to intravenous injection of GNs, the GNs remained cohesive and confined within muscle tissue 3 h after intramuscular injection (Figure 4, d). The nanospheres were distributed throughout the muscle tissue near the injection site, as reflected by the

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*Figure 3.* Co-localization of gelatin nanospheres (GNs, red) and macrophages (green) can be observed 24 h after intravenous injection. (a) Lateral view of the embryo. (b-c) Lateral views of the selected areas from (a). Arrows indicate the co-localization of GNs and macrophages. (d-f) Top view of 3D reconstruction of the distribution of GNs and macrophages. Scale bars represent 100 μm in (a), 50 μm in (b-c) and 500 μm in (d-f).
stripe pattern of the muscle tissue (Figure 4, a-d). The original cohesive mass of GNs was disintegrated into separate clusters of GNs 24 h after intramuscular injection (Figure 5, a-d).

Macrophages were attracted to the injection site 3 h after intramuscular injection, while more macrophages were observed in muscle tissue of zebrafish upon intramuscular injection of GNs (Figure 4, e-f) as compared to injection of nanosphere-free PBS control (Figure S1, e-f). More macrophages were observed at the injection site 24 h after intramuscular injection compared to 3 h after injection (Figure 4, e-f; Figure 5, e-f). GNs and macrophages clearly co-localized in the zebrafish 24 h after intramuscular injection of GNs (Figure 5, a-c, white arrows in b-c). Moreover, the GNs were intimately surrounded by macrophages (Figure 5, f), which might suggest that GNs were occasionally internalized by macrophages (Figure 5, g, white arrow).

### 3.4 *In vitro* macrophage culture studies

To study the interactions between GNs and macrophages in more detail,
we cultured human macrophages derived from the monocytic cell line THP-1 in the presence of fluorescently labeled GNs to allow imaging using CLSM. The nuclei and cytoplasm of the macrophages were stained blue.

**Figure 5.** Gelatin nanosphers (GNs, red) co-localized with macrophages (green) 24 h after intramuscular injection of GNs. (a) Lateral view of the embryo. (b-c) Lateral views of the selected areas from (a). Arrows indicate the co-localization of GNs and macrophages. (d-f) Top view of 3D reconstruction of the distribution of GNs and macrophages. (g) Higher magnification of selected area in (f) where the white arrow indicates that GNs were intimately surrounded by macrophages. Scale bars represent 100 μm in (a, d-f), 50 μm in (b-c) and 20 μm in (g).

**Figure 6.** Cellular internalization of gelatin nanospheres (GNs) by THP-1 macrophages after 5h (a-d) and 24 h (e-h) of macrophage culture in the presence of GNs. Scale bars represent 20 μm. Nuclei of the cells were stained with DAPI/blue (a and e), cytoplasm was stained with Cell-Tracker™ CMFDA/green (b and f) and GNs were labeled with far-red dye/red (c and g).
Injection of Gelatin Nanospheres into Zebrafish

(Figure 6, a and e) and green (Figure 6, b and f), respectively. GNs labeled with a far-red fluorescent dye were observed clearly as well (Figure 6, c and g). Internalization of GNs into the macrophages was confirmed after 5h (Figure 6, a-d; video S1) and 24h of macrophage culture (Figure 6, e-h; video S2).

4 Discussion

The aim of the present study was to monitor the spatial distribution as well as macrophage response to GNs in a zebrafish model. Although the suitability of GNs for application in regenerative medicine has been confirmed in various animal models [8, 9], tracking the fate of GNs in these animal models is not yet possible. Monitoring the spatial distribution of GNs and the associated immune response \textit{in vivo} is, however, prerequisite for the clinical translation of GNs. By using transgenic zebrafish embryo model, we were able to image the spatial distribution of GNs in real-time in a living vertebrate using a relatively simple and straightforward method. In addition, we were able to monitor the response of macrophages to the injected GNs by using zebrafish macrophages expressing green fluorescent proteins.

Upon injection of artificial particulate material \textit{in vivo}, the response of the material to biological environment is determined by a plethora of factors including blood circulation, ionic strength and protein adsorption. Accordingly, the immune response to the presence of foreign body material in zebrafish is initiated via its innate immune system. This immune system is highly comparable to the one of mammals [28, 35, 36] and mainly comprised of the two major types of innate immune cells, i.e., neutrophils and macrophages, which are already present and functional at 3 d post fertilization [28]. Neutrophils respond first, and macrophages are subsequently recruited to phagocytose pathogens and tissue debris [28, 37]. Macrophages have been shown to play a central role in this immune reaction [38, 39]. Therefore, we used transgenic zebrafish expressing GFP in macrophages to study the response of macrophages to injected GNs.

In the present study, the GNs were spread throughout the blood vessels 3 h after intravenous injection (Figure 2, a and d) caused by blood circulation, whereas GNs were largely confined at the intramuscular depot following intramuscular injection. The individual GNs agglomerated in the blood stream 3 h after intravenous injection (Figure 2, c-d), whereas the
nanospheres were not agglomerated before tissue injection as confirmed by reproducible size measurements using DLS of about 566 ± 6 nm in 5 mM HEPES buffer (Figure 1 and Table 1). This agglomeration may be caused by the high ionic strength of the blood, which screens the repulsive interactions between the negatively charged GNs [5]. This charge screening was confirmed by the decreased charge of the GNs in PBS buffer (which has an ionic strength comparable to blood, Table 1). A similar phenomenon was observed 24 h after intramuscular injection. Although the majority of the GNs remained cohesive and confined in the muscle tissue close to the injection site (Figure 5, d and f), the original bulk of the GNs disintegrated into separate clusters (Figure 4, a and d; Figure 5, a and d). In addition to the ionic strength, adsorption of proteins onto the negatively charged GNs in vivo might also have contributed to the agglomeration of GNs. Although the exact mechanism behind agglomeration and disintegration of GNs remains to be elucidated, our observations confirmed that the zebrafish model is suitable for the real-time monitoring of the spatial distribution of the GNs in vivo, which will be highly instrumental for further research on the therapeutic efficacy of GNs as carriers for local drug delivery.

Three hours after intravenous or intramuscular injection of GNs, the macrophages were observed close proximity to the GNs in the bloodstream or at the injection sites (Figure 2, c and f; Figure 4, c and f). Over time, an increasing number of macrophages was observed 24 h after injection (Figure 3, f; Figure 5, f), which may be caused by chemotaxis as reported previously [27, 40]. Consequently, macrophages surrounded the GNs, resulting into co-localization of GNs and macrophages 24 h after injection of GNs in the blood (Figure 3, a-c and f) or muscle tissue (Figure 5, a-c and f-g). Nevertheless, internalization of the GNs by macrophages could not be confirmed unambiguously using CLSM to observe the zebrafish. Therefore, macrophage culture studies were performed to study if GNs could be internalized in vitro.

Internalization of GNs by macrophages was indeed observed in vitro upon culture of THP-1-derived macrophages in the presence of fluorescently labeled GNs. We found that the GNs can be internalized by macrophages after 5 h of macrophage culture (Figure 6, a-d; video S1), while a much larger number of GNs was observed inside the macrophages after 24h of culture (Figure 6, e-h). This internalization of GNs by macrophages suggests that GNs can be used for local delivery of antibacterial agents to combat
macrophage-associated intracellular infections.

5 Conclusion
A novel \textit{in vivo} method was developed for the biological evaluation of gelatin nanospheres using transgenic zebrafish expressing GFP-labeled macrophages. Upon intravenous or intramuscular injection of the fluorescently labeled gelatin nanospheres into zebrafish embryos, the real-time spatial distribution of gelatin nanospheres as well as interaction between gelatin nanospheres and macrophages could be studied using confocal laser scanning microscopy.

The method presented herein provides a valuable tool for the biological evaluation of gelatin nanospheres, which can be useful for investigation on the effect of local delivery of biomolecules from gelatin nanospheres.

Acknowledgement
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References

Supplementary information

Figure S1. Distribution of macrophages (green) in zebrafish embryos 3 h after intramuscular injection of 3 nl of nanosphere-free PBS. (a) Lateral view of the embryo. (b–c) Lateral views of the selected areas from (a). (d–f) Top view of 3D reconstruction of the distribution of macrophages. Scale bars represent 100 μm in (a, d–f) and 50 μm in (b–c). Autofluorescence caused by pigments (a and d–f, white arrows) of the zebrafish embryos appeared as yellow (f, white arrows) upon merging of far-red and green channels. The results in this figure confirmed that the color conversion of the Kaede protein did not impede visualization of the macrophages in the zebrafish.
Chapter 7

Antibacterial effects of electrospun chitosan/poly(ethylene oxide) nanofibrous membranes loaded with chlorhexidine and silver

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1 Introduction

Percutaneous medical devices, such as external fixation devices, urinary catheters, peritoneal dialysis catheters, bone-anchored hearing aids, voice prostheses and intravenous catheters for haemodialysis, have become indispensable in clinical practice [1-3]. These external devices, however, are particularly susceptible to bacterial infections due to their penetration through the skin, the body’s primary defence to infection. The open space created around the implant provides an entrance for bacteria migrating into the body and subsequently colonizing on the implanted medical devices followed by formation of a biofilm [4, 5]. Within the biofilm, organisms are protected from a matrix consists of self-produced extracellular polymeric substances, which enables the bacteria less susceptible to host defense mechanisms and to the treatment of antibiotics [2, 5]. Consequently, the development of such infections may results into life-threatening complications, which yields high morbidity and mortality rates, and high costs to the health care system [6]. Although decades of research, including hospital and patient hygiene measures and development of antimicrobial coatings, has been done, no ubiquitously accepted clinical solution to address the problem of percutaneous device associated infections (PDAIs) has been forwarded.

Instead of treating the PDAIs, development of methods or techniques to prevent PDAIs is of enormous value. Recently, the use of an effective chlorhexidine (CHX)-impregnated dressing at the exit-site of catheters with weekly replacement was reported [7]. This dressing had high efficiency both in reducing colonization of bacteria on the catheter and in reduction of catheter-related bloodstream or central nervous system infections. Therefore, the development of active antibacterial dressing, which can deliver antimicrobial agents locally in a sustained manner, can be an efficient approach for the prevention of PDAIs.

Antimicrobial agents involve both antibiotics and antiseptics [8], which are chemical substances that prevent the growth and development of the microorganisms. Nevertheless, compared with antiseptics, antibiotics are only effective against bacteria and have a higher risk to develop antibacterial resistance [9, 10]. In fact, drug-resistant pathogens are emerging dramatically, leading to strongly increased global healthcare cost [11, 12]. Unfortunately, the development rate of antimicrobial resistance
exceeds the development rate of new drugs by far [12]. Furthermore, clinical therapy based on single drug is often not sufficiently effective to counteract infections, which has stimulated the development of combinatorial multidrug approaches to combat infections more effectively [12-14]. To this end, combination of antiseptics can be an effect method to kill bacteria without inducing antibacterial resistance. Among the various types of antiseptics, silver nanoparticles (AgNPs) are particularly attractive thanks to their broad-spectrum antibacterial activity. As a consequence, AgNPs have been widely used in various medical products [15, 16]. More recently, it has been demonstrated that silver ions (Ag+) are the active bactericidal agent that is released from AgNPs, and the antibacterial activity of AgNPs could be controlled by modulating Ag+ release through manipulation of oxygen availability, particle size as well as shape of AgNPs [17]. Besides AgNPs, CHX is another commonly used antiseptic, which has both bacteriostatic and bactericidal properties against gram positive and negative bacteria [18]. Recent studies showed that the combination of CHX with silver reduced the incidence of catheter infections and prolonged the antibacterial efficacy against a wide range of clinically significant potential pathogens [19, 20].

To achieve the dual delivery of Ag+ and CHX, a suitable drug carrier is required for the fabrication of antibacterial dressing. More recently, chitosan and chitosan-based nanofibrous matrices have gained interest due to their large specific surface area, high drug encapsulation efficiency, superior biocompatibility, and ease of processing by using the electrospinning technique [21-23]. Furthermore, chitosan is a stabilizing material that can protect AgNPs from oxidization [24], while chitosan itself has intrinsic antibacterial properties [23, 25].

Based on the aforementioned, the objective of this work was to develop a chitosan-based nanofibrous antibacterial dressing containing AgNPs for the dual release of Ag+ and CHX. We hypothesized that the most effective approach to prevent PDAIs would involve fast release of CHX to achieve a powerful initial bactericidal effect and avoid the development of bacterial resistance, followed by a sustained release of Ag+ to prevent any latent bacterial infections. To this end, we electrospun chitosan/poly(ethylene oxide) (PEO) in acetic acid solution containing silver nitrate (AgNO₃) to obtain uniform and defect-free nanofibres embedding AgNPs. Additionally, we post-loaded CHX onto the nanofibrous membrane by a diffusion method.
and then investigated the release of CHX and Ag+ in deionized water. Finally, we examined the antibacterial effect of the membranes loaded with different amount of CHX and AgNO₃ and the long-term antibacterial effect of the membrane loaded with 5 wt% of AgNO₃ using the zone of inhibition test against *Staphylococcus aureus* (**S. aureus**), which is one of the most common pathogens associated with PDAIs [1].

2 Methods

2.1 Electrospinning of nanofibres containing AgNPs

Chitosan (degree of deacetylation = 90%, molecular weight = 200-400 kDa, Heppe Medical Chitosan) and PEO (molecular weight = 900 kDa, Sigma-Aldrich®) were mixed at a weight ratio of 75:25 and dissolved in 35 v/v% acetic acid solution to form a 3 w/v% polymer solution. Subsequently, 0.1, 1 and 5 wt% (to polymer) of AgNO₃ (Boom BV, The Netherlands) were added and the mixture was stirred overnight before electrospinning. The electrospinning setup was described previously [26]. The electrospinning process was conducted at 27 kV and the polymer solution was ejected at a flow rate of 4 ml/h controlled by a syringe pump (BD scientific Inc., USA), while the distance between the spinneret and collector was fixed at 15 cm. The electrospun membranes were then punched into discs with a diameter of 11-12 mm, a surface area of approximately 1 cm² and a thickness of approximately 135 µm. The punched discs were cross-linked by glutaraldehyde (25 wt% in water) vapour for 2 h and then left in a fume hood overnight to remove the residual glutaraldehyde. Thereafter, the samples were stored in a desiccator at room temperature for further use.

2.2 Characterization of electrospun fibres

The electrospun discs were sputtered coated with gold (approximately 10 nm in thickness) to make the nanofibres conductive and the morphology of the discs were observed by scanning electron microscopy (SEM, JEOL 6301) at an accelerating voltage of 10 kV. Diameter distributions of the fibres were determined from the SEM micrographs obtained at random locations using the software Image J (National Institutes of Health, Bethesda, USA). One hundred fibres per membrane were analyzed. The formation of AgNPs was examined by transmission electronic microscopy (TEM, JEOL 1010) and TEM-energy dispersive X-ray spectroscopy (TEM-EDX, JEOL 2100 TEM with a Bruker Quantax EDX detector). To investigate the distribution of AgNPs
inside the fibres, cross-sectional samples of the fibres were prepared by embedding of the fibres in Epofix™ cold-setting embedding resin (Struers, The Netherlands) followed by microtome cutting. The cross-sectional samples were then mounted on 200-mesh carbon-coated copper grids (Agar Scientific, United Kingdom) before TEM observation.

2.3 Drug loading and release

A diffusional post-loading method was used to load CHX (chlorhexidine-digluconate, Sigma-Aldrich®) onto cross-linked nanofibrous discs. Briefly, 5 µl of CHX solution in deionized water containing 20, 60 or 100 µg of CHX was dripped on each membrane disc. This volume of CHX solution was selected to allow for total adsorption of the drug solution in the membranes according to our water adsorption test. The membranes were then freeze dried for later use.

Nine groups of loaded membranes were used for the release study of Ag+ and CHX (Table 1, group 2-10). Each piece of membrane disc was placed into a 1.5 ml Eppendorf® tube (n = 5) containing 1 ml deionized water. The tubes were then incubated at 37 °C with gentle agitation. At each predetermined time point, 900 µl supernatant was collected for further analysis and then 900 µl of deionized water was added.

<table>
<thead>
<tr>
<th>Group</th>
<th>Amount of AgNO₃ (wt% to polymer)</th>
<th>Amount of chlorhexidine (µg per membrane)</th>
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CHX in supernatant was detected by reverse phase high performance liquid chromatography (RP-HPLC) using a Hitachi HPLC machine, which consists of a Hitachi L-2130 pump, a Hitachi L-2400 UV detector and a Hitachi L-2200 auto sampler. The specimens were analyzed by a previously
described method [27] with some modifications. Briefly, the mobile phase consisted of acetonitrile/water (40:60, v/v, containing 0.1 v/v% trifluoroacetic acid and 0.1 v/v% triethylamine) which was eluted through a LiChrospher® RP-18 endcapped HPLC column (125 mm × 4 mm, particle size 5 μm) at a flow rate of 1 ml/min. Fifty microliter of collected supernatant was injected and the effluent was monitored at 260 nm. A standard calibration curve was prepared from CHX solution with concentrations ranging from 0.2 to 100 μg/ml. The Ag+ content was determined by using inductively coupled plasma mass spectroscopy (ICP-MS, Thermo Scientific, X series I). The collected supernatant was diluted 20 times in 1 wt% nitric acid for ICP-MS analysis of silver. A standard calibration curve was prepared with concentrations of Ag+ ranging from 8 to 1000 ng/ml.

2.4 Cytotoxicity

The alamarBlue® Cell Viability Assay was used to evaluate the cytotoxicity of the membranes. All groups of membranes listed in Table 1 (n = 3) were immersed in 2.5 ml αMEM cell culture medium supplemented with 10% Fetal Bovine Serum (FBS, Sigma F7524, Taukirchen, Germany) and 1% Penicillin/Streptomycin (Gibco, Invitrogen Corp., Paisley, Scotland) for 24 h to obtain the release medium. Human Foreskin Fibroblasts (HFFs, isolated from foreskin specimens from a healthy donor by following national guidelines for working with human materials) were cultured in αMEM medium. The cells were trypsinized and reseeded onto a 24-well plate with a cell density of 50,000 cells per well. After 24 h, the culture medium was replaced with 1 ml of the release medium. Fresh culture medium and 5% DMSO were used as positive and negative control, respectively. Additionally, a series of CHX solution from 0.2 to 100 μg/ml and Ag+ from 0.2 to 50 μg/ml were prepared in fresh medium and tested simultaneously. After 20 h, the culture medium was refreshed with 1 ml culture medium containing 10 v/v% alamarBlue dye. After 4 h of incubation, a 200 μl aliquot of metabolized medium from each well was transferred into a 96-well plate in duplicate and the absorbance was measured with emission wavelength of 530 nm and excitation wavelength of 590 nm in a Bio-Tek® FL600 microplate fluorescence reader.

2.5 Antibacterial effect

The antibacterial effect of the membranes against S. aureus (ATCC®
25923TM, Manassas, USA) was examined by a zone of inhibition test [28] recommended by the European Society of Clinical Microbiology and Infectious Disease, with the EUCAST (European Committee on Antimicrobial Susceptibility Testing) standard tablets containing 30 µg of cefoxitin as the positive control. In brief, the bacterial suspensions for inoculations were firstly prepared by dispersing the isolated bacterial colonies in sterile saline (0.85% NaCl w/v in water) at a density of a McFarland 0.5 standard. The bacterial suspension was then diluted to a concentration of approximately $6 \times 10^7$ colony-forming units (CFU) per ml and spread evenly over the entire surface of an agar plate by using a sterile cotton swab. The electrospun discs were then placed on the agar plate. After incubation at 35 °C for 20 h, the diameter of the inhibition zone was measured from the centre point of the disk to the border of the inhibition zone.

The zone of inhibition test was also used to examine the long-term antibacterial effect of the membrane. Membranes containing 5 wt% AgNO$_3$ were first incubated with in deionized water under the same conditions as described in section 2.3. The membranes were collected at day 1, 2, 3 and 4 (n = 3) and used for the zone of inhibition test as described above.

**2.6 Statistics**

Statistical analyses were conducted using GraphPad Prism and all results were reported as mean ± standard deviation. Differences among groups were analyzed by one way analysis of variance followed by a Tukey post-hoc test, and a p-value of < 0.05 was considered as a statistically significant difference.

![Scanning electron micrographs and fibre diameter distributions of chitosan/PEO nanofibrous membranes containing 0 (A and E), 0.1 (B and F), 1 (C and G) and 5 wt% (D and H) of AgNO$_3$, with average size of 97 ± 20 nm (E), 99 ± 21 nm (F), 120 ± 26 nm (G) and 121 ± 25 nm (H), respectively.](image)
3 Results

3.1 Characterization of the electrospun fibrous membranes

Chitosan/PEO nanofibrous membranes containing 0, 0.1, 1 and 5 wt% of AgNO₃ were successfully prepared. The nanofibres were uniform and defect-free (Figure 1 A to D), with an average size of approximately 100 nm. Differences in size distributions of the fibres containing different amount of AgNO₃ were statistically insignificant.

The in-situ formation of AgNPs inside the nanofibres was confirmed by TEM (Figure 2). Electron-dense nanoparticles of approximately 4 nm were detectable when AgNO₃ levels were equal or higher than 1 wt% (Figure 2C), and the number of these nanoparticles increased with increasing amount of AgNO₃ (Figure 2D). Moreover, the distribution of the particles throughout the fibres was shown to be homogeneous by means of TEM analysis of the fibre cross-sections (Figure 2 E and F). Elemental analysis of the fibres using TEM-EDX confirmed that these nanoparticles were composed of silver, and the homogeneous distribution of silver inside the organic matrix of the nanofibres could be clearly demonstrated by means of elemental mapping (Figure 3). Furthermore, it was shown that the AgNPs were formed prior to electrospinning (Figure S1).

Figure 2. Transmission electron micrographs of chitosan/PEO nanofibres containing 0 (A), 0.1 (B), 1 (C) and 5 wt% (D) of AgNO₃, and transmission electron micrographs of the cross-section of nanofibres with 0.1 (E) and 5 wt% (F) of AgNO₃.

Figure 3. Transmission electron micrograph (A) combined elemental mapping using energy dispersive X-ray spectroscopy (B) of chitosan/PEO nanofibres containing 5 wt% of AgNO₃. Distribution of silver and carbon is shown in red and green, respectively.
3.2 Release of chlorhexidine and Ag+

Significantly different release profiles were observed for CHX and Ag+. A burst-type release pattern was observed for CHX, irrespective of the amount of CHX or AgNO₃ loaded on each membrane (Figure 4A and 4B). In contrast, Ag+ was released in a sustained manner for more than 28 days for the membranes containing different amount of AgNO₃, irrespective of the presence of CHX (Figure 4C). Moreover, the Ag+ was released relatively fast during the first 4 days followed by slow release during the following time period. After 28 days of release, AgNPs were hardly observed anymore inside the nanofibres (Figure S2).

3.3 Cytotoxicity

To examine the cytotoxicity of the membranes loaded with different amount of CHX and AgNO₃, we immersed the membranes in cell culture medium for 24h, and subsequently collected the release medium and tested the cytotoxicity of the release medium. Significant cytotoxicity was observed for membranes containing ≥ 60 µg CHX (Figure 5A), and no cytotoxicity was observed for membranes loaded with 20 µg CHX or those with AgNO₃. To
examine and compare the cytotoxic level of Ag+ and CHX, a serial dilution of AgNO$_3$ and CHX in cell culture medium was tested. It was shown that Ag+ had no cytotoxic effect at concentrations of AgNO$_3$ up to the highest concentration tested (50 µg/ml). However, obvious cytotoxicity of CHX was observed when the concentration of CHX was higher than 25 µg/ml.

3.4 Antibacterial effect

A zone of inhibition test against *Staphylococcus aureus* was used to examine the antibacterial effects of the membranes loaded with different amount of AgNO$_3$ and CHX (Figure 6). Membranes were surrounded by an obvious inhibition zone when the amount of incorporated AgNO$_3$ was increased to 1 wt% (Figure 6C) and a significantly larger zone of inhibition was observed for samples containing 5 wt% AgNO$_3$ as compared to the samples containing 0.1 and 1 wt% AgNO$_3$ (Figure 6E). Both membranes loaded with 20 µg CHX (Figure S3) and 60 µg CHX (Figure 6F) induced the formation of distinct inhibition zones. However, the zones of inhibition for membranes loaded with 60 µg CHX and different amounts of AgNO$_3$ were of similar diameter (Figure 6F to 6J). Moreover, all membranes loaded with 5 wt% AgNO$_3$ had obvious comparable antibacterial effects (Figure 6K to 6N) against *S.
*aureus* after release of silver for different periods (Figure 6O). Although the differences in the diameter of zone of inhibition were statistically different between day 0, 1 and 4, the average diameter of the inhibition zone of these three groups differed less than 1 mm. It is noted that the diameter of the membranes is different from the size of the EUCAST standard control samples [28], the results in this study were not compared with the EUCAST standard control.

4 Discussion

The aim of this study was to develop an antibacterial dressing with local dual delivery of antiseptics, i.e. Ag+ and CHX, for the prevention of PDAIs. To this end, defect-free chitosan/PEO nanofibres containing homogeneously dispersed AgNPs were successfully prepared.

The traditional method for the fabrication of chitosan/AgNPs nanofibres involves a two-step process to prepare the solution for electrospinning, in which the chitosan/AgNPs complex was prepared prior to mixing with chitosan and/or polymer solutions for electrospinning [29-33]. This method, however, involves the use of toxic reductive chemical agents and complicates the dispersion of chitosan/AgNPs complexes in the viscous electrospinning solution. Although reductive reagents were not used by Wang et al. [34], UV irradiation was introduced to induce the formation of AgNPs after preparation of the solution. Herein, we have developed a novel and simple one-step electrospinning method, in which the electrospinning solution containing AgNPs was prepared by simple dissolution of chitosan and PEO into acetic acid solution containing AgNO₃, without adding any reductive reagent. The in-situ formation of AgNPs prior to electrospinning was confirmed by investigating the electrospinning solution using TEM (Figure S1). Chitosan is a natural antioxidant and the reducing power of chitosan has been confirmed previously [35]. In this study, we proved that the reducing power of chitosan was strong enough to reduce Ag+ into neutral Ag without the addition of reductive reagents or UV irradiation. Furthermore, the AgNPs were distributed homogeneously without aggregation throughout the nanofibres, as confirmed by the TEM micrographs (Figure 2 and 3). These results indicated that the novel one-step method used in this study is efficient and effective for the preparation of chitosan-based nanofibres containing AgNPs.

To improve the chemical and mechanical stability of chitosan, the
nanofibrous membranes were cross-linked by glutaraldehyde vapour. Two possible mechanisms have been suggested for this cross-linking process: glutaraldehyde reacts with chitosan either through Schiff base formation which results into imine-type functionality or through Michel-type adducts with terminal aldehydes which leads to the formation of carbonyl groups [36]. After cross-linking, the dissolution of the nanofibres was avoided when contacting with neutral or basic aqueous solutions.

We hypothesized that the ideal dual release profile would correspond to an initial burst release of a drug to efficiently kill the invaded bacteria but not toxic to the surrounding host cells, followed by a sustained release of a drug at a concentration sufficient to kill any latent bacteria [12]. In this study, CHX was loaded onto the membrane through a diffusional post-loading method, and 70 to 85 % of CHX was released within 2 days (Figure 4A and 4B). There were no strong interactions between CHX and nanofibres since only burst-type release of CHX was observed (Figure 4A). Besides, silver had no influence on the release of CHX since the release profiles of CHX from nanofibrous membranes containing different amounts of AgNO₃ (Figure 4B) or vice versa (Figure 4C) were identical. On the other hand, sustained release of Ag⁺ for more than 28 days was achieved. AgNPs were oxidized upon contact with water resulting into subsequent release of Ag⁺ from the nanofibrous membranes. This process was shown to be time- and oxygen-dependent [17]. AgNPs were depleted almost completely after 28 days release (Figure S2), which indicated that all the AgNPs were oxidized and released as silver ions. The differences of the release kinetics between CHX and Ag⁺ were induced by the release mechanisms of these two antibacterial drugs from the nanofibrous membranes. CHX was released from the membranes mainly through a diffusion-based manner since no strong interactions existed between CHX and nanofibres, while Ag⁺ were released from the matrices via the oxidization of AgNPs, which was time- and oxygen-dependent [17]. Compared to recent studies [31, 32, 34] using chitosan nanofibres containing AgNPs for antibacterial applications, which obtained only 3 to 7 days of release of Ag⁺, we were able to obtain sustained release of Ag⁺ during significantly longer periods.

The cytotoxicity test showed that CHX was the main toxicant and toxicity of CHX appeared to be dose-dependent (Figure 5A), which was in agreement with other studies [37, 38]. Therefore, it is of critical importance to find the toxic level of CHX for practical applications. To characterize the toxicity of
Chapter 7

CHX in more detail, we tested the cell viability as a function of CHX dose and found that the threshold level for cytotoxicity of CHX was 25 µg/ml in the current study (Figure 5B). In comparison, although AgNPs were reported previously to be toxic even at low concentrations [39], they had no obvious cytotoxic effect upon incorporation into the nanofibrous membranes (Figure 5A), while dissolved AgNO₃ was not toxic up to a concentration of 50 µg/ml (Figure 5B).

According to the cytotoxicity results, several groups were selected for the zone of inhibition test against *S. aureus* to examine the antibacterial effectiveness of the antiseptics loaded membranes. Membranes loaded with 20 µg CHX killed *S. aureus* effectively (Figure S3) and the membranes killed *S. aureus* effectively when the amount of incorporated AgNO₃ was 1 wt% or higher. The killing effectiveness increased with increasing AgNO₃ content (Figure 6D and 6E). Moreover, the long-term antibacterial test showed that membranes containing 5 wt% AgNO₃ could kill bacteria effectively and constantly for more than 4 days (Figure 6K to 6O). However, when the amount of loaded CHX was increased to 60 µg, the antibacterial effect of the membrane was dominated by CHX since there were no significant differences among groups loaded with 60 µg CHX and with or without AgNO₃ (Figure 6F to 6J). This result may be related to the release kinetic of CHX from the nanofibrous membranes. Large amount of CHX was released in the first 6h and killed the surrounded bacteria, which dominated the antibacterial effect.

5 Conclusion

A novel one-step electrospinning method was successfully developed for the preparation of chitosan/PEO nanofibrous antibacterial dressing containing silver nanoparticles that facilitate local and dual delivery of two antiseptics, i.e. chlorhexidine and silver ions, at the site of infection for the prevention of percutaneous device associated infections. The in-situ formation of silver nanoparticles was confirmed by TEM-EDX and the silver nanoparticles were distributed homogeneously throughout the nanofibres. Chlorhexidine was released in a burst-type manner within 2 days, while silver ions were released in a sustained manner for more than 28 days were observed. Silver nanoparticles had no cytotoxicity in our experimental conditions while chlorhexidine was toxic when the loaded amount was higher than 25 µg per membrane. The membranes exhibited
obvious antibacterial effect upon loading with either chlorhexidine (20 µg or more per membrane) or silver nitrate (1 and 5 wt% to polymer) and long-term antibacterial effects were observed for membranes containing 5 wt% silver nitrate. Consequently, chitosan/PEO/silver nanoparticles nanofibrous membranes can be used as a local/topical alternative for the prevention of *S. aureus* associated percutaneous devices associated infections.

**Acknowledgement**

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References


Supplementary information

Figure S1. Transmission electron micrograph of chitosan/poly (ethylene oxide) solution containing 5 wt% AgNO₃ prior to electrospinning.

Figure S2. Cross-sectional transmission electron micrograph of chitosan/poly (ethylene oxide) nanofibre initially loaded 5 wt% AgNO₃ after 28 days of Ag⁺ release.

Figure S3. Optical photo of zone of inhibition test against Staphylococcus aureus for membranes loaded with 20 µg CHX.
Summary, closing remarks and future perspectives
1 Summary

Bone infections are a major problem in clinical healthcare since the treatment of these infections is associated with huge medical cost and severe clinical complications. Traditional treatment modalities focus on systemic administration of antibiotics, which may result into systemic toxicity, antibiotic resistance and recurrence of infections. A powerful strategy to address these problems involves local and sustained delivery of antibacterial drugs to treat and/or prevent infections. Therefore, this PhD thesis has focused on the development of various nanostructured drug delivery systems for the local and sustained delivery of antibacterial drugs. To this end, a general introduction on bone infections as well as a brief description of the objectives of this thesis was provided in Chapter 1.

Since gelatin-based materials have been widely used for the delivery of biomolecules, Chapter 2 provided an overview of the use of various types of gelatin carriers for application in bone regeneration, with specific emphasis on the relationship between carrier properties and delivery characteristics. Generally, biomolecules can bind to gelatin matrices through the formation of polyion complexes with gelatin macromolecules following diffusional post-loading, thereby retaining their biological activity. Upon enzymatic degradation of gelatin carriers, the loaded biomolecules are released at rates which can be precisely tuned by adjusting the cross-linking density of gelatin. This mechanism has been confirmed for large biomolecules such as growth factors and nucleic acids, but it is unknown yet if small molecules such as antibiotics can also be bound to gelatin matrices. To facilitate clinical translation and commercialization of gelatin-based carriers for applications in regenerative medicine, carrier manufacturing should be upscaled under good manufacturing practice conditions, while proper preclinical animal models should be selected to correlate the physicochemical properties of the gelatin carriers with the in vivo release kinetics of loaded biomolecules. The possibility to use gelatin nanospheres as carriers for the sustained delivery of antibiotics was studied in Chapter 3. In more detail, this chapter investigated the relationship between the physicochemical characteristics of selected antibiotics and the kinetics of their release from gelatin nanospheres. We found that the release of antibiotics loaded onto GNs by simple post-loading depended strongly on the physicochemical properties of the selected antibiotics. Antibiotics of high molecular weight (colistin and
vancomycin) were released in a sustained manner for more than 14 days, whereas antibiotics of low molecular weight (gentamicin and moxifloxacin) were released in a burst-type manner. The interactions between antibiotics and gelatin carriers were mainly dominated by i) strong electrostatic forces for colistin, ii) strong hydrophobic and electrostatic forces for vancomycin, iii) weak electrostatic and hydrophobic forces for gentamicin, and iv) weak hydrophobic forces for moxifloxacin. The release kinetics of these four selected antibiotics strongly correlated to the binding affinity of antibiotics to gelatin carriers, which decreased in the order colistin (high molecular weight and charge) > vancomycin (high molecular weight and neutral) > gentamicin (low molecular weight and charge) > moxifloxacin (low molecular weight and neutral).

Various drug-loaded coatings have been applied onto orthopedic and dental implants to prevent infections. Control over the release of antibacterial drugs from these coatings is, however, still a challenge. To overcome this problem, Chapter 4 focused on the deposition of implant coatings composed of a chitosan matrix containing gelatin nanospheres loaded with antibiotics onto stainless steel plates by means of the electrophoretic deposition technique. The coatings adhered tightly to the stainless steel substrates, while gelatin nanospheres were distributed homogeneously throughout the chitosan matrix of the coatings. The surface roughness and hydrophobicity of the coatings was fine-tuned by adjustment of the weight ratio between gelatin nanospheres and chitosan in the suspensions. Vancomycin and moxifloxacin were released in a sustained vs. burst-type manner, respectively, while the coatings were highly cytocompatible and exhibited antibacterial efficacy. These results suggest that the antibacterial capacity of metallic implants can be tuned by orthogonal control over the release of (multiple) antibiotics from electrophoretically deposited chitosan-gelatin nanospheres coatings, which offers a new strategy to prevent implant-associated infections.

In Chapter 5 we showed that gelatin nanospheres can also be used to modulate the biological and antibacterial properties of fibrous membranes. Specifically, fibrous silk fibroin membranes were studied since these nanofibers have been widely studied for drug delivery purposes. Nevertheless, control over the release of biomolecules from this type of nanofibers is generally poor due to a lack of interaction between biomolecules and silk fibroin. To combat this problem, we incorporated
oppositely charged gelatin A and B nanospheres into the silk fibroin nanofibers by using the electrospinning technique. Novel silk fibroin nanofibrous membranes containing gelatin nanospheres with a nano-in-nano structure were successfully fabricated. Both oppositely charged gelatin A and B nanospheres were incorporated into the nanofibers. The distribution of gelatin A and B nanospheres could be fine-tuned at the nanoscale by adjustment of either the weight ratio between the nanospheres (using single nozzle electrospinning) or the relative feeding rate of core and shell solutions by using co-axial nozzle electrospinning. A more sustained release of vancomycin was achieved from membranes containing gelatin B nanospheres as compared to nanosphere-free membranes. Gelatin-modified membranes were highly cytocompatible and exhibited antibacterial efficacy against Staphylococcus aureus. Additionally, these membranes supported the attachment and spreading of periodontal ligament cells. These results indicate that the nano-in-nano fibrous silk fibroin membranes containing gelatin nanospheres are promising candidates for controlled delivery of (multiple) biomolecules. The suitability of gelatin nanospheres for local delivery of biomolecules has been confirmed in various preclinical studies. Traditional animal models are, however, not suitable for real-time tracking of gelatin nanospheres and monitoring of the immune response to gelatin nanospheres. Therefore, we have evaluated a novel in vivo method in Chapter 6 to monitor the spatial distribution of and immune response to gelatin nanospheres by using a transgenic zebrafish embryo model with fluorescently labeled macrophages. Upon intravenous or intramuscular injection of the fluorescently labeled gelatin nanospheres into zebrafish embryos, the spatial distribution of gelatin nanospheres as well as the interaction between gelatin nanospheres and macrophages could be monitored real-time using confocal laser scanning microscopy. This method could become a valuable new tool for biological assessment of gelatin nanospheres and the application of gelatin nanospheres for local delivery of various bioactive molecules.

A specific type of infection relates to the use of percutaneous devices. To prevent these infections, antimicrobial dressings can be a promising treatment strategy. However, the use of antibiotics is associated with development of antibacterial resistance, while single drug therapy is often not sufficiently effective to counteract infections. Therefore, we proposed
dual release of antiseptics (such as chlorhexidine, CHX) and silver as an effective strategy to combat percutaneous device-associated infections. In Chapter 7, we prepared electrospun chitosan/poly(ethylene oxide) (PEO) nanofibrous membranes containing silver nanoparticles as an implantable delivery vehicle for the dual and local release of CHX and silver ions. The in-situ formation of silver nanoparticles was confirmed by transmission electron microscopy combined with energy dispersive X-ray spectroscopy, which indicated that the silver nanoparticles were distributed homogeneously throughout the nanofibers. CHX was released in a burst-type manner within 2 days, while silver ions were released in a sustained manner for more than 28 days. Silver nanoparticles did not cause any cytotoxicity under the current experimental conditions while CHX was toxic when the membranes were loaded with more than 25 µg of CHX. The membranes exhibited obvious antibacterial efficacy upon loading with CHX (20 µg or more per membrane) and/or silver nitrate (1 and 5 wt% to polymer). Long-term antibacterial effect up to 4 days was observed for membranes containing 5 wt% of silver nitrate. In summary, it was concluded that chitosan/PEO nanofibrous membranes containing silver nanoparticles and CHX can be used as a local drug delivery device to prevent infections caused by percutaneous devices.

2 Closing remarks and future perspective

This thesis described the development of various nanostructured antibacterial drug delivery systems to treat and/or prevent infections. These nanosphere- and nanofiber-based systems were shown to facilitate local and sustained delivery of antibiotics and antiseptics to kill bacteria. As a result, these antibacterial delivery systems are suitable to treat and/or prevent bone infections, as evidenced by our positive in vitro results. However, several challenges remain to be addressed in future studies. First, the exact amount of antibacterial drugs needs to be determined before clinical translation of these delivery systems can be considered. Ideally, an initial burst release of a high amount of antibacterial drugs (to kill invaded bacteria) should be followed by a sustained release of antibacterial drugs (to prevent latent infections) [1, 2]. At the sustained release stage, the concentration of released antibacterial drugs should be higher than the minimal inhibition concentration or minimal bactericidal concentration (MIC or MBC) for specific bacteria. In vitro models need to be
developed which can mimic the *in vivo* conditions for bacterial growth to study the effects of antibacterial drug treatment and optimize the *in vitro* drug dosage and release kinetics. Subsequently, the release kinetics and therapeutic efficacy of the antibacterial effect should be studied in suitable animal models of infection to further optimize the delivery systems.

Second, treatment of infection using antibiotics may result into the development of antibacterial resistance. Unfortunately, the development rate of antibacterial resistance exceeds the development rate of new drugs by far [1]. Consequently, antibacterial drug delivery systems need to be developed that allow local release of alternative antibacterial agents such as antiseptics and antimicrobial peptides (AMPs) which have less possibility to induce antibacterial resistance [2-4]. Although nanofibrous membranes loaded with antiseptics (chlorhexidine and silver) were successfully developed in Chapter 7, only burst-type release of chlorhexidine was observed due to a lack of interaction between chlorhexidine and chitosan, while potential side effects of silver delivery are still debated [5, 6]. Therefore, delivery of positively charged AMPs from negatively charged gelatin nanospheres can be a solution to increase the mutual interaction between drugs and carriers as well as reduce toxicity.

AMPs are endogenous polypeptides produced by a wide variety of organisms as an essential component of their innate immune response to protect a host from pathogenic microbes [4, 7, 8]. These AMPs can be found among all classes of life ranging from prokaryotes to humans [7]. Generally, AMPs have less than 100 amino acids residues with an overall net charge of +2 to +9 caused by charged lysine and arginine residues [4, 8]. Moreover, AMPs can be active against both Gram-positive and Gram-negative bacteria, yeast and some viruses [3]. Positively charged AMPs of molecular weight higher than 2 kDa are hypothesized to form polion complex with negatively charged gelatin nanospheres through electrostatic and hydrophobic interactions. Consequently, the possibility of using gelatin nanospheres for the sustained delivery of AMPs can be investigated in the future to overcome issues related to antibacterial resistance.

Third, intracellular infections especially intracellular infections induced by medical devices are typically highly persistent infections which are difficult to treat. These bacteria exhibit reduced susceptibility to antibiotics and may develop intracellular persistence. The capability of the delivery systems developed in this thesis to target intracellular infections was not yet studied.
In Chapter 6, by culturing THP-1-derived macrophages in the presence of gelatin nanospheres, we confirmed that the gelatin nanospheres can be internalized by macrophages. Therefore, future studies should focus on the possibility to use gelatin nanospheres to combat intracellular infections. To this end, the transgenic zebrafish model as developed in Chapter 6 could be used to study intracellular delivery of antibacterial agents from gelatin nanospheres in vivo by using fluorescently labeled macrophages, gelatin nanospheres and antibacterial agents.
References
Chapter 9

Samenvatting, slotopmerkingen en toekomstperspectieven
1 Samenvatting

Botinfecties zijn een groot probleem in de klinische zorg omdat de behandeling van deze infecties gepaard gaat met enorme medische kosten en ernstige klinische complicaties. Traditionele behandelingen tegen botinfecties gaan uit van systemische toediening van antibiotica, wat kan leiden tot systemische toxiciteit, resistentie tegen antibiotica en terugkeer van infecties. Een alternatieve strategie behelst lokale en langdurige afgifte van antibacteriële middelen waarmee infecties behandeld dan wel voorkomen kunnen worden. Dit proefschrift borduurt voort op dit concept door een bijdrage te leveren aan de ontwikkeling van diverse nanogestructureerde dragersystemen ten behoeve van lokale en langdurige afgifte van antibacteriële middelen.

Na een algemene inleiding over botinfecties werden in Hoofdstuk 1 de doelstellingen van dit proefschrift beschreven. Aangezien gelatine-gebaseerde materialen op grote schaal toegepast worden om biomoleculen af te geven, werd in Hoofdstuk 2 het gebruik van verschillende soorten gelatine dragermaterialen ten behoeve van de regeneratie van botweefsel beschreven. Daarbij lag de nadruk op de relatie tussen de eigenschappen van de dragermaterialen en het afgifteprofiel van de diverse biomoleculen. In het algemeen kunnen biomoleculen aan gelatine matrices binden door zogenaamde polyion complexen met gelatine macromoleculen te vormen waardoor hun biologische activiteit behouden blijft. Doordat gelatine enzymatisch degradeert kunnen de geladen biomoleculen vrijkomen met een snelheid die afhankelijk is van de crosslink-dichtheid van gelatine. Dit mechanisme is bevestigd voor grote biomoleculen zoals groeifactoren en nucleïnezuren, maar het is nog onbekend of kleine moleculen zoals antibiotica eveneens binden aan gelatine matrices. Om de klinische vertaling en commercialisering van gelatine-gebaseerde dragers voor toepassingen in de regeneratieve geneeskunde mogelijk te maken, zal de productie van dragermaterialen moeten worden opgeschaald onder GMP condities. Daarnaast dienen geschikte preklinische diermodellen ontwikkeld te worden om de fysisch-chemische eigenschappen van de gelatine dragers te kunnen correleren met de in vivo afgifte van biomoleculen.

De mogelijkheid om gelatine nanosferen als dragers te gebruiken voor de langdurige afgifte van antibiotica is onderzocht in Hoofdstuk 3. Dit hoofdstuk richtte zich met name op de relatie tussen de fysisch-chemische
eigenschappen en afgiftekinetiek van enkele veelgebruikte antibiotica. Hierbij bleek dat de afgifte van antibiotica vanuit gelatine nanosferen sterk afhing van de fysisch-chemische eigenschappen van de specifieke antibiotica. Antibiotica met een hoog molecuulgewicht (colistine en vancomycine) werden langdurig afgegeven gedurende 14 dagen, terwijl antibiotica met een laag molecuulgewicht (gentamicine en moxifloxacine) veel sneller en abrupter werden afgegeven. De interacties tussen antibiotica en gelatine nanosferen werden bepaald door i) sterke elektrostaticke krachten voor colistine, ii) sterke hydrofobe en elektrostatische krachten voor vancomycine, iii) zwakke elektrostatische en hydrofobe krachten voor gentamicine, en iv) zwakke hydrofobe krachten voor moxifloxacine. De afgiftekinetiek van deze vier specifieke antibiotica bleek sterk te correleren met de affiniteit van antibiotica aan gelatine die afnam in de volgorde colistine (hoog molecuulgewicht en sterke lading) > vancomycine (hoog molecuulgewicht en neutrale lading) > gentamicine (laag molecuulgewicht en zwakke lading ) > moxifloxacine (laag molecuulgewicht en neutrale lading).

Diverse deklagen zijn na belading met geneesmiddelen aangebracht op orthopedische en tandheelkundige implantaten ter voorkoming van infecties. Het blijft echter een uitdaging om de afgiftekinetiek van antibacteriële geneesmiddelen uit deze deklagen te kunnen gecontroleerd te laten plaatsvinden. Dientengevolge richtte Hoofdstuk 4 zich op het aanbrengen van deklagen bestaande uit een chitosan matrix en met antibiotica (vancomycine en moxifloxacine) beladen gelatine nanosferen op roestvast stalen implantaten met behulp van de elektroforetische depositietechniek. De deklagen hechten stevig aan de roestvrij stalen substraten, en de gelatine nanosferen waren homogen verspreid door de chitosan matrix van de deklagen. De oppervlaktemeruwheid en hydrofobiciteit van de deklagen kon worden beïnvloed door de gewichtsverhouding tussen gelatine en chitosan in de suspensies te variëren. Vancomycine en moxifloxacine werden afgegeven in een langdurige respectievelijk kortdurende wijze. De deklagen waren cytocompatibel en vertoonden antibacteriële activiteit. Deze resultaten suggereren dat de antibacteriële capaciteit van metallische implantaten kan worden gemodificeerd door controle over de afgifte van (meerdere) antibiotica uit elektrofoetisch aangebrachte chitosan-gelatine deklagen. Deze nieuwe methode vormt daarmee een nieuwe strategie voor de bestrijding van implantatie-
geassocieerde infecties.

In *Hoofdstuk 5* hebben we aangetoond dat gelatine nanosferen eveneens kunnen worden gebruikt om de biologische en antibacteriële eigenschappen van vezelige membranen te modijfieren. Hiertoe werden nanovezelmembranen op basis van zijde geselecteerd omdat dit type vezels veelvuldig is onderzocht ten behoeve van gecontroleerde afgifte van geneesmiddelen. Ondanks deze populariteit is de controle over de afgifte van biomoleculen vanuit zijde nanovezels matig, wat te wijten valt aan een gebrek aan interactie tussen biomoleculen en de zijde dragermaterialen. Om dit probleem op te lossen, werden gelatine nanosferen in de zijdemembranen aangebracht met behulp van de electrospinning techniek. Zowel positief geladen gelatine A als negatief geladen gelatine B nanosferen werden met succes ingebouwd in de zijde nanovezels. De verdeling van gelatine A en B nanosferen kon op nanoschaal worden ingesteld door de gewichtsverhouding tussen de beide typen nanosferen of de relatieve toevoersnelheid te variëren. De afgifte van vancomycine was langduriger vanuit membranen beladen met gelatine B nanosferen ten opzichte van membranen zonder ingebouwde nanosferen. De gelatine-gemodifyeerde zijdemembranen waren cytocompatibel en vertoonden antibacteriële werkzaamheid tegen Staphylococcus aureus bacteriën. Bovendien ondersteunden deze membranen de hechting en spreiding van parodontale ligamentcellen. Deze resultaten bewijzen dat de “nano-in-nano” nanovezelmembranen veelbelovende eigenschappen bezitten voor het bewerkstelligen van gereguleerde afgifte van (meerdere) biomoleculen. De geschiktheid van gelatine nanosferen voor lokale afgifte van biomoleculen is in verschillende preklinische studies bevestigd. Traditionele diermodellen zijn echter niet geschikt voor real-time tracking van gelatine nanosferen en het monitoren van de immuunrespons op gelatine nanosferen. Daarom hebben we in *Hoofdstuk 6* een nieuwe *in vivo* methode onderzocht om de ruimtelijke verdeling van en immuunrespons op gelatine nanosferen te monitoren in transgene zebravisembryos met fluorescent gelabelde macrofagen. Na intraveneuze of intramusculaire injectie van fluorescent gelabelde gelatine nanosferen in zebravisembryo’s kon de ruimtelijke verdeling van gelatine nanosferen en de interactie tussen gelatine nanosferen en macrofagen real-time worden gevolgd met behulp van confocale laser scanning microscopie. Deze methode kan een waardevol hulpmiddel vormen bij de biologische evaluatie van gelatine nanosferen.
ten behoeve van lokale afgifte van bioactieve moleculen.
Het gebruik van percutane medische devices kan eveneens tot infectie leiden. Om dit type infecties te voorkomen, kunnen antimicrobiële dressings worden ingezet. Het gebruik van antibiotica leidt echter tot de ontwikkeling van antibacteriële resistentie, terwijl de toediening van slechts één medicijn vaak niet voldoende effectief is om infecties te bestrijden. Daarom hebben wij in deze studie de gelijktijdige afgifte van zowel een ontsmettingsmiddel (chloorhexidine, CHX) en antibacterieel zilver als een mogelijke strategie om infecties rondom percutane devices te bestrijden. In Hoofdstuk 7 zijn chitosan / poly (ethyleen oxide) (PEO) nanovezelmembranen gesynthetiseerd die zilver nanodeeltjes bevatten om daarmee lokale afgifte van zowel CHX en zilverionen te bewerkstelligen. De in-situ vorming van zilver nanodeeltjes werd bevestigd door transmissie elektronenmicroscopie gecombineerd met energie dispersieve röntgenspectroscopie, waaruit bleek dat de zilver nanodeeltjes homogeen door de nanovezels verspreid waren. CHX werd kortstondig afgegeven binnen 2 dagen, terwijl zilverionen langduriger werden afgegeven gedurende meer dan 28 dagen. Zilver nanodeeltjes veroorzaakten geen cytotoxiciteit onder de huidige experimentele, terwijl CHX toxisch werd wanneer de membranen beladen waren met meer dan 25 μg CHX. De membranen vertoonden antibacteriële effectiviteit bij belading met CHX (20 of meer μg per membraan) en / of zilvernitraat (1 en 5 gewichtsprocent ten opzichte van chitosan). Membranen die 5 gewichtsprocent zilvernitraat bevatten, vertoonden langdurige antibacteriële effectiviteit tot 4 dagen. Samenvattend kon uit deze studie worden geconcludeerd dat chitosan / PEO nanovezel membranen zeer bruikbaar zijn voor de lokale afgifte van medicijnen tegen infecties die veroorzaakt zijn door percutane devices.

2 Slotopmerkingen en toekomstperspectieven
Ten eerste dient de vereiste dosering van de antibacteriële geneesmiddelen vastgesteld te worden voordat klinische translatie van de afgiftesystemen kan worden overwogen. Idealiter zou een initiële kortstondige afgifte van een grote dosering antibacteriële geneesmiddelen (ter bestrijding van binnengedrongen bacteriën) gevolgd dienen te worden door een langdurige afgifte van antibacteriële geneesmiddelen (om latente infecties te bestrijden) [1, 2]. Tijdens deze tweede, langdurige fase van de afgifte dient de concentratie van afgegeven antibacteriële geneesmiddelen hoger te zijn dan de minimale bactericidale concentratie (MIC of MBC) die voor de desbetreffende bacteriën geldt. *In vitro* modellen moeten worden ontwikkeld die de *in vivo* omstandigheden voor bacteriële groei zoveel mogelijk nabootsen om de effecten van antibacteriële geneesmiddelbehandeling te kunnen bestuderen, waarmee de *in vitro* dosering en afgiftekinetiek van deze geneesmiddelen geoptimaliseerd kan worden. Vervolgens dient de afgiftekinetiek en therapeutische werkzaamheid van de antibacteriële middelen bestudeerd te worden in geschikte diermodellen voor infectie om de uiteindelijke afgiftesystemen verder te kunnen optimaliseren.

Ten tweede kan de behandeling van infectie met behulp van antibiotica leiden tot de ontwikkeling van antibacteriële resistentie. Helaas is de ontwikkelingssnelheid van antibacteriële resistentie veel hoger dan de ontwikkelingssnelheid van nieuwe geneesmiddelen [1]. Derhalve dienen afgiftesystemen ontwikkeld te worden die lokale afgifte van alternatieve antibacteriële middelen zoals antiseptica en antimicrobiële peptiden (AMPs) mogelijk maken, aangezien dergelijke middelen minder snel tot antibacteriële resistentie leiden [2-4]. Hoewel nanovezel membranen beladen met antiseptica (chloorhexidine en zilver) met succes in Hoofdstuk 7 zijn ontwikkeld, blijft de kortstondige afgifte van chloorhexidine - veroorzaakt door een gebrek aan interactie tussen chloorhexidine en chitosan - een probleem. Daarnaast kunnen mogelijke bijwerkingen van zilver niet uitgesloten worden [5,6]. Afgifte van positief geladen AMPs uit negatief geladen gelatine nanosferen zou een mogelijke oplossing kunnen zijn om de affiniteit tussen geneesmiddelen en dragermaterialen te versterken en de toxiciteit te verminderen.

AMPs zijn endogene polypeptiden die geproduceerd worden door een grote verscheidenheid aan organismen als een essentieel onderdeel van hun aangeboren immuunsysteem ter bescherming tegen pathogene micro-organismen [4, 7, 8]. Deze peptiden komen voor in alle klassen
van leven variërend van prokaryoten tot de mens [7]. AMPs bestaan gewoonlijk uit minder dan 100 aminozuurresiduen en dragen een totale nettolading van +2 tot +9 als gevolg van positief geladen lysine en arginine residuen [4, 8]. Bovendien zijn AMPs tegen zowel Gram-positieve als Gram-negatieve bacteriën, gisten en sommige virussen [3]. Positief geladen AMPs met een moleculair gewicht hoger dan 2 kDa zouden wellicht polion complexen kunnen vormen met negatief geladen gelatine nanosferen dankzij elektrostatische en hydrofobe interacties. Dientengevolge kan de mogelijkheid worden onderzocht om gelatine nanosferen te gebruiken voor de langdurige afgifte van AMPs om uitdagingen met betrekking tot antibacteriële weerstand te overwinnen.

Ten derde zijn intracellulaire infecties – en dan met name intracellulaire infecties veroorzaakt door medische devices – gewoonlijk zeer hardnekkige infecties die moeilijk te behandelen zijn. Deze bacteriën vertonen een verminderde gevoeligheid voor antibiotica en kunnen intracellulaire persistentie ontwikkelen. De mogelijkheid om de afgiftesystemen zoals ontwikkeld in dit proefschrift in te zetten tegen intracellulaire infecties is echter nog niet eerder onderzocht. In Hoofdstuk 6 is middels in vitro studies aangetoond dat gelatine nanosferen kunnen worden geïnternaliseerd door in THP-1 afgeleide macrofagen. Toekomstige studies dienen zich te richten op de mogelijkheid om intracellulaire infecties te bestrijden met gebruikmaking van gelatine nanosferen. Hiertoe kan het transgene zebrafismodel worden gebruikt zoals ontwikkeld in Hoofdstuk 6. Dit model kan worden ingezet om in vivo intracellulaire afgifte van antibiotica uit gelatine nanosferen te bestuderen door gebruik te maken van fluorescent gelabelde macrofagen, gelatine nanosferen en antibacteriële middelen.
References

Acknowledgements
List of publications
Curriculum Vitae
Acknowledgements

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List of Publications

Publications related to this PhD thesis:


Other publications


*Authors contributed equally.
Curriculum Vitae

Jiankang Song (宋建康) was born on Mar 11, 1987 in Huojia County of China. In 2005, he was enrolled in Henan University of Technology, majoring in Food Science and Engineering. He obtained his Bachelors degree with honors in June 2009. In the same year, he started his Masters at State Key Laboratory of Pulp and Paper Engineering, South China University of Technology, under the supervision of Dr. TANG Aimin. After 3 years of the Masters program, he was supported by the China Scholarship Council to pursue his PhD in the Department of Biomaterials at Radboud University Medical Center in October 2012. Under the supervision of Prof. John A. Jansen, Dr. Sander C.G. Leeuwenburgh and Dr. Fang Yang, his project focused on the development of nanostructured antibacterial drug delivery systems. In 2015, he went to University of Erlangen-Nuremberg (Germany), Institute of Biomaterials, as a visiting researcher for one month under the supervision of Prof. Aldo R. Boccaccini. The results of the performed studies are described in this thesis and are presented as separate publications in scientific journals.