The incorporation of silver to enhance the antibacterial properties of biomaterials
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JINLONG SHAO
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The incorporation of silver to enhance the antibacterial properties of biomaterials

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The incorporation of silver to enhance the antibacterial properties of biomaterials

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Roel op 't Veld
To my beloved parents, grandma, and Bing

谨以此书献给我的父母、奶奶和兵
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Chapter 1

General introduction
1 Biomaterials

Severe injuries can damage human tissues beyond repair. Nevertheless, such an irreparable damage to the human body does not necessarily imply the functional loss or reduction of the quality of life. Tissue restoration can be achieved by the surgical installation of a medical device, like an artificial heart, orthopedic or dental implant, artificial blood vessel, catheter, etc. Medical devices are made of biomaterials, which are defined as “materials of natural or manmade origin that are used to direct, supplement, or replace the functions of living tissues.”

2 Biomaterials-associated infections

Although the application of medical devices improves the patients’ quality of life, they are also associated with problems. For example, the biomaterials, as used for the manufacturing of the medical device, can attract microorganisms and represent a niche for infection, which can be a risk for their human application. Currently, biomaterials-associated infections (BAI) comprise a substantial clinical incidence of infections. For instance, 95% of cases of urinary tract infections are catheter-related, 87% of cases of bloodstream infection originate from an indwelling vascular catheter, and 86% of cases of pneumonia are associated with mechanical ventilation. The initiation of BAI is usually due to microbial adhesion to the used biomaterial. Once adhesion has occurred, the subsequent microbial proliferation leads to the development of a biofilm, which is resistant to most therapeutic agents at achievable concentrations. Subsequently, device removal is then required to ensure eradication of infection or to avoid relapse.

3 Status of antibacterial strategies to combat BAI

BAI can result from the multifaceted interaction of bacterial, device, and host factors. Among them, the bacterial factors are probably the most important in the pathogenesis of device-associated infection, whereas device factors are the most amenable to modification with the objective of preventing infection. Clinically, antibiotics are most commonly chosen to be active against bacterial infections and antibiotic-releasing biomaterials have been developed to prevent infections. However, the occurrence of resistance against antibiotics has evoked a lot of concerns since the incidence of antibiotic-resistant pathogens has increased dramatically. Long-term local application of the antibiotics can contribute to an amplified rate of antibiotic resistance. Unfortunately, the increase of antibiotic resistance surpasses the pace at which new antibiotics are developed. Therefore, as an alternative, the use of antiseptics, which
are chemical substances that prevent the growth and development of microorganisms, has attracted a lot of attention due to their decreased tendency to develop resistance.

4 The use of silver to enhance the antibacterial properties of biomaterials

Among the various types of antiseptics, the use of silver has got significant research interest to prevent infections. Silver was used in ancient civilization for its purity and to make jewelry. There are also some early reports that silver was used to maintain the quality of stored water [7]. Pharmaceutical application of silver was first recognized with the use of silver nitrate in the early 1800s for the treatment of ulcers. Later, due to the discovery of penicillin and its potent antibacterial properties, the interest of using silver to combat infections decreased and completely disappeared around the second world war [8]. It was not until the 1960s that silver was reintroduced in the form of 0.5% silver nitrate solution to treat burn wounds [9]. At present, silver has reemerged as a viable option for infection treatment [10]. Silver can be applied in the form of silver compounds, e.g. silver nitrate and silver sulphadiazine or as silver nanoparticles [11]. It has been demonstrated that: (1) metallic silver only causes minimum damage to living organisms, and (2) the silver ions are the major biologically active species in the application of silver compounds and silver nanoparticles. The antimicrobial properties of silver ions are mainly attributed to its ability to interact with proteins and enzymes, block cellular respiration and electron transfer, intermingle with nucleic acid and induce reactive oxygen species production [9].

Silver can be incorporated into or coated on polymers or metals by e.g. lyophilization [12,13], dip coating [14,15], electospinning [16], layer-by-layer deposition [17] and additive manufacturing [18]. These techniques provide a great potential for the use of silver to enhance the antibacterial property of biomaterials. However, to the best of our knowledge, only several studies are available that evaluate the efficacy and biocompatibility of silver incorporated biomaterials in vivo, i.e. silver-incorporated dressings to treat burns [19,20], silver-coated mega-endoprostheses or pins [21,22], polyurethane-silver nanocomposites for the manufacturing of catheters [23], silver-coated implants [14], and silver-coated Dacron vascular grafts [24]. The in vivo evaluation of the antibacterial efficacy of silver can only be done by using a valid and reliable experimental animal infection model. Currently, following initial in vitro bacteriological analysis, a subcutaneous infection model has been well established as a secondary test to evaluate antibacterial efficacy of a biomaterial [25]. The subcutaneous model involves the bacterial inoculation around an implanted biomaterial and provides overall information of the efficacy of antibacterial additives. However, a more dedicated
model for testing the antibacterial efficacy of the biomaterial is needed when used for a specific application, for example, percutaneous devices are implants made of a biomaterial that penetrates through the skin. Percutaneous implants are used amongst others as external fixator in complicated bone fractures and for the fixation of an ear as well as limb prosthesis. Percutaneous implants are associated with the occurrence of bacterial infections at the skin exit-site, which can result in failure of the device. However, despite the previous attempts to constructing a convincing infection model for percutaneous applications [26-30], such a model is still not available.

5 The objective of this thesis

The aim of this thesis is to explore the efficacy of using silver to enhance the antibacterial properties of polymeric biomaterials. More specifically, this thesis aimed:

- to evaluate the existing animal models for percutaneous device-related infection, with a specific focus on the inoculation of bacteria,
- to incorporate silver nanoparticles into polymers and evaluate their potential as the membrane for guided tissue regeneration,
- to exploit the silver nanoparticles incorporated polymeric membranes as antibacterial wound dressing,
- to explore the efficacy of silver incorporated membranes as antibacterial percutaneous pin sleeve,
- to develop 3D printed polycaprolactone scaffold loaded with silver and lidocaine to prevent bacterial infection as well as provide pain relief using a one-step technique.

As scientific approach for these (sub)aims, we addressed the following research questions:

1. Which animal models in literature are available to evaluate the antibacterial properties related to percutaneous devices (Chapter 2)?
2. Do silver incorporated membranes hold potential for application in guided tissue/bone regeneration (Chapter 3)?
3. Possess silver incorporated membranes antibacterial properties and wound healing ability when used as wound dressing (Chapter 4)?
4. Can silver nanoparticles incorporated chitosan-based membranes effectively prevent infection as pin sleeves (Chapter 5)?
5. Can silver and lidocaine be loaded into materials by 3D printing technique to prevent bacterial infection as well as provide pain relief (Chapter 6)?
References


Animal models for percutaneous device-related infections: a review

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\textsuperscript{b} Department of Medical Microbiology, Radboud University Medical Centre, Nijmegen, The Netherlands.
1 Introduction

A percutaneous device is an object made from a synthetic material, which penetrates the skin through a surgically created defect [1]. Presently, percutaneous devices are widely used in clinical treatment, and applications include, amongst others, indwelling catheters, external fixators, electrical connection of sensors, vascular access devices, dental implants, auditory prostheses, and orthopaedic prostheses [2]. Although the application of percutaneous devices greatly improves the quality-of-life of patients, there are still considerable drawbacks. Specific problems, as reviewed previously [3], include marsupialization (i.e. the process of epidermal migration along a percutaneous implant resulting in the formation of an epidermis-lined pouch in which the implant rests), per-migration (the process by which epidermal cells migrate through the pores of a percutaneous implant, eventually filling the pores with epidermal maturation products), bacterial infection or abscess formation, avulsion (mechanical disruption of the tissue/implant interface), and extrusion (destruction of tissue-implant continuity with loss of the implant function). In particular, the occurrence of an infection with bacteria colonizing the percutaneous device is a prelude to marsupialization, per-migration, and avulsion. Thus, infection is the most common reason for the final failure of a percutaneous device. Infection rates around percutaneous devices are rather high in comparison with other categories of permanently implanted devices such as prosthetic joint infections (<2%) [4]; with reported rates ranging from 5%–30% in bone-anchored prostheses [5,6] to over 50% for external fixators [7,8].

To prevent infection of percutaneous devices, researchers have explored different strategies. First of all, proper surgical procedures, e.g. hand hygiene and aseptic techniques, and systemic antimicrobial prophylaxis are applied as a standard in clinics for last decades. Furthermore, studies have been concentrating on the development of a dynamic interface between the skin tissue and the percutaneous device, for example by using biomaterials, by modifying the implant shape, or by applying specific micro- and nano-conformations on the surface [9-14]. For the evaluation of those strategies, animal models without bacterial inoculation were usually employed. However, more recently the local application of antibacterial materials or antibacterial drugs has attracted more and more attention in the field of percutaneous devices, to prevent the occurrence of infections [15-17]. The efficacy of this new generation of antibacterial-containing devices can only be verified when a reliable percutaneous device-related infection model exists. Previous attempts of constructing a reliable infection model have been conducted [15,17-20], but at present, the animal species, the strain of bacteria, amount and method of inoculation, and the methods for subsequent evaluation of the infection, are still quite variable. Therefore, the purpose of this review is to evaluate the existing animal models for percutaneous device-related infection, with a specific focus on the inoculation of bacteria.
2 Animal models with spontaneous infection

Different animals have been used in studies related to percutaneous devices. First, it can be questioned whether a spontaneously occurring infection can reliably be constructed, i.e. without the intentional inoculation of bacteria. In Table 1, such spontaneous infection rates are listed, which are the results from the control groups or groups without any treatment.

Table 1 | Spontaneous infection rates around percutaneous devices in different animal models, not employing the inoculation of bacteria

<table>
<thead>
<tr>
<th>Species</th>
<th>Position</th>
<th>Study duration</th>
<th>Infection rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>Dorsum</td>
<td>3 days - 6 months</td>
<td>0%</td>
</tr>
<tr>
<td>Rats</td>
<td>Dorsum</td>
<td>3 days - 9 weeks</td>
<td>0%[26-29], 3%[12]</td>
</tr>
<tr>
<td></td>
<td>Scalp</td>
<td>1-3 weeks</td>
<td>0%[9,30], 8%[31]</td>
</tr>
<tr>
<td>Rabbits</td>
<td>Dorsum</td>
<td>3 weeks - 8 months</td>
<td>0%[32-35], 12.5%[36], 50%[37]</td>
</tr>
<tr>
<td></td>
<td>Tibia</td>
<td>3 - 8 months</td>
<td>0%[35,38,39], 7%[36]</td>
</tr>
<tr>
<td></td>
<td>Proximal tibia metaphysis</td>
<td>4 weeks</td>
<td>58%[40], 75%[41], 90%[42]</td>
</tr>
<tr>
<td></td>
<td>Scalp</td>
<td>3 weeks - 8 months</td>
<td>0%[35,38,39]</td>
</tr>
<tr>
<td>Guinea pigs</td>
<td>Tibia</td>
<td>3 - 7 weeks</td>
<td>0%[35,38]</td>
</tr>
<tr>
<td></td>
<td>Dorsum</td>
<td>3 - 7 weeks</td>
<td>0%[35,38]</td>
</tr>
<tr>
<td>Micro-pigs or pigs</td>
<td>Limbs</td>
<td>14 days</td>
<td>44%[43]</td>
</tr>
<tr>
<td></td>
<td>Dorsum</td>
<td>1 - 15 months</td>
<td>0%[44, 13%[45], 17%[46]</td>
</tr>
<tr>
<td></td>
<td>Dorsum</td>
<td>90 days</td>
<td>25%[47]</td>
</tr>
<tr>
<td>Dogs</td>
<td>Dorsum</td>
<td>4 - 32 weeks</td>
<td>33%[48]</td>
</tr>
<tr>
<td>Goats or sheep</td>
<td>Abdominal wall</td>
<td>4 months</td>
<td>8%[49]</td>
</tr>
<tr>
<td></td>
<td>Dorsum</td>
<td>14 weeks</td>
<td>0%[50]</td>
</tr>
<tr>
<td></td>
<td>Tibia</td>
<td>3 weeks - 9 weeks</td>
<td>0%[51,52], 20%[53]</td>
</tr>
<tr>
<td></td>
<td>Proximal tibia metaphysis</td>
<td>24 weeks</td>
<td>85%[54]</td>
</tr>
</tbody>
</table>

In general, it can be concluded that spontaneous infection rates are relatively low (<25%), and moreover, authors describe a significant variability. Despite this variability, it seems that the highest natural infection rate might be achieved in investigations regarding the proximal tibia metaphysis in rabbits and sheep. Further analysis showed that the reason behind such higher infection rate was likely due to biomechanical factors, i.e. a relatively high mobility of the skin/soft tissues at the proximal tibia metaphysis area [54]. Therefore, the infection rates would largely depend on chance, and this circumstance would substantially increase the variability of the experiment,
and thus result in the necessity of using much more animals to evaluate treatment efficacy. All in all, it is concluded that achieving a reliable natural infection around percutaneous devices is not yet obtainable in an animal model.

3 Animal models with bacterial inoculation

3.1 Infection rates of animal models with bacterial inoculation

A common alternative way to obtain a more reliable rate of infection is to inoculate bacteria into the surgical wound. Microorganisms, primarily bacteria are responsible for most device-related infections. Indeed, ample explorations have been conducted to study the performance of percutaneous devices after deliberate infection (Table 2).

For most of the studies, the infection rate in the control groups was quite reliable and (close to) 100%, even despite the fact that the animal models varied in many aspects, e.g. animal species, implant position, inoculated bacteria, and time points. Different from the natural infection process, where infection occurs when the increasing bacterial load eventually is too high to be combated by the immune system, the controlled infection from bacterial inoculation can precisely standardize some important variables such as host, type of implant, inoculated bacterium, inoculated load, wound size, and duration of infection. Therefore, the inoculation of bacteria is a very effective way of constructing and investigating an infection around a percutaneous device.

3.2 The function of inoculated bacteria

To achieve a reliable infection at the exit site of a percutaneous device, researchers need to identify the exact effects of different bacteria on the formation of such an infection. In 1999, Clasper et al. inoculated S. aureus in a sheep external fixator model and detected the microbes present in the contaminated area on day 7 and 14. They described that in addition to the applied S. aureus, nine other species of microbes were detectable in the superficial implant tract or medulla [67]. Oka et al. [57] and Gimeno et al. [17] confirmed this finding in rabbit and sheep models, respectively. Later, Williams et al. provided more evidence to corroborate those results in a rabbit model [59]. In their experiment, a certain strain of S. aureus was repeatedly inoculated from week 2 and weekly thereafter. Finally, the collected samples were analysed to ascertain the bacteria strains existing in the infection. It was found that the present pathogens were not only S. aureus but also other bacteria strains; however, the ratio of different bacteria was not described. More interestingly, the sub-strain S. aureus detected at the end of the experiment was representative of the normal skin flora, rather than the applied sub-strain of bacteria [59].
Table 2 | Infection rates around percutaneous devices in different animal models, when making use of the inoculation of bacteria

<table>
<thead>
<tr>
<th>Species</th>
<th>Position</th>
<th>Bacteria</th>
<th>Bacteria dose in solution volume</th>
<th>The way of inoculation</th>
<th>Inoculation time post-operation</th>
<th>Duration after inoculation</th>
<th>Infection rate (control group)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice[55]</td>
<td>dorsum</td>
<td>S. aureus, coagulase-negative staphylococcus</td>
<td>--</td>
<td>Swabbed painted onto entry site three strokes in the same direction</td>
<td>Immediately, or 7 days</td>
<td>1, 3, 6, 12, 24, 48 hours</td>
<td>100%, but diminished with longer time</td>
</tr>
<tr>
<td>Rats[56]</td>
<td>femur</td>
<td>S. aureus ATCC 25923</td>
<td>1×10⁷ CFU in 0.1ml</td>
<td>Placed into implant-skin interface</td>
<td>Immediately</td>
<td>14 days</td>
<td>76.70%</td>
</tr>
<tr>
<td>Rats[57]</td>
<td>femur</td>
<td>MRSA ATCC 43300</td>
<td>1×10⁴ CFU in 1ml</td>
<td>Placed into implant-skin interface</td>
<td>Immediately</td>
<td>7 days</td>
<td>100%</td>
</tr>
<tr>
<td>Rabbits[56]</td>
<td>tibia</td>
<td>S. aureus ATCC 25923</td>
<td>3×10⁶ CFU in 1ml</td>
<td>Subcutaneously injected into the implant site</td>
<td>5 days</td>
<td>9 days</td>
<td>100%</td>
</tr>
<tr>
<td>Rabbits[58]</td>
<td>tibia</td>
<td>S. aureus ATCC 25923</td>
<td>1×10⁷ CFU in 0.2-0.8ml</td>
<td>Placed into implant-skin interface</td>
<td>Immediately</td>
<td>14 days and weekly after</td>
<td>72 weeks</td>
</tr>
<tr>
<td>Rabbits[59]</td>
<td>tibia</td>
<td>S. aureus ATCC 49230</td>
<td>1×10⁶ CFU in 0.2 ml</td>
<td>Placed into implant-skin interface</td>
<td>Immediately</td>
<td>14 days and weekly after</td>
<td>72 weeks</td>
</tr>
<tr>
<td>Rabbits[60]</td>
<td>dorsum</td>
<td>S. aureus ATCC 49230</td>
<td>1×10⁶ CFU</td>
<td>Placed into implant-skin interface</td>
<td>Immediately</td>
<td>4 weeks and weekly after</td>
<td>100%</td>
</tr>
<tr>
<td>Rabbits, pigs[61]</td>
<td>dorsum</td>
<td>S. aureus or E. coli</td>
<td>6×10⁵ CFU in 0.6 ml</td>
<td>Swabbed paint in 5 cm² area surrounding the implant</td>
<td>&gt; 10 days</td>
<td>48 month</td>
<td>0%</td>
</tr>
<tr>
<td>Micro-pigs[62]</td>
<td>dorsum</td>
<td>S. aureus and P. aeruginosa</td>
<td>1 ml</td>
<td>Swabbed paint in 1 cm² area surrounding the implant</td>
<td>5 weeks</td>
<td>24, 48, 72 hours</td>
<td>0%</td>
</tr>
<tr>
<td>Goats[63]</td>
<td>iliac crest</td>
<td>S. aureus ATCC 25923</td>
<td>7.6×10⁶ CFU in 1ml</td>
<td>Placed into implant-skin interface</td>
<td>Immediately</td>
<td>16 days</td>
<td>100%</td>
</tr>
<tr>
<td>Goats[64]</td>
<td>tibia</td>
<td>S. aureus ATCC 25923</td>
<td>2.4×10⁷ CFU in 0.1ml</td>
<td>Placed into implant-skin interface</td>
<td>Immediately (keep for 1h)</td>
<td>14 days</td>
<td>100%</td>
</tr>
<tr>
<td>Goats[65]</td>
<td>tibia</td>
<td>S. epidermidis HBH276</td>
<td>3×10⁷ CFU in 0.1ml</td>
<td>Placed into implant-skin interface</td>
<td>Immediately</td>
<td>21 days</td>
<td>88.90%</td>
</tr>
<tr>
<td>Goats[66]</td>
<td>tibia</td>
<td>S. aureus ATCC 25923</td>
<td>3×10⁴ CFU in 0.03ml</td>
<td>Applied to the pin threads before final insertion</td>
<td>In operation</td>
<td>14 days</td>
<td>95.80%</td>
</tr>
<tr>
<td>Sheep[67]</td>
<td>tibia</td>
<td>S. aureus ATCC 25923</td>
<td>2.5×10⁴ CFU in 0.1ml</td>
<td>Placed into implant-skin interface</td>
<td>Immediately</td>
<td>7, 14 days</td>
<td>70% (day 7); 100% (day 14)</td>
</tr>
<tr>
<td>Sheep[68]</td>
<td>tibia</td>
<td>S. aureus ATCC 6538</td>
<td>5×10⁷ CFU in 0.2 ml</td>
<td>Placed into implant-bone interface with/without suspension retention</td>
<td>Immediately</td>
<td>1 hour</td>
<td>77.8%; 0%</td>
</tr>
<tr>
<td>Sheep[17]</td>
<td>tibia</td>
<td>S. aureus ATCC 25923</td>
<td>5.5×10⁷ CFU in 0.1ml</td>
<td>Placed into implant-skin interface</td>
<td>Immediately</td>
<td>7 days</td>
<td>100%</td>
</tr>
</tbody>
</table>
In contrast, Koseki et al. constructed an infection model on the femur of rats by bacterial contamination and found that the bacteria isolated from the purulence or drainage were of the same strain as was inoculated\cite{56}. However, this discrepancy might be explained by the fact that the latter study used specific-pathogen-free (SPF) animals.

Based on the overall consensus, the mechanism of percutaneous infection after bacterial inoculation may be as follows (Figure 1). First, a local infection is initialized by the inoculation of bacteria. Thereafter, the properties of the percutaneous device, the host immune response, and the bacteria present in the microenvironment, are determining the final formation of an infection. If the infection is successfully constructed, after a prolonged period of time the final composition of the pathogens present depends on the interaction of the inoculated bacteria and the commensal bacteria.

![Figure 1](image1.png)

**Figure 1** | The function of bacterial inoculation in percutaneous infection. (A) The bacteria are inoculated into the interface between percutaneous device and tissues. (B) In a bacteria-free environment, the pathogen in the formed infection is originating from the inoculated bacteria. (C) When the bacteria are inoculated in an environment with commensal bacteria of the host, the infection is initiated by the inoculated bacteria, but over time are mixed or replaced with commensal bacteria.

### 4 The construction of an animal model with bacterial inoculation

As mentioned, the initial infection can be achieved by bacterial inoculation; however, the final infection formation can still be influenced by various factors, i.e. the host immune response, bacterial inoculation load, the properties of the percutaneous device, and the environment around percutaneous devices. A successful infection model can only result from a balance among those factors. In this section, a detailed discussion on percutaneous device design, animal species, and bacteria, is conducted to facilitate the construction of an animal model.
4.1 Percutaneous device design

4.1.1 Anchor design of implant

The anchor design of percutaneous devices not only is a major consideration to keep them at the implant site, but also can prevent physical avulsion at the skin-implant interface, by transferring the mechanical forces from the percutaneous area to the deeper subdermal tissues [21,32]. Various anchoring configurations have been proposed, ranging relatively from simple to more complex structures (Figure 2). According to the tissue composition of the anchoring site, percutaneous animal models can be divided into two major groups, namely soft tissue-anchored and bone-anchored percutaneous models. Irrespective of the anchoring design of the implant, sharp corners are to be avoided, since they cause stress peaks in the skin when external forces are applied to the protruding component [69].

Figure 2 | Common applied percutaneous devices in animal models

The most common design for soft-tissue anchored percutaneous implants is the flange shape, and less commonly rod, cuff, sheet, or irregular shapes [23,35,38,70]. The flange-shaped implant mainly includes a large subcutaneous part with a relatively small percutaneous pole, where the large subcutaneous part serves as a secure anchor to the soft tissue [32,35]. The rod-shaped implant can be anchored using surgical methods, e.g. the internal part is fixed in a tissue tunnel (leaving both ends of implants exposed in the air), or by suturing the inside part of devices directly to the adjacent skin to improve the stability [23,24]. Cuff-shaped implants are anchored by the supposed ingrowth of soft tissue into the porosity of the cuff, and are commonly used for the manufacturing of catheters, like the Tenckhoff catheters [43]. Sheet-shaped implants are usually composed of soft materials (e.g. Dacron® velour), which can be easily sutured to the skin [71]. A limited number of irregularly shaped implants are also described in animal models. For instance, Chehroudi et al. used a U-shaped design and in his studies to the effect of implant surface topography on marsupialization [70].
The most common anchoring designs used in bone anchored percutaneous animal models are either screw type (threaded) or cylindrical (rod-shaped without thread) [72]. Screw type produces excellent initial stability, whereas stability a cylindrical shape depends on an exact fit within the bone [73,74]. Regarding rod-shaped implants, Chou et al. used a cerclage wire to further stabilize the cylindrical implant [58].

4.1.2 Exposure of implant

As the name speaking for itself, a percutaneous device always consists one part extruding through the skin. A percutaneous device fails when the skin covers the whole implant. Swelling and skin mobility appear to contribute to overgrowth of the skin. The problem might be solved by increasing the length of the exposure part of percutaneous implants [58]. In a mice dorsum percutaneous study, porous poly (2-hydroxyethyl methacrylate) implants with 2.5 mm to 5 mm long exposure were used [22-25]. The failure rate of 2.5 mm long exposure was 8/28 due to the overgrowth of the skin, but no failure was reported with 5 mm long exposure [22, 23]. In another study, the implant is designed with an enlarged cap, where the diameter of the exposed part is larger than that of the soft tissue surrounded part. Although the length of the enlarged cap was as short as 3 mm, only 2/40 implants were found to fail due to the overgrowth of skin [74]. Moreover, in several catheter studies with exposed lengths of 5 mm to 30 mm in different animals, no implants failed due to skin overgrowth [27,43,55,71]. From a histological perspective, the exposure of a percutaneous device is related to the proliferation and migration of epithelial cells. The rate of proliferation and migration varies among different animal species. In rabbits this rate was fastest with a speed of 2.0 mm/week; in dogs intermediate with a speed of 1.6 mm/week; and in goats slowest with a speed of 1.5 mm/week [71]. Given that, the exposure length of the implant is closely related to its final status and this should be carefully considered when designing an experimental animal study.

4.2 The selection of the animals

Infection models have been constructed in various animal species, such as mice, rats, rabbits, micro-pigs, pigs, goats and sheep. Among them, rabbits, goats, and sheep seem more commonly used compared to mice, rats, or pigs. This may be firstly attributed to the fact that most infection models consider bone-anchored percutaneous devices, and thus rabbits and sheep have a more favourable size of the bone for common surgical positions located at the tibia, femur, and iliac crest. From this perspective, it could be argued that also micro-pigs or pigs are suitable models. However, only limited studies are available considering those species [61,62]. Further studies are needed to prove whether certain species of pig are indeed suitable as an animal model in this field.
Small rodents (mice, rats) are not widely used as percutaneous device-related infection models. Cooper et al. tried to create an infection around soft tissue-anchored percutaneous devices by inoculating bacteria in mice. The infection was successfully induced, but the intensity of the infection subdued during the implantation time [55]. More recently, a femoral infection in rats was explored by inoculating a relatively large dose \((1 \times 10^7 \text{ CFU})\) of \textit{S. aureus} into the implant-tissue interface. Even under such harsh conditions, the infection rate in the control group only reached 76.7% [56]. It is difficult to construct a reliable infection in small rodents, and therefore small rodents can only be used for preliminary studies. To this end, device-related infection models in the larger animals are preferable and recommended.

4.3 Bacteria inoculation

4.3.1 The selection of the bacterial strain

The major aim of animal studies is to investigate the mechanism of various diseases in human beings for the purpose of preventing and treating diseases. Therefore, common pathogens related to human diseases are preferentially considered in percutaneous animal models (Table 2). The pathogens reported most commonly around percutaneous devices are bacteria such as \textit{S. aureus}, \textit{Staphylococcus epidermidis}, \textit{Escherichia coli}, \textit{Pseudomonas aeruginosa} [6,7, 75-77]. Especially, the bacteria shared between human and animal hosts are preferred. The most commonly selected and important bacterium for animal studies, \textit{S. aureus}, causes most device-related infections in both humans and animals, such as rabbits and sheep [20]. Last but not least, the selection of bacterial strains is largely determined by the study purpose or to facilitate the identification. For example, studies on antibiotic resistance or biofilm formation usually require certain strains of \textit{S. aureus} or \textit{Pseudomonas} with specific characteristics. Moreover, to facilitate the detection of the inoculated bacterium, an \textit{S. aureus} sub-strain, which has been genetically selected to be resistant to streptomycin, is often used [66].

Both laboratory strains, as well as swabs obtained directly from patients, can be employed as a resource of bacteria for inoculation. Still, most studies choose laboratory strains, e.g. \textit{S. aureus} American type culture collection (ATCC) 29213, 25923 and 6538. \textit{S. aureus} ATCC 49230 was selected for several studies as well, as they originate from a patient with osteomyelitis [58-60]. Less commonly, \textit{E. coli}, \textit{P. aeruginosa}, and \textit{S. epidermidis} are selected. It is noteworthy that the \textit{S. epidermidis} is usually chosen due to its ability to form biofilms and close relationship with late-onset prosthesis-related infections, compared to \textit{S. aureus}, which is associated with early-onset infections [17]. Approximately 99.9% of the bacteria in nature are the inhabitant
of a biofilm, and biofilms can play a critical role in resistance to antibiotics by serving as a protective barrier [19,56,78-81].

4.3.2 The load of bacteria

In 1982, Werner Zimmerli et al. published a key paper on the effect of foreign materials on the formation of infections [77]. By inoculating different loads of *S. aureus* to subcutaneous tissue cages, $10^2$ CFU of *S. aureus* strain Wood 46 was sufficient to cause 95% infection, whereas $10^8$ CFU did not produce any abscesses without tissue cages implantation. Thus, the existence of a foreign material can greatly facilitate occurrence of infections. A summary of the infection rates and inoculated loads used in literature is presented in Table 2. One study inoculated different loads of *S. aureus* ($2.4 \times 10^7$ CFU vs. $3 \times 10^4$ CFU) to the skin-implant interface immediately after surgery in a goat tibia model, and then assessed the effect on day 14 post operation. Although the difference in inoculated bacterial loads was large, the discrepancy in infection rates was small (100% vs. 95.8%) [64,66]. In a rabbit model, a high load ($1 \times 10^8$ CFU) of *S. aureus* was inoculated at the skin-implant interface on day 14 post operation and then weekly after resulting in 100% infection [58,59]. In contrast, a low load ($2.5 \times 10^4$ CFU) of *S. aureus* inoculated in a sheep tibia model resulted in the occurrence of only 70% animals [58-60,67]. Even though these experiments were done with different settings, e.g. animal species, inoculation methods, and experimental duration, it can be speculated that the infection rate is related to the load of bacterial inoculation. A proper load of bacteria is a balance between creating an infection and giving the chance of combating the infection by animal and the antibacterial materials.

As there seems to be no common consensus, for each study design the inclusion of a pilot experiment is recommended. Such a pilot can elucidate the optimal bacterial dosage, the inoculation method, the timing, and inoculation frequency, to create a persistent infection over the entire experimental period, without producing systemic bacterial sepsis [15].

Further, it should be emphasized that in a percutaneous animal model, by definition, the wound is always exposed to the surrounding environment, even with the protection of a wound dressing. Thus, a load of bacteria, besides the inoculated ones, are always present in the microenvironment around the wound sites. Therefore, attention should be paid to both the inoculated bacteria and the environmental bacteria by using techniques such as aseptic operation procedures.
4.3.3 Inoculation methods

An efficient bacterial inoculation method is a prerequisite for the success of a percutaneous infection model. There are two frequently used methods for inoculation (Figure 3): 1) direct injection into the implant sites, 2) colonizing bacteria on the implant *in vitro* before implantation [76].

![Figure 3](image.png)

**Figure 3** Two common ways of bacterial inoculation. (A) The bacteria suspensions are inoculated into the interface between percutaneous device and tissue to construct an infection. (B) The percutaneous device is first immersed into a bacteria suspension to form a biofilm. Thereafter, the biofilm-covered device is implanted percutaneously into the tissue to develop an infection.

Directly injecting bacteria at the implant-skin interface has been validated to be efficient in forming and maintaining an infection in many animal models [15,17,57-60,63-65,67]. Clasper *et al.* applied a pin tract infection in sheep and found that the bacteria could rapidly (within 1 hour) pass the implant-bone interface into the medullary canal despite the fact the pins were well-fixed into the bone [68]. Such bacterial spreading into the medullary canal might be caused by micro-fractures from the drilling process [82]. Alternatively, efforts have been made by not injecting but rather swabbing the bacteria around the implant. Yet, in both rabbit and pig models, infections failed to form using this swabbing method [61,62]. Evidently, an animal model is only successful when the infection is maintained around the percutaneous exit-site for at least 1 hour [64], which the swabbing method cannot guarantee.

Unlike the method of injecting, the method of colonizing bacteria onto the implant *in vitro* is less common. However, it is undeniable that this is a promising method to
inoculate bacteria, as there is more resemblance to some clinical conditions since this is the only way that a biofilm can reliably be formed on the implant \cite{39}. The efficacy of this method has been verified by applying bacteria to the implant threads before inserting the implant into the femur of goats, resulting in a 95.8% infection rate \cite{66}. In addition, mature biofilms of S. aureus have been created on the surface of membranes using a modified biofilm reactor \textit{in vitro}. This biofilm-covered membrane can be potentially used in future to study biofilm-related infection \cite{19}. In conclusion, colonizing bacteria on a device on beforehand contributes to future animal models on the relation between biofilms and percutaneous device-related infections, and warrant further studies.

Besides the bacterial inoculation approach, the time point of inoculation is another critical factor. The currently selected time points are quite different (Table 2). In most studies, the bacteria have been added to the wounds during surgery, or immediately after surgery. Other investigators started the inoculation after a given period of wound healing, ranging widely from 5 days to 5 weeks after surgery \cite{16,58,62}. Also, repeated infection is applied. For instance, one rabbit model describes inoculation of bacteria from week 2 to 10 on a weekly basis \cite{58}. Apparently, a single injection mimics the situation as occurs during surgery, whereas repeated inoculation mimics the clinical situation of a post-operative infection. Finally, since multiple implants are frequently installed in one animal, care should be taken to prevent contamination of adjacent (control) implants \cite{57}.

\subsection*{4.4 Collection of samples}

The reliable collection of the specimens at the end point of an animal study is a prerequisite for subsequent microbiological and histological detection. Any retrieval method should contain steps for sterilization of instruments and implant site, collection, and bacteria detachment \cite{63}. During harvesting, great care should be taken to avoid cross-contamination.

In some cases, the whole implant with its surrounding tissue are removed for evaluation. The entire explant including the percutaneous device is placed into sterile saline, minced, sonicated, and then homogenized by a vortex shaker at high speed \cite{57,83}. This method is an efficient way for further bacterial counts, isolation, and identification \cite{66,83}.

However, as an infection usually develops from outside to inside, the development stage of the infection, e.g. only soft tissue involved or osteomyelitis in the case of bone-anchored implants, are beneficial information for the treatment. For this purpose, more complicated retrieving methods are used as described below (Figure 4).
Figure 4 | Methods of sample collection for microbiological detection. For soft tissue, there are several methods described like (A) incision exposure method, (B) needle fluid harvesting method, (C) punch harvesting method. For hard tissue, commonly described methods include (D) advancing the tip of the percutaneous device to expose sample site, (E) applying force to form transverse fracture to expose sample site, (F) resecting the lower part of the bone marrow cavity by reaming, to expose the sample site.

For the identification of the infection in soft tissue, swabs from the tissue-implant interface are preferred to be collected. The most simple and popular method is to expose the tissue-implant interface by making incisions (Figure 4 A) [67]. Alternatively, the tissue-implant interface is accessed by using a biopsy punch from 5 mm distal to the skin/implant interface to take out a sub-dermal tissue for swabs (Figure 4 B) [18,59]. These two methods are quite applicable for post-mortem animals. For the live animals, the samples can be harvested several times by a needle without termination of the experiment (Figure 4 C) [62]. Here, 1 ml of sterile saline is directly injected from distal skin to the tissue adjacent to the implant, after 1 min, 0.1 ml of liquid is withdrawn for subsequent microbiological detection.

Likewise, to identify infection in bones, there are also several methods to expose bone-implant interface or bone marrow surrounding the implant. Advancing tip method is relatively straightforward. The implant is pushed through the bone to show the tip for further examination (Figure 4 D) [84]. Alternatively, the whole bone-interface can be exposed by creating a transverse fracture in the same plane of the implant (Figure 3 E) [67]. Moreover, the entire medullary canal can be opened by aseptically resecting
the lower half of the bone to show the bone marrow surrounding the implant (Figure 4 F)\[18,59\]. Such an approach maximally avoids contamination from exposure operation, and thus gives a very reliable result of medullar status; however, it is particularly applicable to very thick bones.

### 4.5 Evaluation of infection

There are three main methods to detect and qualitatively or quantitatively evaluate infection, i.e. (1) to score clinical signs of infection, (2) to apply a microbiological detection, and/or (3) to perform a histological assessment.

#### 4.5.1 Clinical signs of infection

To score clinical signs associated with infection is the most popular way to evaluate the infection status. Upon visual inspection, grading is employed. Most widely adopted is the guideline for clinical signs of infection in accordance with the study of Checketts et al.[85]. This grading scale is defined as follows:

- Grade I level of infection: slight redness of skin with slight discharge around the implant, with one or more of the following symptoms: appetite suppression, limited water consumption, lethargy, distress/limping, and/or pain and tenderness at the implant site.
- Grade II level: redness of skin, discharge from the implant site, and pain and tenderness on palpation of the soft tissues.

It is noteworthy that the grade II is not only regarded as a sign of infection but also widely accepted as a standard ‘humane endpoint’ to avoid the excessive suffering of animals\[17,59,60\].

#### 4.5.2 Microbiological detection

A variety of standard microbiological procedures can be used for classification (qualitative culture) or measurements (quantitative counting) of the present strains of bacteria. Bacterial culture is the most common method to screen and quantify bacteria. Moreover, matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) can be applied as an accurate and rapid technique to identify bacteria [86]. Furthermore, pulsed field gel electrophoresis is applied to identify bacteria [59]. Besides, there is another emphasis on using a morphologically recognizable or certain antibiotics-resistant bacteria to facilitate the identification of bacteria or on applying more advanced genotyping techniques [66].
As biofilms are currently attracting increased attention in the infection study, it is worthwhile to mention that the interactions of biofilms with materials can be detected with imaging techniques. Confocal microscopy, electron microscopy, single particle tracking microscopy and fluorescent correlation spectroscopy are all possible techniques to elucidate these interactions [65,87-90].

4.5.3 Histology/ histomorphometry

In histology routine, gram staining is the most commonly selected method to detect the presence of bacteria [19,91]. Although histology does not guarantee 100% accuracy, it is still described to be the most reliable tool for detection in animal models [58]. The scoring of only clinical parameters of infection can be misleading, as not all infections show obvious clinical symptoms [67,68]. Also, using only microbiological detection is not always reliable due to the relatively low culture sensitivity of bacteriological swabs [76]. Therefore, it is recommended that the occurrence of the infection is determined by at least two positives among clinical signs of infection, microbiological cultures, and histology [16,58,59,66]. Moreover, to facilitate the statistical analysis, the parameters above should preferentially be assessed in a quantitative way [92,93]. A well-designed quantification method, avoiding the excessive influence of any single parameter, could contribute to the comparison among study groups, or the relation to other studies.

5 Conclusions and future perspectives

Infections related to the use of percutaneous devices remain a prevalent complication in many clinical applications. This comprehensive review of all currently available animal models evidences that there is still no accepted animal model to study percutaneous device-related infections. Still, overall it seems that the models that apply standardized inoculation of laboratory bacterial strains are more reliable and repeatable than models in which spontaneous infection has to occur. Regarding the animal species, large animals seem to be more reliable. Even though the inoculated bacteria strains vary, the standard laboratory strains are preferred as they are more easily available and more reproducible. Moreover, a pilot study is recommended to elucidate the optimal bacterial dosage, the inoculation method, the timing, and the inoculation frequency. To obtain reliable results, aseptic surgical procedures should be strictly followed. The various sample collection methods are dependent on the specific application, and all are well-described regarding applicability and reliability. It is advisable that conclusions are always drawn from positive signs based on at least two detection methods of clinical symptoms, microbiological detection, and/or histology. A major challenge for the future is to design acceptable animal models in
which all aspects are standardized and specialized for certain areas of research, such as catheter-related infections and pin tract infections. Moreover, further emphasis should be placed on models which regard biofilm formation on percutaneous devices.
References

Animal models for percutaneous device-related infections


Animal models for percutaneous device-related infections


Animal models for percutaneous device-related infections


Biological evaluation of silver nanoparticles incorporated into chitosan-based membranes

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1 Introduction

Periodontitis is a chronic inflammatory disease of the tooth supportive tissues [1]. It begins as an infection caused by the microflora of the dental plaque that forms adjacent to the teeth. With dental plaque developing, the number of gram-negative anaerobic bacteria (i.e. mainly *Porphyromonas gingivalis* (Pg) and *Fusobacterium nucleatum* (Fn)) increases [2,3]. The subsequent inflammation leads to the destruction of periodontal tissues as well as alveolar bone and can eventually result in tooth loss. Periodontitis is highly prevalent, e.g. from 2009 to 2012, 46% of US adults representing 64.7 million people had periodontitis, with 8.9% having severe destructive periodontitis [4].

The treatment of destructive periodontitis involves surgical intervention, including flap surgery, bone grafting as well as guided tissue regeneration (GTR) [5]. The strategy of GTR is to isolate the periodontal defect from the faster-growing connective tissue using an occlusive membrane so that the slower-growing periodontal tissues can occupy the defect area [6,7]. From a clinical point of view, the GTR membranes must function for at least 4 to 6 weeks to allow the periodontal tissues to regenerate [8,9]. However, during this time period the GTR membranes face the risk of bacterial colonization when exposed to the bacteria-rich oral cavity, e.g. via soft tissue dehiscence [10]. Therefore, the development of a GTR membrane that combines structural integrity with antibacterial properties is of great importance to improve the success rate of the GTR procedure.

Various biomaterials have been investigated for GTR application. Among them, chitosan, a naturally derived biopolymer, seems to be quite suitable due to its excellent biological performance, i.e. non-toxicity, biodegradability, and inherent antibacterial properties [11,12]. To meet clinical requirements, the antibacterial property of chitosan-based materials is commonly enhanced with addition of antimicrobial agents [13,14]. However, the incorporation of classical antibiotics risks development of bacterial resistance [15]. On the contrary, agents, like silver, have a much lower tendency to induce bacterial resistance [16,17]. Especially the use of silver nanoparticles (AgNPs) is promising, as AgNPs have 1.4 – 1.9 times stronger antibacterial properties compared to silver ions [18]. Nevertheless, AgNPs have been observed to induce toxicity (to mammal cells) at elevated concentrations [19], although AgNPs are reported to be non-toxic in most studies [20].

In our previous study, we developed AgNPs incorporated into electrospun chitosan-based membranes, which facilitated a sustained release of silver for at least 28 days [21]. Therefore, this study aimed to explore the biological performance of these membranes, in terms of antibacterial effect, *in vitro* cytotoxicity, and *in vivo*
biocompatibility. To this end, three groups of membranes with different amount of AgNPs were tested for antibacterial properties against Pg and Fn for up to 28 days. The cytotoxic effect of the membranes was explored by culturing human periodontal ligament cells (PDLCs). Finally, the tissue response of the membranes was evaluated *in vivo* by subcutaneous implantation in rabbits.

## 2 Materials and methods

### 2.1 Material preparation

Chitosan (degree of deacetylation = 90%, molecular weight = 200-400 kDa, Heppe Medical Chitosan, HMC+, Halle, Germany), poly (ethylene oxide) (PEO, Molecular weight = 900 kDa, Sigma-Aldrich Chemie GmbH, Steinheim, Germany), glutaric dialdehyde (GA, 25 wt. % solution in water, Merck KGaA, Darmstadt, Germany), acetic acid (99.9%, Boom BV, Meppel, Netherlands), and silver nitrate (AgNO₃, Sigma-Aldrich) were used as received. Ultrapure water was made by Milli-Q system (Millipore, Merck).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ago</th>
<th>Ag1</th>
<th>Ag5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitosan (g)</td>
<td>0.72</td>
<td>0.72</td>
<td>0.72</td>
</tr>
<tr>
<td>PEO (g)</td>
<td>0.48</td>
<td>0.48</td>
<td>0.48</td>
</tr>
<tr>
<td>AgNO₃ (µg)</td>
<td>0</td>
<td>12</td>
<td>60</td>
</tr>
<tr>
<td>Total polymer:AgNO₃</td>
<td>100:0</td>
<td>100:1</td>
<td>100:5</td>
</tr>
<tr>
<td>35% Acetic acid (mL)</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
</tbody>
</table>

Three solutions were prepared for electrospinning and named as AgX (X = 0, 1 or 5) based on the AgNO₃: polymer ratio as shown in Table 1. The electrospinning setup was the same as previously described [21]. In brief, solutions were stirred overnight to ensure complete dissolution of the polymers and silver salts. The solutions were loaded into a 10 mL syringe (BD Plastipak; Becton Dickinson S.A., Madrid, Spain). A Teflon tube (Eriks B.V., Alkmaar, Netherlands) was connected to the syringe and an 18 G spinneret. A syringe pump ((KD Scientific, Inc., Holliston, MA) was used to control the flow rate at 4 mL/h, and the distance between the spinneret and collector was set to 18.5 cm. The electrospinning voltage was set to 28 – 30 kV. After electrospinning, the obtained membranes were cross-linked in GA vapor for 2h as described previously [21], and then left in a fume hood overnight to remove solvent residuals. The membranes were cut into discs (12 mm in diameter) for all the *in vitro* tests and square membranes (15 mm × 15
mm) for the *in vivo* study. All the samples were sterilized by γ-radiation at a dose of 25 kGy (Synergy Health Logistics B.V., Ede, Netherlands) before the further experiment.

### 2.2 Morphology characterization

The morphology of the electrospun fibrous membrane was observed by field emission scanning electron microscopy (SEM; Sigma 300, Zeiss, Zeiss, Oberkochen, Germany). The fiber diameters were measured at 100 random locations from 5 different images using Image J (National Institutes of Health, Bethesda, MD) [22]. The formation and distribution of AgNPs in the electrospun fibers were evaluated by transmission electron microscopy (TEM; JEOL 1010, Tokyo, Japan).

### 2.3 Antibacterial test

The disk samples (12 mm in diameter, ~ 2.0 mg in weight) were first incubated in 1mL phosphate buffer saline (PBS, Gibco, Paisley, UK) and agitated gently at 37°C. The PBS was refreshed every 2 to 3 days. On day 0, 7, and 28 the disk samples were lyophilized for further usage. The antibacterial properties were evaluated against two typical periodontal pathogenic bacteria, i.e. Pg (ATCC 33277) and Fn (ATCC 25586) using a disk diffusion test or modified Kirby-Bauer test [23,24]. In brief, aliquots of $3 \times 10^8$ CFU of Pg or Fn in physiological saline were spread evenly over the entire surface of Brucella agar plate (Becton Dickinson, Franklin Lakes, NJ). Lyophilized disk samples described above (n = 3) were placed on the Brucella agar plate with penicillin and metronidazole tablets as positive controls. All agar plates were transferred into GasPak jars (Becton Dickinson). An anaerobic environment with 0.2% O₂, 9.9% CO₂, 5% H₂ and 84.9% N₂ was achieved by Anoxomat Mark II (Mart Microbiology B. V., Drachten, Netherlands). The zone of inhibition was measured after incubation of 48 h for Fn and 72 h for Pg at 36 °C.

### 2.4 Cell viability

Silver and membrane cytotoxicity was tested using human periodontal ligament cells (PDLCs) [25-27]. All experiments were done by following national guidelines for working with human materials (Dutch Federation of Biomedical Scientific Societies, human tissue and medical research: code of conduct for responsible use. Available at http://www.federa.org/). PDLCs were harvested from an extracted third molar from a healthy adult patient (18 years, female). The cell culture medium was Dulbecco's Modified Eagle Medium (DMEM; Gibco, Paisley, UK) supplemented with 10% fetal calf serum (FBS; Sigma-Aldrich) and 100 U/mL penicillin/streptomycin (Gibco). The cells were cultured in culture medium under a CO₂ (5%) atmosphere at 37 °C for 3 passages
and then frozen in liquid nitrogen in DMEM containing 20% FBS and 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich) until use. After thawing, cells were expanded until passage 6, and then seeded on 24 well culture plates or coverslips (Thermanox Nunc, Naperville, IL) at a density of 5×10⁴ cells per well for 24 h before the assays.

To determine the cytotoxic effect of AgNO₃, the culture medium was replaced with 1 mL of culture medium containing a series of AgNO₃ ranging from 0 – 100 µg/mL (n = 3). Fresh culture medium and 5% DMSO in culture medium were used as positive and negative controls, respectively.

To assess the cytotoxic effect of AgNPs incorporated membranes, membranes weighing 0.5, 1.0, and 2.0 mg from each group (resulting in Ag1 with 5, 10, 20 µg AgNO₃, and Ag5 with 25, 50, 100 µg AgNO₃ in the membranes, respectively) were placed in trans-wells with 0.4 µm pores (Greiner Bio-One GmbH, Frickenhausen, Germany), and then transferred to the cell culture wells with 1 mL fresh culture medium. The experiment was performed in triplicate.

After 20 h, the culture medium, for both the AgNO₃ samples and the AgNPs incorporated membrane samples, was replaced with 1 mL culture medium containing 10% Alamar Blue dye (Bio-Rad, Hercules, CA). After 4 h of incubation, a 200 µl aliquot of metabolized medium from each well was transferred into a 96-well plate (Greiner Bio-One) in duplicate and the absorbance was measured at 570 nm and 600 nm in a Synergy HTX multi-mode reader (BioTek Instruments, Winooski, VT).

For the Live/Dead assay, samples on coverslips (n = 2) were exposed to calcein AM/ethidium homodimer-1/PBS working solution (Invitrogen, Carlsbad, CA) for 30 min at 37 °C according to the manufacturer's instruction, and then observed at wavelength of 488 nm for live cells (green) and 568 nm for dead cells (red) by an automated fluorescent microscope (Axio Imager Microscope Z1; Zeiss).

2.5 In vivo implantation

For the in vivo evaluation, based on the antibacterial and cytotoxic test results and following the 3R rules (replacement, reduction, and refinement) of animal experiments, only Ag0 and Ag5 membranes (measuring 15 mm × 15 mm, ~ 5.5 mg) were compared. The protocol was approved by the Institutional Animal Care and Use Committee of Innoheart (2015/022), Singapore. Fourteen 12-month-old male New Zealand white rabbits (Oryctolagus cuniculus), each weighing 3 – 4 kg, were used. The dorsum was chosen as implant location. The procedure included subcutaneous implantation of membranes alone or membranes contained in a tissue cage (Figure 1). The tissue cages
were made of stainless steel 304 meshes with the size of 1.5 cm in length and 0.8 cm in diameter and were prepared as described previously [28]. All the samples were labeled by one investigator and were implanted and analyzed blindly by the other investigators to avoid bias.

Figure 1 | Implantation scheme for in vivo evaluation. (A) Five subcutaneous pockets were made per rabbit. The anterior sites (1, 2) were selected to implant membranes alone, and the posterior sites (3, 4, 5) were adopted to implant membranes contained in tissue cage and empty tissue cage as sham control. (B) Photograph of the implants. From top to bottom, a piece of electrospun membrane (15 mm × 15 mm), an empty tissue cage, and a membrane-containing cage. Scale in cm with mm subdivisions.

The rabbits were anesthetized with isoflurane, and their backs were shaved and disinfected with alcohol and iodine scrub. Five 2 cm long incisions per rabbit were made on the dorsum skin and subcutaneous connective tissues were blunt dissected (Figure 1). The membranes (Ag0 and Ag5) were randomly distributed to the two anterior pockets, and three tissue cages, of which two maintained membranes (Ag0 and Ag5) and one was an empty tissue cage as sham control, were randomly distributed to the three posterior pockets according to numbers generated from https://www.random.org/. After implant insertion, the skin was closed with 4-0 absorbable VICRYL® sutures. The wound healing status and post-operative condition were recorded every week. After 4 and 12 weeks, 7 rabbits were euthanized by an overdose of Nembutal. After shaving and disinfection of the skin with 70% ethanol, a central incision was created to expose the subcutaneous tissues.
The anterior specimens with surrounding tissues were retrieved, labeled, fixed in 10% phosphate-buffered formalin for 48 h, and stored in 70% ethanol. Then, the specimens were dehydrated in a graded series of ethanol and embedded in paraffin, after which 5µm sections from at least three arbitrary regions were cut perpendicular to the long axis of the tissue capsule using a standard microtome (Leica RM 2165, Leica Microsystems, Nussloch, Germany). The sections were stained with hematoxylin and eosin (H&E) for general evaluation, elastica van Gieson (EVG) for measurement of the thickness of the fibrous tissue capsule, and tartrate-resistant acid phosphatase (TRAP) for observation of macrophages and foreign body giant cells (FBGCs). The thickness of the fibrous tissue capsule surrounding the anterior specimens (Figure 2) was measured at 6 evenly distributed points per histological section and for three sections of each sample.

For the posterior samples, the exudates inside the tissue cage implants were collected using 25 gauge needles. The collected exudates were centrifuged at 2400 g at 4 °C for 10 min to precipitate cell pellets. All the cell pellets from the same group were pooled together for RNA extraction prior to PCR array analysis. Due to blood contamination in the 4-week samples, only the samples collected at week 12 were tested. Rabbit inflammatory cytokines and receptors RT2 profiler PCR array (QIAGEN Inc., Valencia, CA) was conducted to profile the expression of 84 key genes mediating the
inflammatory response. The relative fold change of gene expression between groups was calculated and then showed in a heat map using a web tool: http://biit.cs.ut.ee/clustvis/. A fold change of more than 4 was arbitrarily regarded as significant.

### 2.6 Statistical analysis

All data were reported as mean ± standard deviation, and were analyzed using one-way analysis of variance followed by Tukey’s multiple comparison test (for more than two groups) or t test (for two groups) by Graphpad Prism 5.0 (GraphPad Software Inc., San Diego, CA). Differences were considered statistically significant at $p < 0.05$ or less.

### 3 Results

#### 3.1 Morphological characterization

The electrospun membranes showed a uniform fibrous structure (Figure 3). The average fiber diameter was approximately 175 nm, and the addition of AgNO$_3$ in the electrospinning solution had no significant effect on the fiber diameter.

![Figure 3 | Scanning electron micrographs and fiber diameter distributions of Ag0 (A, B), Ag1 (C, D), and Ag5 (E, F), with average size of 177 ± 60 nm, 185 ± 58 nm, and 168 ± 58 nm, respectively. Scale bar: 1 µm.](image)

The distribution of AgNPs in the fibers was assessed by TEM (Figure 4). Homogenous electro-dense areas, which indicated the formation of AgNPs, was detected in the Ag1 and Ag5 groups, while there was no sign of electron dense areas in the fibers of the Ago group.
3.2 Antibacterial test

The long-term antibacterial effect of the membranes was evaluated against the typical oral pathogenic gram-negative anaerobic bacteria Pg and Fn (Figure 5). Ag0 specimens showed no inhibition zone and the bacteria grew under the membranes. Ag1 and Ag5 displayed an obvious inhibition zone around the membranes against both Pg and Fn even after 28-day immersion in PBS. Further quantification showed that Ag5 exhibited a significantly larger inhibition zone than Ag1.

Figure 5 | Zone of inhibition test against Porphyromonas gingivalis (Pg) and Fusobacterium nucleatum (Fn). The membranes were pre-immersed for 0, 7, and 28 days in PBS. The optical photos of membranes are shown in the left panel. The corresponding diameters of inhibition zone in the right panel, with dot lines indicating the original diameter of the discs (12 mm). * indicates no inhibition zone. † indicates p < 0.05, and ‡ p < 0.01.
3.3 Cell viability

Cell viability in culture medium containing different amounts of AgNO₃ is shown in Figure 6A. An AgNO₃ concentration below 3 µg/mL in DMEM medium showed no cytotoxicity, and the concentrations of AgNO₃ showing 50% reduction in cell viability (IC₅₀ = half maximal inhibitory concentration values) was 5µg/ml.

The cytotoxicity of AgNPs incorporated membranes are displayed in Figure 6B-D. Figure 6B illustrates the PDLC viability in the presence of membranes. No cytotoxicity could be observed for all the samples, including the group of 2.0 mg Ag5 membranes, corresponding to 100 µg total AgNO₃ incorporation. The results of the fluorescent Live/Dead staining (Figure 6C-6D) confirmed that all the cells were viable with the presence of membranes (green color) and no dead cells (red color) could be found.
3.4 Descriptive histology of host tissue response

After surgery, all animals remained in a good health condition and showed no sign of wound complications including infection or adverse tissue response (i.e. redness, swelling). At retrieval, all specimens (direct or cage implantation) were found to be surrounded by a tissue capsule, without indication of serious inflammation or pus formation.

Figure 7 | Histology (H&E staining) of explanted tissues. The left images are the overview of the capsule, and the right images are the corresponding magnified local view. m: membranes, i: area infiltrated with inflammatory cells, f: fibrous tissue capsule. Scale bar in left images: 1000 µm, in right images: 100 µm.
Representative HE stained images are depicted in Figure 7. All implanted membranes did stain red and were found to be intact after both 4- and 12-week implantation. The membranes did not show any visual signs of degradation. The tissue response from 4 and 12 week samples showed a similar histological appearance. All membranes were folded-up into a wave-like structure and surrounded by a tissue capsule, which was featured by the presence of connective tissue and inflammatory cells (mainly leucocytes). The inflammatory cells mostly aggregated in the vicinity of the membrane, which was displayed in a dark purple color. Over the implantation time period, the capsule thickness appeared to decrease, while the presence of inflammatory cells appeared unchanged. Higher magnification indicated that the membranes had a layered structure, and the marginal area of the membranes were in a loose structure with cells infiltration; however, no cells had infiltrated into the center of the membranes. EVG staining revealed that no significant differences existed in capsule thickness between Ag0 and Ag5 after either 4 or 12 weeks of implantation. However, the fibrous capsule after 12 weeks of implantation was significantly thinner compared to those after 4-weeks implantation (Figure 8).

![Figure 8](image.png)

**Figure 8** | Quantification of the thickness of the fibrous tissue capsules after 4 and 12 weeks of implantation. Significance: * indicates p < 0.05, ** indicates p < 0.01.

TRAP staining of the light microscopic sections (Figure 9) indicated the infiltration of macrophages into the various materials and FBGC formation. Macrophages and FBGCs were stained red and were observed to be mainly located in an interfacial area between the membrane and the surrounding fibrous capsule. No differences in appearance could be detected between Ago and Ag5 samples after both 4 weeks and 12 weeks of implantation.
Figure 9 | Histology (TRAP staining) of the explanted samples. The red spots indicated by small black arrows are the macrophages or the foreign body giant cells. Scale bar: left image 1000 µm, right image 100 µm.
Figure 10 | Gene expression analysis in the *in vivo* study. The fold change of detected inflammatory cytokines are shown after 12 weeks. The empty cage was used as control. A significant up-regulation of gene expression is labeled red, and down-regulation blue.

### 3.5 Expression of inflammatory cytokines/chemokines

Gene expression for the inflammatory cytokines/chemokines of exudates from explanted 12-week tissue cage samples were assessed. The results of 19 out of the 84 tested cytokines/chemokines were discarded because their average threshold cycles were higher than 30, meaning the gene expression was too low to be detected accurately. The relative fold change of gene expression for the remaining 65 cytokines/chemokines are shown in Figure 10. In general, compared to the sham control, both Ago...
and Ag5 showed high inflammatory profiles as demonstrated by the overexpression of inflammatory cytokines/chemokines (higher than 4-fold). Specifically, the cytokines/chemokines related to the presence of macrophages, e.g., CCL4, CCR1, CCR5, CSF1, CX3CR1, were all significantly up-regulated. Also IL10, a common cytokine to inhibit inflammation, was significantly up-regulated, whereas the cytokines to promote inflammation, IL17A and IL18, were not significantly changed. When Ag5 and Ag0 were compared, there were no significant changes in gene expression for the majority of the cytokines, except for IL6 and CCL4. For Ag5, the expression of IL6, a pro-inflammatory cytokine, was down-regulated compared to Ag0, whereas CCL4, a pro-inflammatory chemokine attracting monocytes and other immune cells, was up-regulated.

4 Discussion

In the current study, we aimed to evaluate the biological performance of chitosan-based membranes, which contained silver nanoparticles. Our hypothesis was that AgNPs: (1) would enhance the antibacterial properties of the membranes, and (2) had no adverse effect on the soft tissue to the membranes. Our main findings showed that the incorporation of AgNPs in the chitosan-based membranes produced a dose-dependent antibacterial effect against Pg and Fn for at least 28 days. The chitosan-based membranes were not cytotoxic in vitro and the silver incorporation did not affect the evoked in vivo tissue response.

With many bacteria developing resistance to conventional antibiotics, silver is widely used for disinfection by functioning in forms of silver ions or AgNPs. AgNPs were widely explored mostly because they can act as the depot to continuously release silver ions, and the released silver ions are commonly accepted as the biochemically active agent [29]. In the antibacterial process, the positively charged silver ions destroy the negatively charged bacterial membranes and lead to damage of the bacterial respiratory chain enzymes as well as their DNA. Eventually, this mutual effect induces bacterial death through cell lysis and leakage [30]. In our previous studies, AgNPs incorporated electrospun membranes resulted in a sustained release of silver for a period of 28 days [21]. Besides, the TEM images of the cross-section of membranes showed that the AgNPs almost disappeared after 28-day immersion [21]. In agreement with the release study, the current study demonstrated that the persistent antibacterial property of AgNPs-GTR membranes against Pg and Fn lasted also for at least 28 days, with Ag5 showing a stronger antibacterial effect than Ag1. This long-term antibacterial effect is consistent with the sustained release of silver ions. The stronger effect of Ag5 over Ag1, indicates that the antibacterial properties of the membranes can be adjusted by controlling the silver concentration.
Although the antibacterial property of silver is evident, the cytotoxicity of silver nanoparticles is a major concern for its clinical application. Cytotoxicity of AgNPs may be considered from two aspects, i.e. silver ions by dissolution of AgNPs and the “Trojan horse” effect of AgNPs. The silver ions have been explosively accepted as having a dose-dependent toxicity. In a study by Vrcek [31], the toxicity of silver ions was determined. It was shown that silver ions displayed a dose-dependent cytotoxicity to the human hepatoblastoma (HepG2) cells, and the IC50 value of silver ions was 0.5 µg/mL. In the current study, the IC50 of silver ions was found to be 5 µg/mL to PDLCs. Here, PDLCs showed a higher cytotoxic threshold compared to HepG2 cells, this discrepancy may indicate that different cell types or different experimental setups may induce a different cytotoxic threshold to silver agents. One the other hand, the membranes containing AgNPs showed no obvious cytotoxicity to the PDLCs. This suggests that the silver ions released from the membranes did not reach the cytotoxicity threshold in this study. The other most widely spread theory is the “Trojan horse” mechanism of biocidal activity of AgNPs [32,33]. In that theory, AgNPs could act as a Trojan horse to enter the cells, release silver ions in the cytosol and consequently induce cellular apoptosis. To understand the cytotoxicity mechanism of the AgNP-incorporated membrane, it would be ideal to distinguish the form of the released silver, i.e. particles or ions. However, the current analytical tools have difficulties to achieve this goal [34,35]. It deserves more research efforts in future to distinguish AgNPs and silver ions.

Clinically, the membranes for biomedical application must not interfere with the tissue healing response, which is characterized by a mild inflammatory response, followed by fibroblast proliferation, granulation tissue formation, and tissue remodeling [36]. In literature, only a few reports are available dealing with the subcutaneous tissue response to silver-containing materials. Zhang et al. [37] evaluated implanted nAg–HA–TiO2/PA66 membranes and e-PTFE membranes in a rat subcutaneous model for 1, 4 and 8 weeks and found that the membranes were surrounded by granulation tissue. The granulation tissue layer was thicker for the e-PTFE group at 1 and 4 weeks than for the silver composite group. However, the thickness of the granulation tissue decreased to a thin layer for both composite group and e-PTFE group at 8 weeks. Similarly, Takamiya et al. [38] implanted subcutaneously polyethylene tubes filled with a pure fibrin sponge or a fibrin sponge loaded with various types of silver nanoparticles for 7, 15, 30, and 90 days. It was observed that the different groups showed a similar inflammatory response at all experimental periods, i.e. the formation of granulation tissue and a fibrous capsule. In the current study, the main aim was to evaluate whether the incorporation of AgNPs would have an effect on the tissue response. Therefore, we only included the chitosan based membranes without AgNPs as a control/reference material, and the Ag5 membrane with the strongest antibacterial efficacy in vitro. To reduce the number of experimental animals, Ag1 membrane was not included in the in
The subcutaneous implantation of membranes alone in the rabbits caused a fibrous capsule formation, which became thinner from 4 to 12 weeks for both groups. Besides, the distribution of macrophages and FBGCs were similar between groups. Nevertheless, when critically regarding the thickness of the fibrous tissue capsules, all chitosan based membranes induced a relatively thick capsule. As reported from our previous study [39], this relatively thick capsule may be due to the mismatch of the mechanical property between implanted membranes and the surrounding tissues. The fact that the membranes were folded up due to capsule contraction indicates that the membranes were lacking capacity to remain a stable shape. The folding up of the membranes might have resulted in an increase of inflammatory cells at the interface of membranes and tissues; and indeed, the macrophages and FBGCs were mostly distributed in these areas. Still, our main question could be answered positively as the incorporation of AgNPs did not result in a larger capsule thickness. Therefore, we can still conclude that the incorporation of AgNPs does not have an effect on tissue response.

The current study further analyzed the effect of the incorporation of AgNPs on in vivo cytokine gene expression profiles. For this purpose, tissue cages were implanted that were either empty or containing membranes with or without silver. All the cages containing membranes showed a significantly up-regulated gene expression of most cytokines, especially the cytokines related to macrophages, likely as a normal foreign body response which occurs to any implanted biomaterial. However, there was no significant alteration in the gene expression of most detected cytokines for the membranes with vs. without the silver. Still, two cytokines, i.e. IL6 and CCL4, were changed significantly in gene expression as a result of the AgNPs. The expression of IL6, commonly identified as a pro-inflammatory cytokine [40], was significantly down-regulated. However, the expression of CCL4, the other pro-inflammatory chemokine that attracts activated T cells, monocytes/macrophages, NK cells, and eosinophils [41], was significantly up-regulated. Together, the results from the PCR array were inconclusive regarding the effect of silver on inflammatory status.

Furthermore, for biomedical applications, such as GTR, the membranes should stay intact for at least 4 to 6 weeks to allow the periodontal tissues to regenerate [8,9]. In this study, although the loose structure of marginal area of the membranes showed some cells infiltration, the middle part of the membranes stayed intact and without cell infiltration even after 12-weeks of implantation. This appears to be a promising characteristic of the membranes and can support the formation of a physical barrier to stop the ingrowth of unwanted cells. This absence of degradation may be due to the cross-linking treatment of the chitosan based membrane and the relatively high degree of deacetylation of chitosan, i.e. 90% [42].
Despite the benefits of AgNPs, special concerns should be raised to their safety for biomedical applications. The most common effect of the chronic exposure is permanent bluish gray discoloration of the skin (argyria) or the eyes (argyrosis) [43]. Fortunately, this adverse effect rarely happen and has mostly been reported after the intake of a large amount of AgNPs [35,43-45]. Furthermore, the exposure to silver compounds may produce toxic effects to organs like liver and kidney and to respiratory system and circulation system [43]. Nevertheless, pre-treatment of AgNPs, such as washing or coating, has been reported to be able to lower the toxicity to a safe level for skin application [46]. In this study, the incorporation of AgNPs in the polymers could be another efficient way to lower their cytotoxicity both in vitro and in vivo.

5 Conclusion

This study shows that the incorporation of silver nanoparticles can provide a sustained antibacterial assistance to chitosan-based membranes in a dose-dependent manner. Further in vitro cell culture revealed that the incorporated amount of AgNPs did not cause a noticeable cytotoxic effect on periodontal ligament cells. The in vivo study demonstrated that the incorporation of silver nanoparticles into the chitosan based membranes did not alter their soft tissue response. This warrants the further investigation to their usefulness in biomedical field, such as GTR procedures.
References


Antibacterial effect and wound healing ability of silver nanoparticles incorporation into chitosan-based nanofibrous membranes

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1 Introduction

Silver has been widely explored to enhance the antibacterial property of medical devices, such as wound dressings, catheters, and external fixators [1-3]. In topical burn treatment, silver sulfadiazine (SSD) is even regarded as the gold standard [4]. Silver can be applied in the form of silver salts, e.g. silver nitrate and silver sulphadiazine, or as silver nanoparticles (AgNPs) [2]. Compared to silver salts, AgNPs have a prolonged release of silver and this feature contributes to the reduced frequency of dressing changes, and thus the reduction of pain caused by dressing changes and the associated costs [4].

However, the toxicity of AgNPs is still under debate. Several studies postulate that AgNPs can be more toxic towards bacteria than towards mammalian cells, leading to a therapeutic window that can kill bacteria without harm to mammalian cells [5,6]. In contrast, other studies have reported AgNPs had the same cytotoxic threshold towards bacteria and human cells [7]. This discrepancy might lie in the used culture medium for assessing toxicity. Most studies assess antibacterial effects in culture medium without serum, which is known to interact with nanoparticles [8]. Furthermore, the inorganic ions in body fluid might also interact with AgNPs [9], but their effects on silver release are often overlooked.

Wound dressings, one of the medical products often with AgNPs incorporation, are designed to remove exudates, to inhibit invasion of exogenous microorganisms, and improve skin appearance [10]. For wound dressings, it is critical to evaluate the relevant property of a biomaterial in a more clinically relevant condition. Interestingly, despite the large amounts of studies on AgNPs related materials, there are only limited studies available about the antibacterial evaluation of AgNPs in vivo and those studies are mostly evaluating the antibacterial property indirectly through the wound healing rate [11,12]. Furthermore, there is no consensus among the studies about the effect of silver-containing dressings on wound healing, as evidenced by reports about delayed [13,14], accelerated [1,10,15-17], and no changed wound healing rate [18,19].

In this study, we therefore aimed to elucidate first the mechanism of proteins and inorganic ions on the silver release from the AgNPs incorporated chitosan-based membranes in vitro, to explore their antibacterial efficacy both in vitro and in vivo and the wound healing ability in vivo. To this end, we first conducted a silver release study in phosphate buffered saline (PBS) to reflect the ionic microenvironment and in fetal bovine serum (FBS) to further elucidate the influence of proteins. After that, we evaluated the antibacterial property of membranes after immersion using the zone of inhibition against Staphylococcus aureus. A rat subcutaneous intra-operative
contamination model was selected to further test antibacterial efficacy in vivo. Finally, the wound healing ability was evaluated using a rat excisional wound splinting model.

2 Materials and methods

2.1 Material preparation

The samples were fabricated using an electrospinning technique. Briefly, 40 ml electrospinning solutions containing 26 ml ultrapure water (Millipore Corp, Bedford, MA, USA), 14 ml acetic acid (99.9%, Boom BV, Netherlands), 0.72 g chitosan (degree of deacetylation = 90%, molecular weight = 200-400 kDa, Heppe Medical Chitosan), 0.48 g polyethylene oxide (PEO, Molecular weight = 900 kDa, Sigma-Aldrich®) with 0 (Ag0 group), 12 mg (Ag1 group), or 60 mg (Ag5 group) silver nitrate (AgNO3, Sigma-Aldrich®) were stirred overnight. The solutions were electrospun to membranes at a flow speed of 4 ml/h, a distance of 18.5 cm between collecting plate and spinneret, and a voltage of 28-30 kV. The membranes were crosslinked with glutaric dialdehyde (25 wt. % solution in water, ACROS Organics™) vapor at room temperature for 2 hours. After the cross-linked membranes were dried, they were cut into 12 mm-diameter disks for further study. All the disk samples were sterilized by 25 kGy γ-radiation (Synergy Health, Ede, The Netherlands).

2.2 Morphology characterization

The morphology was observed by field emission scanning electron microscopy (SEM; Zeiss Sigma 300, Germany) with X-ray energy-dispersive spectrometry (EDS; Bruker, Billerica, MA). All samples were sputter-coated with a thin layer of chromium before the detection. The fiber diameters were measured at 100 random locations from 5 different images using Image J (National Institutes of Health, Bethesda, USA) [20]. The distribution of AgNPs was detected by JEOL 1010 transmission electron microscope (TEM; JEOL, Tokyo, Japan).

2.3 Silver release behavior in different biological solutions

The disk membrane of 4 mg from each group (n = 5) was placed into 1.5 ml Eppendorf® tubes containing either 1 ml phosphate buffer saline (PBS, Gibco™) or fetal bovine serum (FBS, Sigma F7524, Taufkirchen, Germany). The tubes were incubated at 37 °C with gentle agitation. At each predetermined time point, 900 µl superna on day 0, 4, 7, 11 and 14 after surgery on day 0, 4, 7, 11 and 14 after surgery taut was collected for further analysis and then refreshed with 900 µl PBS or FBS correspondingly. The silver
concentration in the collected supernatants was determined by using inductively coupled plasma mass spectroscopy (ICP-MS X series I, Thermo Scientific, Waltham, MA). The collected supernatant was diluted 20× with 1% nitric acid before the ICP-MS analysis. A standard calibration curve was prepared with silver concentrations ranging from 8 to 1000 ng/ml. Furthermore, to further elucidate the alteration of PBS and FBS immersed samples, 2 extra samples were retrieved at each predetermined timepoint and lyophilized for SEM-EDS detection.

2.4 In vitro antibacterial test

The disk samples were incubated in 1 ml of PBS with gentle agitation at 37°C. PBS was refreshed every 2 to 3 days on day 0, 4, 7, 14, 21, and 28, the disk samples were lyophilized for further evaluation (n = 3). A zone of inhibition test (modified Kirby–Bauer test) was adopted to evaluate the antibacterial property against typical pathogenic bacteria in skin wound, *Staphylococcus aureus* (*S. aureus* ATCC® 25923™, Manassas, USA) [21]. Standard tablets containing 30 µg of cefoxitin were used as the positive control. In brief, the bacterial suspension was prepared by mixing the *S. aureus* ATCC 25923 strain with sterile saline (0.85% w/v NaCl in water) to McFarland Standard value of 0.5 and further diluted for 10 times. The bacterial suspension was smeared evenly on Mueller Hinton agar plate (BDTM, Becton Dickinson, Franklin Lakes, NJ). Then the disk samples were placed in the center of agar plates, using a different agar plate for each disk sample. After inoculation for 20 hours at 36 °C, the diameters of the transparent zones of inhibition were measured.

2.5 In vivo evaluation

The animal study was approved by the Animal Experiments Commission of Radboud University and the national Central Animal Experiments Committee (CCD No. AVD10300 2015-241) according to the legal regulations as stipulated in the amended Animal Testing Act in the Netherlands and Directive 2010/63/EU of the European Parliament and of the Council.

Blinding and randomization were applied throughout the animal study. All the samples in the animal experiments were labelled and the group information were kept by an independent investigator, who did not participate the following surgery, histological and microbiological detection until all the data were collected. Randomization was achieved using a computerized random sequence generator (www.random.org).

The animals were housed under standard conditions and received food and water *ad libitum*. All animals were acclimatized for two weeks before the surgery. All surgery
was conducted by inhalation of 5% (isoflurane/oxygen) and maintained by inhalation of 2.5% (isoflurane/oxygen). After surgery, Buprenorphine (Temgesic®, Reckitt Benckiser, Slough, United Kingdom) and carprofen (Rimadyl®, Pfizer, New York City, NY) were subcutaneously injected for analgesia immediately and also on day 1 postoperatively.

2.5.1 *In vivo* antibacterial test

42 male specific-pathogen-free (SPF) Charles river Wistar Hannover [CRL: WI (Han)] rats (weight 250–300g) were randomly assigned to 3 study groups with 2 sacrifice time points, i.e. day 7 and day 28. After anesthesia, the hair on the back of the rats was shaved. Two 1.5 cm-long linear incisions were created bilaterally on rat dorsum. Disk samples were placed into the pockets. Following implantation, 50 µL bacterial suspension containing $1 \times 10^6$ CFU of *S. aureus* in PBS were immediately inoculated into the pocket directly above the implant. The incisions were then closed with staples. After 7 or 28 days, the rats were euthanized by inhalation of a mix of CO$_2$ and O$_2$. The capsulated samples, i.e. tissue capsules and embedded disk samples, were collected. The collected samples on the right dorsum were used for enumeration of bacteria, and those on the left dorsum were treated by histology.

For microbiological detection, the capsulated samples were incised to separate the implanted disk samples from the surrounding tissues, and the bacterial amount on the disk samples and the tissue samples were counted separately. The samples were then cut to small pieces, added into 1 ml of 0.85% saline, vortexed for 30 seconds, ultrasonically treated for 5 mins, and vortexed again for 30 seconds to isolate the bacteria from the tissues or the membranes [22]. The bacterial suspensions were then diluted in series of 10 times and quantified by bacterial counting method with countable colonies of 10-200 per agar plate after inoculated on Columbia Blood Agar with 5% Sheep Blood plates (CBP, BD™), Columbia CNA Agar with 5% Sheep Blood plates (CNA, BD™), and MacConkey Agar without Salt plates (MCP, BD™). The CBP is rich agar plate to culture all the bacteria in the suspension, CNA is used to selectively culture gram-positive cocci (streptococcus/ staphylococcus), and MCP is selected to culture gram-negative rods like *E. coli*. Based on the colony morphology, the bacterial colonies were identified primarily by smearing the bacteria in 2 drops of physiological saline or rabbit plasma, where *S. aureus* was dispersed in saline (-), but clustered in plasma (+). For suspicious colonies, matrix-assisted laser desorption/ionization with time-of-flight mass spectrometer (MALDI-TOF, Billerica, MA) was further applied to identify the bacteria. After the bacterial counting, the silver content in the tissue samples was evaluated by ICP-MS.
After retrieval, the collected samples on the left dorsum were fixed in 4% buffered formaldehyde for 48 hours, immersed in 70% ethanol for at least 2 days, then dehydrated in series of ethanol, and embedded in paraffin. Thereafter, 5 µm-thick sections were cut using a Leica RM 2165 Microtome (Leica Biosystems, Wetzlar, Germany). For each sample, sections from 6 sequences of parallel layers of samples were collected and stained with routine hematoxylin and eosin (H&E) staining and Brown-Brenn modified Gram staining. For H&E staining, paraffin sections were deparaffinized in xylene, rehydrated to water, stained with a Delafield hematoxylin, dehydrated again to 96% ethanol, and stained with Eosin. For Gram staining, paraffin sections were deparaffinized in xylene, rehydrated to 96% ethanol, treated with ammonium ethanol, rinse in water, stained with filtered Hucker-Conn solution, rinsed with water again, treated with Lugol, rinsed in water and dried, treated with 60°C acetate acid ethanol, rinsed with water, and stained with Twort solution. After staining, the sections were sequentially rinsed in ethanol and xylene, and mounted with DPX (BDH Laboratory Supplies, Dorset, UK).

2.5.2 In vivo wound healing evaluation

An excisional wound splinting rat model was selected to prevent wound closure caused by skin contraction in order to heal the wounds through granulation and re-epithelialization [23]. 16 male SPF CRL: WI (Han) rats (weight 250–300g) were assigned to 4 balanced Latin square for 4 groups, i.e. sham control, Ag0, Ag1, and Ag5 group. The donut-shaped splints (Inner Ø12 mm, Outer Ø 22 mm) were punched from red silicone sheets (JTR-SA-1.0-Silicone Isolator Sheet Material, 0.9 mm Thick, Grace Bio-Labs, Bend, OR). Then, the splints were sequentially ultrasonically washed in 10% liquinox detergent (Alconox, New York, NY, USA), isopropanol and ultrapure water (Millipore) for 2 min, respectively. Before surgery, the splints were autoclaved at 121°C for 15 min. The surgical procedure is performed as described [24,25]. In brief, after anesthesia, 4 of 8 mm-diameter circular wounds were punched bilaterally on the dorsum with disposable biopsy punches (Integra™ Miltex®, Plainsboro, NJ). The splints were glued and sutured to the surrounding skin by 4-0 suture (Coated VICRYL®, Ethicon Inc., Somerville, NJ). The disk samples were placed to cover the whole wounds, and further stabilized with transparent film dressing (3M™ Tegaderm™, St. Paul, MN). Subsequently, the dressings were secured with bandages (Petflex® No-Chew Cohesive Pet Bandages, Portsmouth, NH) by wrapping to the front part of the fore leg to prevent damage from paw movement or chewing. The samples were changed and standardized digital wound photographs were taken with a ruler as calibrator on days 0, 4, 7, 11, and 14 with the bandages being reapplied. The animals were euthanized on day 4 and 14 by inhalation of a mix of CO₂ and O₂. The wounds with the surrounding skins were retrieved for histological and histomorphometrical analysis.
The wounds reduction was quantified with a method adapted from a previous publication \cite{24}. After the image scale was calibrated with the ruler on each photograph, the wounds areas were measured. Then wound reduction was expressed as % original wound area using the following equation:

\[
\% \text{ original wound area} = \frac{\text{wound area at day } X}{\text{wound area at day } 0} \times 100\%
\]

The paraffin sections were prepared using the same method as described in 2.5.1. In addition to routine HE staining, the collagen was stained with Elastine van Gieson (EvG) staining, neovascularization was assessed by $\alpha$-smooth muscle actin ($\alpha$SMA) staining, and macrophages and foreign body giant cells (FBGCs) were identified by ED1 staining. For EvG staining, the paraffin sections were dehydrated with xylene, rehydrated to 96% ethanol, stained with Lawson solution, rinsed in 96% ethanol, stained the nuclear with Weigert A + B, rinsed in water, and stained with van Gieson's picrofuchsin. For $\alpha$SMA staining, the sections were treated with 3% $\text{H}_2\text{O}_2$ in methanol to inactivate endogenous peroxidase, and post-fixed with 4% formaldehyde in PBS. After rinsing with 0.075% glycine in PBS (PBSG), the sections were incubated in citrate buffer (pH 6.0) at 70°C for 10 min followed by incubation with 0.075% trypsin in PBS at 37°C for 5 min for antigen retrieval. The sections were pre-incubated with 10% normal donkey serum (NDS, Chemicon, USA) followed by overnight incubation of 1\textsuperscript{st} antibody mouse-anti-rat $\alpha$SMA (1:51200; Serotec, DPC, the Netherlands) at 4 °C. Subsequently, the sections were incubated with secondary antibody donkey-anti-mouse IgG (1:750; Jackson Labs, USA) for 60 min in dark, treated with avidin biotin complex (ABC) for 45 min in dark, and treated with 3,3-diaminobenzidine (DAB) until dark brown staining. Until now, PBSG was always used to rinse the sections between the treatments. Then the sections were rinsed in water, and the staining was intensified with 0.5 CuSO\textsubscript{4} in 0.9% NaCl. After rinsing with water, the sections were counterstained with Delafield hematoxylin. After staining, the sections were dehydrated sequentially with ethanol and xylene and mounted with DPX (BDH Laboratory Supplies). For ED1 staining, the procedure is like $\alpha$SMA staining. There were only two differences. First, the antigen retrieval was achieved by incubation in 0.075% trypsin in PBS at 37°C for 15 min. Second, the first antibody was mouse-anti-rat ED1 (1:500; Serotec, DPC, the Netherlands).
Figure 1 | The quantification by using macros of Image J. (A) (D) and (G) The typical images from EvG, αSMA, and ED1 staining. (B) (E) and (H) The region of interest after threshold using macros. (C) (F) and (I) The amplified local area after threshold.

All slides were digitized with the same parameter using a 3DHistech Panoramic 250 Flash II scanner (3DHistech, Budapest, Hungary). After exporting the images, the complete wound area was selected as the region of interest (ROI) by hand. The ROIs were then quantified with macros created by image J as shown in Figure 1. The collagen formation, vessel density, and macrophage density were calculated by using the following formulas.
% collagen = \frac{\text{collagen area (B)}}{\text{total area of region of interest}} \times 100\% \\
\text{vessel density} = \frac{\text{vessel number (E)}}{\text{total area of region of interest}} \\
\text{macrophage density} = \frac{\text{macrophage number (H)}}{\text{total area of region of interest}}

### 2.6 Statistical analysis

All data were reported as mean ± standard deviation and were analyzed using one-way analysis of variance followed by Tukey’s multiple comparison tests or Kruskal-Wallis test followed by Dunn’s multiple comparisons test by GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, USA). Differences were considered statistically significant at \( p < 0.05 \).

### 3 Results

#### 3.1 Morphological characterization of AgNPs incorporated membranes

The fibrous membranes with different amounts of AgNPs were fabricated by electrospinning, and their morphologies were characterized by SEM and TEM. SEM images (Figure 2A-C) showed a uniform fibrous structure for all groups with a similar average fiber diameter of \( \approx 200 \text{ nm} \) (\( P > 0.05 \)). TEM image (Figure 2D) demonstrated that the electron-dense AgNPs were formed within the fibers.

#### 3.2 In vitro interaction with biological solutions

After immersion, the PBS did not alter the thickness of the membranes but the FBS resulted in an obvious increase of the thickness by visual observation. Figure 3A showed the morphological changes after immersion in PBS or FBS. The membranes kept their fibrous structure after immersion in PBS, while more and more white spots appeared on the fibers with the increase of silver amount and immersion time. Further identification using EDS revealed that the white spots had a high amount of silver and chloride. For the FBS-immersed membranes, a thick layer of protein was clearly observed on the fibrous membranes since day 4.

EDS identification (Figure 3B) demonstrated that silver was detectable on the surface of membranes throughout the test period after PBS immersion whereas no silver was present on the surface after FBS immersion except for the Ag5 membrane after 28-day immersion.
Figure 2 | The SEM images of the electrospun membranes of (A) Ag0, (B) Ag1, and (C) Ag5 groups. (D) the TEM image of single fiber from Ag5 group, where the electron-dense areas were silver nanoparticles.

The released silver from the chitosan-based membranes in either PBS or FBS was quantified by ICP-MS as shown in Figure 3C. The PBS immersed groups resulted in a slow but sustained silver release manner and that the amount of released silver was independent on the amount of AgNPs incorporated in the membranes. By contrast, the FBS immersed groups showed a fast silver release of up to 1 day and 7 days for Ag1 group and Ag5 group, respectively. Afterward, no more released silver was detectable. The cumulative release percentage relative to the theoretical amount of silver in each membrane was successfully fitted by an allometric power growth equation \( y = kt^n \) (Table 1).

Table 1 | Cumulative release (%) of silver from chitosan membranes and the parameters obtained from curve fitting using Korsmeyer-Peppas model \( (y = kt^n) \).

<table>
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<tr>
<th></th>
<th>PBS-Ag1</th>
<th>PBS-Ag5</th>
<th>FBS-Ag1</th>
<th>FBS-Ag5*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burst release (within 6 h)</td>
<td>1.18 ± 0.16</td>
<td>0.27 ± 0.01</td>
<td>34.76 ± 5.45</td>
<td>3.4 ± 0.57</td>
</tr>
<tr>
<td>Total release (after 28 days)</td>
<td>10.49 ± 0.51</td>
<td>2.16 ± 0.01</td>
<td>46.30 ± 5.82</td>
<td>23.53 ± 1.75</td>
</tr>
<tr>
<td>( k )</td>
<td>0.027</td>
<td>0.06</td>
<td>N.A.</td>
<td>0.105</td>
</tr>
<tr>
<td>( n )</td>
<td>0.40</td>
<td>0.38</td>
<td>N.A.</td>
<td>0.61</td>
</tr>
<tr>
<td>Adjusted R2</td>
<td>0.99</td>
<td>0.99</td>
<td>N.A.</td>
<td>0.95</td>
</tr>
</tbody>
</table>

* Based on the release data during day 0 to day 4; N.A. means not applicable due to not enough data points.
Figure 3 | In vitro interaction with PBS and FBS. (A) The SEM micro-images of membranes after immersed in PBS and FBS. Scale bar: 1µm. (B) The percentage of silver in the immersed membranes as quantified by EDS. (C) The silver release profile of the membranes.
3.3 *In vitro* antibacterial efficacy after interaction with biological solutions

The antibacterial property of membranes after immersion in PBS and FBS was quantified by a zone of inhibition (ZOI) test (Figure 4). All the Ag0 membranes did not have any ZOI as expected. After immersion in PBS for up to 28 days, both Ag5 and Ag1 groups showed ZOI and the diameter of ZOI was decreasing with increased immersion time. On the contrary, the FBS immersed membranes revealed that the Ag5 membranes had a ZOI only until day 4 and Ag1 membranes did not show a ZOI.

![Figure 4](image)
The diameter of zone of inhibition of the membranes against *S. aureus*. The dashed line indicates the sample diameter. Data are shown as mean ± SD.

3.4 *In vivo* antibacterial efficacy

The antibacterial properties of the membranes were evaluated both microbiologically and histologically after 7-day and 28-day subcutaneous implantation challenged by *S. aureus* intra-operatively. The membranes were encapsulated by fibrous capsules, and the samples were collected as membranes and tissue capsules separately. The bacterial identification through MALDI-TOF confirmed that all the bacteria in the samples were *S. aureus*. The bacteria counting results (Figure 5A) showed that both the Ag0 and Ag1 membranes did not kill the bacteria, whereas the Ag5 membranes significantly decreased the number of bacteria in the membranes and the surrounding tissues. Besides, the decrease of the bacterial amount was continued to 0 for most samples in Ag5 group. The gram staining results (Figure 5C) displayed obvious bacterial aggregations in the tissues around Ag0 and Ag1 membranes, but fewer or no
bacteria could be found around the Ag5 membranes. Moreover, a correlation between bacterial counting and the silver amount in each capsule revealed that no bacteria could survive when there was more than 250 ng of silver per capsule (Figure 5B).

Figure 5 | Results from in vivo antibacterial evaluation. (A) The bacteria counting result. The dashed line indicates the original inoculated bacterial amount. Data are shown as mean ± SEM. * indicates P < 0.05, ** indicates P < 0.01. (B) The total residual silver content correlated with the bacterial count result in the tissue capsules. (C) The Gram staining of sections. The red dots are cell nuclei. The green area are cytoplasm structures. M represents the implanted membranes, and the dark purple dots, indicated by pink arrows, are Gram positive bacteria. Scale bar: left 200 µm, right 50 µm.
3.5 *In vivo* wound healing

![Figure 6](image)

Figure 6 | (A) the representative macroscopic images of wounds on day 0, 4, 7, 11 and 14 after surgery. The sizes of redish splints are inner ø12mm and outer ø22mm. (B) the % original wound area quantified from macroscopic images. The quantification of (C) collagen formation, (D) vessel density, and (E) macrophage density. Data are shown as mean ± SD. ** indicates P < 0.01.

The wound healing upon the coverage of rat full-thickness skin wounds with different membranes was evaluated. All the wounds healed in 2 weeks, including the sham group (Figure 6A). The wound healing rate in Figure 6B showed that no significant differences were found among the wounds covered with membranes. However, the Ag1 and Ag5 groups showed a faster healing compared to sham control. Histologically, a similar healing pattern was displayed for all the wounds (Figure 7). Overall, abundant granulation tissue characterized by new connective tissues and microscopic blood vessels were observed by visual inspection of the wound area; epidermis covered the wounds partially on day 4 and totally on day 14. Furthermore, the formation of collagen, new vessels, and the presence of macrophages was histomorphometrically
quantified in Figure 6C-E. No significant differences were found in the wound area among all the membrane groups regarding collagen formation, vessel density, and macrophage density.

Figure 7 | The wounds area after H&E staining. E: epidermis; D: dermis; W: wound area.

4 Discussion

Silver nanoparticles have been widely used in medical devices such as wound dressings thanks to their antimicrobial properties. However, no consensus has been reached on the efficacy and safety of AgNPs incorporation [6,7]. To decipher the potential of the AgNPs, the present study adopted AgNPs incorporated chitosan-based membranes and focused on exploring their antibacterial property and wound healing ability both in vitro and in vivo. It was found that the proteins and inorganic ions had a strong influence on the release of silver, and consequently altered the antibacterial property of the tested membranes. Furthermore, incorporation of AgNPs enhanced the antibacterial property of the chitosan-based membranes both in vitro and in vivo, but did not alter the wound healing effect of the membranes.
In our study, the chitosan-based membranes were successfully electrospun with a uniform nano-fibrous structure, and the AgNPs was confirmed to be homogeneously distributed in the fibers by TEM. This formation of AgNPs can be attributed to the reduction of silver nitrate by chitosan [26].

In biological systems, the presence of ions and proteins, of course, is inevitable. In this study, we selected PBS and FBS as representative solutions to explore the influence of inorganic ions and proteins on the silver release from the AgNPs-incorporated chitosan-based membranes. PBS is composed of 10 mM of \( \text{PO}_4^{3-} \), 137 mM of NaCl, and 2.7 mM of KCl; FBS has a similar ion-composition to PBS but with ample proteins [27]. After immersion, the PBS immersion did not alter the thickness of the membranes and kept the complete fibrous structure with only some silver salts deposition on the surfaces. The silver element can be detectable on the surface of the membranes with EDS and a continuous silver release was detected during the detection period. Subsequently, the AgNPs incorporated membranes showed antibacterial properties through the whole period. Together, this suggests that the inorganic ions, either bonding with silver ions to easily form water-insoluble salts or not, do not compromise the antibacterial property of AgNPs incorporated membranes.

In contrast, the FBS immersion covered the fibrous structure of membranes with a thick layer of proteins, which hid the fibrous structure completely after 7 days. The FBS immersion resulted a significantly larger amount of released compared to PBS immersion. The silver was not detectable on the surface since day 4 and the silver was not detectable in the released medium after certain times (i.e. 1 day for the Ag1 group and 7 days for the Ag5 group) as well. Correspondingly, the AgNPs incorporated membranes in our study lost their antibacterial property after 4-day and 7-day serum immersion for the Ag1 group and the Ag5 group, respectively. Combining the silver release and the antibacterial property, it may suggest that (1) the released silver is responsible for the antibacterial property of incorporated AgNPs and the release profile may well anticipate the antibacterial efficacy of silver incorporated materials, and (2) choosing a release medium similar to biological conditions is pivotal to evaluate the antibacterial potential of AgNPs incorporated materials.

To elucidate the possible mechanism of the silver release from the membranes, an allometric power growth equation \( (y = kt^n) \) was fitted with adjusted \( R^2 \) of more than 0.95 to all the groups except FBS-Ag1 due to an insufficient number of data points (Table 1). The Power law or Korsmeyer-Peppas model \( (M_t/M_\infty = kt^n) \) is a simple semi-empirical equation to describe the drug release from polymeric systems [28,29]. In this equation, \( k \) is a constant relating to the structural and geometric characteristics of the release system, and \( n \) is the release exponent indicating the mechanism of drug
release [28]. Ideally, the release is totally controlled by a Fickian diffusion when n is equal to 0.45. Clearly, when the exponent n takes a value of 0, the total drug release amount is constant which is only dependent on the dissolution of the drug; on the other hand, when n takes a value of 1 indicates that the release rate is independent of time corresponding to zero-order kinetics [28]. The PBS immersed membranes showed a release exponent (n) smaller than 0.45, suggesting that the silver release kinetics better followed the mechanism of diffusion, but were restricted by temporary supersaturation of drugs in the release medium, which happens commonly for poorly water-soluble substances [30]. The sparingly water-soluble salts such as silver chloride and silver phosphate are likely formed due to the bond between the released silver ions and the available chloride or phosphate ions in PBS [9]. Besides, a value of n between 0.45 to 1 can be regarded as an indicator for boundary conditions such as the presence of a stagnant layer or a net on the release surface [29]. The FBS immersed membranes showed a release exponent of 0.61, indicating the formation of a stagnant layer on the membranes and, indeed, a protein layer was displayed in the SEM images. The formation of the protein layer may be explained by a well-known hypothesis called the Vroman Effect, which postulates that small and abundant molecules will be the first to coat a surface, and molecules with higher affinity for that particular surface will replace them over time [31]. Compared to PBS, the relatively faster silver release in FBS could be driven by the consumption of free silver ions due to the strong affinity of silver ions to the thiol groups, which exist abundantly in serum proteins, e.g., cysteine, homocysteine, and glutathione [32-35]. The formation of protein layers around the materials altered the silver release profiles.

Based on the above information, the potential mechanism of the interaction of AgNPs incorporated membranes with biological solutions may be illustrated in Figure 8. When the AgNPs incorporated membranes are exposed to a buffer solution, the silver release is dominated by diffusion, but mostly limited by solubility equilibrium. In contrast, the exposure of silver incorporated membranes to protein abundant solutions can result in the formation of protein layers on the membrane surface and simultaneously the chelation of silver ions by proteins would result in a fast release of silver. Over time, the protein layers grow thicker and eventually become a barrier to slow down and even stop the release of silver. As such, the alteration of protein layers on the silver release may affect the antibacterial efficacy and biological performance of the materials. Together, this suggests that special attention should be paid when applying silver-containing materials in a protein-rich environment, where the antibacterial ability of silver might be blocked by the formation of a thick layer of protein around the constructs.
Figure 8 | A schematic model that proposes a potential mechanism of silver release after PBS and FBS immersion.

A rat subcutaneous intra-operative contamination model was selected to explore the antibacterial efficacy of the AgNPs-incorporated chitosan membranes in vivo. In the present study, we selected the subcutaneous intra-operative contamination model considering that the sealed subcutaneous environment provides a microenvironment with biological fluids, such as tissue fluids and blood from surgery, and a reliable bacterial counting [36]. Despite the large amounts of S. aureus inoculation at the implantation site, AgNPs incorporated membranes showed a dose-dependent antibacterial property evidenced by the bacterial decrease in Ag5 group and no bacterial decrease in Ago and Ag1 group. Most samples of the Ag5 group continuously decreased the bacterial amount to 0 from day 7 to 28, suggesting a continuous antibacterial effect in vivo even after 7 days. Interestingly, our in vitro antibacterial test showed that both the Ag1 group and the Ag5 group had a stronger antibacterial property than the Ago group, and the serum immersion resulted that the Ag5 membranes had a ZOI only until day 7 and Ag1 membranes did not have ZOI. This discrepancy of the sustained antibacterial efficacy may be due to the different protein contents among tissue fluids, PBS and FBS [37]. Therefore, we can speculate that the biological fluids may play an important role in the antibacterial property of silver for biomedical applications.
A rat excisional skin model was used to evaluate the effect of the membranes on the wound healing. Despite all the wounds healed in 2 weeks, the Ag1 and Ag5 groups showed a faster healing compared to sham control, which might be due to a synergistic effect of both chitosan and AgNPs since both are reported to be able to promote wound healing \[10,38-40\]. Although the wounds were macroscopically healed on day 14, the remodeling of wounds was still ongoing: the newly formed tissues in the wound area were mostly granulation tissue, but not structural dermal tissues as in the normal skin. Furthermore, no significant differences were histomorphometrically found in the wound area among all membrane groups regarding collagen formation, vessel density, and macrophage density, which play important roles in the wound healing \[41\]. This result may suggest that the silver incorporation had no negative effect on wound healing. The anti-inflammatory effect still needs to be proven.

5 Conclusions

Silver nanoparticles incorporated chitosan-based membranes have been fabricated. The AgNPs incorporated membranes showed a sustained release of silver ions for at least 28 days in phosphate buffer saline whereas they displayed a silver release profile of fewer than 7 days in the fetal bovine serum. In vitro antibacterial results confirmed that the AgNPs-incorporated membranes had antibacterial properties corresponding to the silver release profile. These results indicated a potential mechanism that the silver bonding ions can restrict the silver release through solubility equilibrium whereas the proteins layer can first accelerate the silver release by chelation the silver ions and then hamper the silver release as the protein layer becomes thicker to be a barrier to block the silver release. Therefore, special attention should be paid to the proteins when the antibacterial efficacy is evaluated in vitro. Indeed, further in vivo evaluation confirmed that a high amount of AgNPs is necessary to achieve in vivo antibacterial efficacy. Moreover, the wound healing model displayed that AgNPs incorporated membranes did not alter the wound healing rate and tissue response. Our results suggest the AgNP incorporation can enhance antibacterial efficacy of biomaterials while having good wound healing ability.
References


Chitosan-based sleeves loaded with silver and chlorhexidine in a percutaneous rabbit tibia model with repeated bacterial challenge

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1 Introduction

External fixation in orthopedics can be used for the treatment of comminuted fractures, open fractures, osteomyelitis, nonunion or pseudarthrosis, lower-extremity deformities, limb length inequality, and arthrodesis [1]. Due to the transcutaneous or percutaneous nature of the external fixators, there is a high risk of pin-tract infection (PTI) [2]. PTI is an almost inevitable problem of external fixation. The rate of PTI, ranging from 10 - 53.3%, is rather high in comparison with other infections associated with permanent implants such as prosthetic joint infections (<2%) [3,4]. The standard treatment of PTI is systemic administration of antibiotics. However, the existence of the pin facilitates the occurrence of infection, which are difficult to eradicate by systemic antimicrobial treatments [5]. Additionally, the systemic use of high doses of very potent antibiotics can cause irreversible damage to organs [6]. The frequent use of antibiotics has also high risk of fostering bacterial resistance, which has become a major global healthcare problem [7].

As PTI initiates with the bacterial adhesion and colonization on the pins [8], various approaches to improve the percutaneous have been proposed [2,3,9-11]. However, as a material, which allows permanent epithelial and connective tissue attachment is not yet available, the prevention of infection by creating an infection-resistant percutaneous seal has still not been successful [12]. The strategy to manufacture pins with antibacterial properties is another approach [1,13]. Incorporation of antibacterial agents to pins requires the pins to be altered either by depositing drugs in a hollow porous design, which inevitably compromises mechanical properties and requires complicated fabrication processes [14-17]. An alternative method is the use of antibacterial pin sleeves, which can function as an antibacterial barrier [18,19]. Voos et al. implanted pins contaminated by Staphylococcus aureus (S. aureus) in the iliac crests of goats and then provided them with tobramycin-impregnated polymethylmethacrylate sleeves [18]. They found that the antibacterial pin sleeves prevented the development of PTI up to 15 days [18]. Although antibiotics are the most frequently used drugs, they are associated with some problems, as mentioned already above. Antibacterial agents, like silver or chlorhexidine, can be an option to avoid the problems with antibiotics [16,20]. Our previous study showed that silver/chlorhexidine-loaded membranes showed excellent antibacterial efficacy in vitro [21], and thus may be a promising candidate for antibacterial sleeves to prevent PTI.

The evaluation of antibacterial efficacy necessitates the use of animal models before going into clinical trials [22-26]. For this purpose, a suitable percutaneous animal model is needed, which must have the following characteristics: (1) a consistent induction of infection, (2) stably fixed implants, and (3) maintenance of the percutaneous passage.
throughout the experimental time. It has been proven that consistent infection rates can only be induced by bacterial inoculation \[14,27\]. We recently reviewed existing percutaneous animal models and found that in most models an implant location was used, where the (sub)cutaneous tissues were very thin, such as the scalp, dorsum, and medial part of tibia. In contrast, clinically external fixators are used at locations with a thicker tissue layer. Moreover, models report experimental periods varying from 3 days to 15 months \[4\], whereas pins clinically have to stay in place for 6-7 weeks \[28\]. Finally, the existing animal models report occasionally the occurrence of bone fractures due to the implant installation \[14\] as well as the loss of percutaneous status due to overgrowth of skin \[27\].

Therefore, the aim of this study was to construct a percutaneous animal model to decrease the large discrepancies between the animal test and clinical situation. We also applied this model to test whether: (1) silver incorporation in sleeves would reduce PTI \textit{in vivo} with repeated bacterial challenges for 6 weeks, and (2) the addition of chlorhexidine would further reduce the PTI as compared to the silver-loaded sleeves alone. To this end, we designed titanium pins and implanted them in a percutaneous rabbit tibial model with repeated bacterial inoculations for 6 weeks. Before the start of the study, a pilot study was done to determine the proper implant location and the length of the enossal part as well as percutaneous part of the implant. The rabbit anterolateral tibia was selected as the implantation location, since there is a thick subcutaneous tissue. After measuring the diameter of the tibia (around 8 mm) and the soft tissue thickness (around 10 mm) on a cadaver, the implants was initially designed with a total length of 23 mm including an enossal part of 8 mm in length. However, such implants were found to be rapidly covered due to the overgrowth of skin. Therefore, the percutaneous part of the implants was extended by connecting 3 mm diameter tubes or rods to elongate the implants. Eventually, a total implant length of 40 mm was shown to be long enough to keep the implants percutaneous and was used for the study.

2 Materials and methods

2.1 Implant materials

The implants in this study were designed to represent external fixator pins. The diameter of the implant was 3 mm. The length of implants was 40 mm based on a pilot study to maintain a percutaneous passage (Fig. 1A&B). An 8-mm long thread of M2.5 \times 0.45 was fabricated at the apical end of the implant, which was based on the measurement of tibial diameter in rabbit cadavers and a 1 mm wide slot was created
on the other end of the implants to facilitate the installation of the implants with a screw driver (Fig. 1C). All implants were made of grade 5 titanium alloy (Ti-6Al4V) using a milling machine and did not receive any further surface treatment (Jansen Machining Technology B.V., Valkenswaard, the Netherlands). The implants were then sequentially ultrasonically washed in 10% liquinox detergent (Alconox, New York, NY, USA), isopropanol, acetone, and ultrapure water (Millipore Corp, Bedford, MA, USA) to remove dirt and oil. After washing, the implants were autoclaved.

The sleeves were prepared as previously reported [29]. Briefly, the chitosan based blend solutions were prepared by dissolving chitosan (degree of deacetylation = 90%, molecular weight = 200-400 kDa, Heppe Medical Chitosan, Halle, Germany), poly (ethylene oxide) (PEO, Molecular weight = 900 kDa, Sigma-Aldrich®, St. Louis, MO, USA), and silver nitrite (AgNO₃, Sigma-Aldrich®) in 35% of acetic acid (99.9%, Boom BV, Netherlands)/ultrapure water (Millipore Corp, Burlington, MA, USA) solution at total solid concentration of 3 wt% with different mass ratios (Chitosan/PEO/AgNO₃ = 60/40/0, 60/40/1, and 60/40/5). After stirring overnight, the solutions were electrospun at a flow speed of 4 ml/h, a distance of 18.5 cm between collecting plate and spinneret, and a voltage of 28-30 kV. The electrospun membranes were further dried and crosslinked with glutaric dialdehyde (25 wt% solution in water, ACROS Organics™, Geel, Belgium) vapor at room temperature for 2 hours. After drying in a fume hood, they were cut into 13 mm × 40 mm rectangular sheets (Fig. 1D) and named as Ag0 and Ag5 corresponding with their silver content, respectively. Thereafter, the sleeves with silver and chlorhexidine were prepared. For this purpose, an aliquot of 60 µl of 20% chlorhexidine digluconate in water (Sigma-Aldrich) was pipetted onto Ag5 sheets. These samples were subsequently air-dried in a fume hood overnight, and thereafter named as Ag5C.

Finally, all samples were sterilized by 25 kGy γ-radiation (Synergy Health, Ede, The Netherlands).

2.2 Animals and experimental design

The animal experiment was approved by the Dutch Central Animal Experiment Committee (project license AVD10300 2015-241) and the work protocol (Protocol No. 2015-0072-004) was approved by the Animal Experiments Committee of Radboud University according to the legal regulations as stipulated in the amended Animal Testing Act in the Netherlands and Directive 2010/63/EU of the European Parliament and of the Council.
Thirty-three 12-week-old New Zealand white rabbits (2.6 - 3.6 kg) were randomly assigned to the three groups with 7, 13, and 13 rabbits for Ag0, Ag5, and Ag5C group, respectively. Randomization was achieved using a computerized random sequence generator (www.random.org). The blinding was achieved by an independent investigator, who labeled the samples and assigned the samples to the animals, but did not participate in the data collection and analysis process. After data analysis, the sample assignment was disclosed.

Before surgery, the rabbits were acclimatized for two weeks, housed in the standard conditions and received food and water *ad libitum*.

### 2.3 Surgical techniques

All rabbits received analgesic before surgery via subcutaneous injection of enrofloxacin (8 mg/kg) and carprofen (4 mg/kg). After 30 min, dexmedetomidine (50 µg/kg) and ketamine (10 mg/kg) were injected into the rabbit’s semitendinosus or semimembranosus muscle. Surgery was performed under general inhalation anesthesia with a combination of isoflurane, nitrous oxide, and oxygen.

The right hind leg was shaved and prepared for surgery by scrubbing with a povidone-iodine scrub for three times. The animal was immobilized on its back. A longitudinal incision (about 10 mm in length) was made on the anterior lateral middle third of the tibia. After exposing the bone with blunt dissection, a pilot hole was drilled completely through the tibia. The bone preparation was performed at 800 revolutions per minute.
with continuous external saline cooling\cite{30}. The implant was screwed into the prepared
hole. The pre-assigned sleeve samples were wrapped around the implant and inserted
in the skin site. The finally applied sleeve consisted of four layers. After suturing
the incision with 4-o sutures (Vicryl, Ethicon Inc., Somerville, NJ, USA), the sleeves
were exposed about 2 mm above the percutaneous exit site (Figure 2C). No further
precautions were taken to cover the exit sites, since regular observation in a pilot
study showed that the rabbits did not try to lick or chew the implants.

To recover the rabbit from anesthesia, atipamezole (500 µg/kg) was injected
subcutaneously into the rabbit's neck region. For postoperative pain management
and infection prevention, carprofen (4 mg/kg) and enrofloxacin (8 mg/kg) were
subcutaneously injected into the neck region after 12 and 24 hours.

2.4 Bacterial challenge

After 2 weeks and weekly thereafter, 50 µl of $\sim 2 \times 10^7$ colony forming units (CFU) of
\textit{S. aureus} (ATCC™ 25923) suspension was injected into the percutaneous exit site of the
implants. The bacterial suspension was always freshly prepared before inoculation
by dispersing \textit{S. aureus} ATCC™ 25923 colonies in sterile saline (0.85% NaCl w/v in
water) at a density of 0.60 McFarland standard value and then diluted 10 times. The
concentration of bacterial suspension was confirmed by bacterial counting on agar
plates.

2.5 Clinical evaluation

The general condition of the rabbits such as weight and activity were evaluated weekly
after surgery and the percutaneous exit site of the implant was inspected weekly for
symptoms of infection. During these inspections, the appearance of the exit site and
percutaneous tunnel was registered using a systematic list (Table 1) modified from a
previous study\cite{31}. 
Table 1 | Grading scale for the appearance of the implant exit sites

<table>
<thead>
<tr>
<th>Exit site</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>skin color</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>1</td>
</tr>
<tr>
<td>Pink</td>
<td>2</td>
</tr>
<tr>
<td>Red irritated</td>
<td>3</td>
</tr>
<tr>
<td>Drainage</td>
<td></td>
</tr>
<tr>
<td>No drainage</td>
<td>1</td>
</tr>
<tr>
<td>Little exudate</td>
<td>2</td>
</tr>
<tr>
<td>Accumulated exudate (fluids) around the implant</td>
<td>3</td>
</tr>
<tr>
<td>The dehiscence of wounds around implants</td>
<td></td>
</tr>
<tr>
<td>No dehiscence with skin tightly surrounding the implant</td>
<td>1</td>
</tr>
<tr>
<td>Irregular dehiscence with small exposed wounds</td>
<td>2</td>
</tr>
<tr>
<td>Large irregular dehiscence with large wet wounds</td>
<td>3</td>
</tr>
<tr>
<td>Swelling</td>
<td></td>
</tr>
<tr>
<td>Not obvious</td>
<td>1</td>
</tr>
<tr>
<td>Obvious</td>
<td>2</td>
</tr>
</tbody>
</table>

2.6 Tissue processing and histology

After 6 weeks, all animals were euthanized by an overdose of pentobarbital. The right hind leg was shaved and the skin around the implant exit sites was disinfected with 70% ethanol. The tibia of 2 rabbits from each group was cross-sectioned at 2 cm distance from the implants using an oscillating bone saw. Subsequently, these specimens were immersed in 10% neutral formaldehyde for 48 hours, dehydrated in a graded series of ethanol (70–100%) and then embedded in polymethylmethacrylate (PMMA). After polymerization, thin sections of 10 µm were prepared in a cross-sectional direction of tibia and along the longitudinal direction of the implants using a diamond blade microtome (Leica Microsystems SP 1600, Nussloch, Germany). Three sections of each specimen were stained with methylene blue and basic fuchsin.

For the other animals, the implants were first unscrewed and immersed into 5 ml physiological saline for bacterial counting tests as described in Section 2.7. The percutaneous implant tunnel was maintained by inserting a wooden stick in the hole as left in the cortical bone after removal of the implant. The tissues with the stick, including tibia and skin, were then retrieved again by cutting at 1.5 cm distance from the implant location. After retrieval, all tissues were immersed in 10% neutral formaldehyde for 48
hours, and then in 70% ethanol for at least 2 days. Then, the tissues were decalcified by a decalcification system (TDE 30, Sakura Finetek Europe B.V, Alphen aan den Rijn, the Netherlands) for 7 days and dehydrated in series of ethanol. After the wooden stick was removed, the specimens were immediately embedded in paraffin. Finally, 5 µm-thick sections were cut through the longitudinal axis of the percutaneous pin tract using a Leica RM 2165 Microtome (Leica Biosystems, Wetzlar, Germany). For each sample, two sections were stained with routine hematoxylin and eosin (H&E) staining for general tissue observation and Brown-Brenn modified Gram staining to show the bacteria. For H&E staining, paraffin sections were deparaffinized in xylene, rehydrated to water, stained with Delafield's hematoxylin, dehydrated again to 96% ethanol, and stained with eosin. For Gram staining, paraffin sections were deparaffinized in xylene, rehydrated to 96% ethanol, treated with ammonium ethanol, rinse in water, stained with filtered Hucker-Conn solution, rinsed with water again, treated with Lugol, rinsed in water and dried, treated with 60°C acetate acid ethanol, rinsed with water, and stained with Twort solution. After staining, the sections were sequentially rinsed in ethanol, xylene, and mounted in DPX (BDH Laboratory Supplies, Dorset, UK).

To quantify the bacterial amounts in the tissues, three images were taken at locations close to the subcutaneous connective tissue, muscle, and bone as shown in Fig 6A. Subsequently, the bacterial amount and tissue area were quantified from the images with macros created in Fiji (NIH, Bethesda, MD, USA). The bacterial number was further normalized by the tissue area as: bacterial density = log (total of counted bacteria) / area of tissue in mm²).

2.7 Microbiological evaluation

Immediately after unscrewing of the implants, these implants were immersed in 5 ml saline and vortexed for 30 s, ultrasonically treated for 5 min, and vortexed again for 30 s to dislodge the bacteria [32]. To count the bacterial number, a serial dilution of the suspensions was inoculated on Columbia Blood Agar with 5% Sheep Blood plates (CBP, Becton Dickinson, Franklin Lakes, NJ, USA), which are suitable for all types of bacteria to grow. For suspicious colonies, matrix-assisted laser desorption/ionization with time-of-flight mass spectrometer (MALDI-TOF, Billerica, MA, USA) was further applied for bacterial identification.

2.8 Statistics

All data were reported as mean ± standard deviation and were analyzed by GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, USA) or R version 3.4.3. The animal weight data were compared with two-way analysis of variance (ANOVA). The grading score of clinical symptoms was compared by a linear mixed model using the lme4
library. Based on the data distributions, the quantified bacterial data from tissue slides and implants were compared by Student’s t-test and Mann Whitney test, respectively. Differences were considered statistically significant at $p < 0.05$.

### 3 Results

#### 3.1 Characterization of the sheets

The chitosan/PEO membranes with or without silver were successfully prepared. Ago and Ag5 sheets displayed uniform and defect-free fibrous structures (Figure 2A&B). With the addition of CHX (Ag5C group), the chitosan-based fibers remained intact but the pores between the fibers were observed to be more filled with chlorhexidine (Figure 2C).

![Figure 2](image1.png) **Figure 2** | The scanning electron micrographs of the sleeves made by electrospinning of chitosan-based solutions: (A) Ago group, (B) Ag5 group, and (C) Ag5C group. Scale bar: 1 µm.

![Figure 3](image2.png) **Figure 3** | General conditions. (A) the implant remained percutaneous at sacrifice. (B) The implant exit site at sacrifice. (C) X-ray image of an implant in the tibia at sacrifice. (D) the collected implant with the sleeve. (E) The weight change of rabbits during the test period.
3.2 General observation of the animal's health

All rabbits survived throughout the entire evaluation period, and all implants stayed stable and percutaneous with approximately 12-mm extrusion above the skin surface (Figure 3A-C). However, the soft tissues around the exit-site were swollen, which made that the sleeve did not longer protrude above the skin surface. The presence of the implants did not disturb normal movement of the rabbits, such as walking and jumping. The first week after surgery, none of the rabbits gained weight, but they continuously gained weight from the second week on (Figure 3E). No significant differences between the various implant groups were detected for the growth rate of the rabbits (P = 0.4269). When the implants were finally retrieved, the sleeves were still found to be present and attached to the implants (Figure 3D).

![Figure 4](image)

Figure 4 | The scores of clinical symptoms based on (A) skin color, (B) drainage, (C) dehiscence, and (D) swollen. The sums of scores are shown in (E). A linear mixed model was adopted to analyze the summed scores. Ag0 vs Ag5 was significant at P = 0.0095; whereas Ag5 vs Ag5C was not significant at P = 0.2489.
3.3 Clinical analysis of the percutaneous exit site

The clinical inflammatory symptoms of the percutaneous exit site of the implants were evaluated according to the grading scale listed in Table 1. The total score of infection symptoms was selected to represent the inflammatory status as shown in Figure 4. The inflammatory status at day 14 was a baseline before bacterial challenge. At day 14, 3 out of 7, 4 out of 13, and 3 out of 13 rabbits in Ago, Ag5, and Ag5C groups, respectively, had a score of 4, meaning no inflammation was seen. Further analysis revealed that no statistical difference was found among the three groups \((P = 0.6634)\). After bacterial inoculation, the total score of the infection symptoms increased and all the percutaneous exit sites in the Ago group were infected as depicted in Figure 4. A significant difference existed between Ago group and Ag5 group \((95\%\text{ confidence intervals (CI)}: [0.228, 1.635], P = 0.0095)\) whereas no significance was found between Ag5 group and Ag5C group \((95\%\text{ CI}: [-0.242, 0.935], P = 0.2489)\).

3.4 Histological observation

Light microscopical images of the PMMA embedded specimens are depicted in Figure 5A-C and reveal that the percutaneous part of the implants is surrounded by about 20-mm thick soft tissues. The screw part was found to penetrate the tibial cortices bilaterally. New bone formation was observed around the enossal part of the implants, which was penetrating the medullar cavity. The newly formed bone as well as the cortical bone were in close contact with the implant surface without an intervening fibrous tissue layer. Furthermore, new bone formation was observed at the apical side of the implant. This newly formed bone could easily be discerned from the native bone. Further observations on the HE stained slides showed that the sleeves could be detected as a small gap in between the implant surface and soft tissue (Figure 5A). The tissue response to the implants wrapped with chitosan-based sleeves was similar for all animals. A representative histological section of the paraffin embedded specimens is shown in Figure 5D. The sections indicated that after implant extraction, no residual sleeve material was observed in the implant tunnels. The sleeves remained attached to the implants (Figure 3D). Further, the tissues surrounding the percutaneous passage were found to be composed of a thick layer of the connective tissue, which was infiltrated with inflammatory cells (Figure 5E). The surface of the pin tract was observed to be covered with epithelial cells. The epithelial downgrowth by visual inspection was usually seen up to halfway the pin tract tunnel. However, it never progressed until the bone surface.
Figure 5 | Cross-sections of PMMA embedded tibia with implant from (A) Ag0 group, (B) Ag5 group, and (C) Ag5C group. i: implant, b: bone. (D) Representative microscopic graph of the tissues around the extracted implant after H&E staining. s: skin, m: muscle, b: bone, e: epidermis, c: connective tissue, t: implant tunnel. Scale bar: 5 mm. (E) The magnified image of connective tissue in the square area of D. The arrow head points to the inflammatory cells. Scale bar: 100 µm.
Figure 6 | (A) Bacterial counting based on the Gram staining. Three images from locations near skin, muscle and bone were made and the bacteria were quantified. S: superficial layer, M: middle layer, D: deep layer. Scale bar: left 2 mm, right 50 µm. (B) Bacterial density of each group as evaluated by Gram staining. Statistical analysis was done by Student’s t-test. * indicates $P < 0.05$ between Ago group and Ag5 groups.
3.5 Bacterial density in the percutaneous passage

After Gram staining, the cytoplasm structures were stained in light green and the bacteria were stained in dark purple. The bacterial density in the tissues was quantified by selecting three representative images from the superficial, middle, and deep part of the tissue tunnel (Figure 6A). An average bacterial density of the three parts was further calculated to represent each sample. Figure 6B showed that a significant difference existed between Ag0 group and Ag5 group (P = 0.0223), but not between Ag5 group and Ag5C group (P = 0.6685).

3.6 The microbiological evaluation

Finally, the bacteria attached to the implants were isolated, identified, and quantified. The bacteria were identified by the colony appearance and the MALDI-TOF. Besides the inoculated bacteria i.e. *S. aureus* ATCC 25923, other bacteria, mostly *Enterococcus faecalis* strains and other *S. aureus* strains, were identified. Based on this finding, counting was divided into two categories, i.e. *S. aureus* vs. other bacteria (Figure 7). Significant differences existed consistently between Ag0 group and Ag5 group regarding inoculated *S. aureus* (P = 0.0444), other bacteria (P = 0.0185) and total bacteria (P = 0.0183); whereas no significant differences were found between Ag5 group and Ag5C group for either inoculated *S. aureus* (P = 0.7369), other bacteria (P = 0.3809) or total bacteria amount (P = 0.2739).

![Figure 7](image.png)

Figure 7 | The number of bacteria found on implants using a CFU plating counting method. Statistical difference was compared by Mann Whitney test. * indicated P < 0.05 between Ago group and Ag5 groups.
4 Discussion

The current study focused on evaluating the efficacy of drug-loaded antibacterial sleeves for the prevention of PTI for 6 weeks in vivo. A percutaneous rabbit tibial model was successfully constructed with the use of bacterial inoculation. The obtained data indicate that the silver loaded chitosan-based sleeves can reduce the inflammatory response of the pin tract significantly even after repeated bacterial challenge. However, the addition of chlorhexidine did not improve the antibacterial efficacy of the silver-loaded sleeves.

Several remarks can be made regarding the implant design. First, the 2.5-mm diameter implant screw survived throughout the study period without compromising the mechanical strength of the rabbit tibia, which corroborates with a previous study [14]. Moreover, the screw with a length of 8 mm proved to be capable to stabilize the implants bilaterally. However, in contrast to a previous rabbit study [6], a 5 mm exposure length above the skin surface appeared not to be enough to keep the implant percutaneous as evidenced by the coverage of implants from the swollen soft tissue in our pilot study. This discrepancy may be attributed to the post-operative swelling of the thicker soft tissue layers (estimated around 13-mm) in the anterior lateral tibia, which differs from the thin soft tissue layer of the medial tibia. Therefore, based on this study, we propose that an exposure length of at least equivalent to the thickness of soft tissue is required to keep implants percutaneous.

The observations of the bone response showed that the enossal part of the implant penetrated the tibia bilaterally and kept the implants stable. The cortex at the apical (implant tip) side apparently became noticeably thicker, as confirmed by histology, whereas the cortex on the other side did not change obviously. The thickening of the cortex at the apex of the implants and the upgrowth of bone along the implant surface at the endosteal side of the cortex is probably caused by damage to the periosteum and endosteum during the drilling procedure and implant installation. It is known that the periosteum and specifically the attached cambium layer play an important role in osteogenesis [33]. Moreover, the newly formed bone and the cortical bone were in close contact with the implant surface without an intervening fibrous tissue layer, which explains the stability of the implants. In addition, the bone formation around the screws was also indicative that the release of silver and chlorhexidine from the sheets did not affect the bone healing, which corroborates previous results [34,35]. Although the intramedullary bone formation and close bone-implant contact was not the main interest of the current study, it verified our success in constructing the rabbit percutaneous model.
The baseline of clinical symptoms at day 14 showed that the percutaneous passage did not spontaneously evoke an inflammatory response in all animals. This proved the necessity of bacterial inoculation to initiate infection, which corroborates previous studies [14,27]. After bacterial inoculation, a 100% bacterial infection rate in the control group (Ag0) was verified by visual observation of inflammatory parameters, microbiological, and histomorphometrical tests. The microbiological identification further substantiated that the final bacteria colonizing a wound do not only include inoculated strains (in this case, the S. aureus) but also other environmental bacteria, which again agrees with previous findings [4,14]. Although the infection rate of 100% in the control group confirmed the effectiveness of the bacterial inoculation, we cannot conclude that the inoculated bacteria are solely responsible for the further development of the infection.

Regarding the study results, the inflammatory symptoms at the percutaneous exit site formed the most important parameter to indicate the occurrence of infection. Overall, all rabbits showed inflammation of the percutaneous passage during the study periods after multiple challenge of large quantities of bacteria and the sleeves were extracted together with the implants at the end of the study (Figure 3D). This maintenance of the sleeves may be attributed to the slow degradation of the chitosan-based membranes as evidenced by that the chitosan based membranes were implanted remained intact after subcutaneous implantation for 12 weeks in rabbits [29]. Despite the repeated inoculation, the silver loaded sleeves did show a significantly lower grade of inflammation than the sleeves without silver. Similar decrease in the numbers of colonized bacteria on the percutaneous implants and the surrounding tissues was confirmed by microbiological and histomorphometrical analysis. This antibacterial effectiveness of the silver loaded sleeves can be attributed to the sustained release of silver, as evidenced in our previous silver release study [21] and previous studies from other groups [36-38]. However, the incorporation of silver in biological systems might be risky in the sense that silver ions are released in the cell membrane and thus may lead to cytotoxicity effects, incite immunological responses and disrupt cell growth activity [39-41]. Indeed, silver showed a dose-dependent cytotoxicity in our previous study, but the silver loaded membranes adopted in this study displayed antibacterial property without noticeable cytotoxicity in vitro [29]. Therefore, the lower grade of inflammatory response evoked by the silver loaded sleeves could be attributed to the fewer bacteria present in the wounds surrounding the silver loaded sleeves. Even without additional wound cleaning, the silver loaded sleeves still succeeded to lower the inflammatory response and decrease the number of the colonized bacteria of the pin tract. It can be hypothesized that this effect of such sleeves will even be more evident in the human clinical application, where daily pin care is routinely carried out and bacterial exposure
is much lower [8]. Therefore, this effectiveness encourages further (pre)-clinical evaluation of the silver loaded sleeves.

In contrast, the further post-loading of chlorhexidine on the sleeves failed to enhance the long-term antibacterial ability of the sleeves. Since the chlorhexidine loaded membrane showed antibacterial effect in vitro [21], this failure can most likely be attributed to the way the chlorhexidine was loaded into the membranes. The SEM pictures indicated that the chlorhexidine was stored in the membrane fibers, which resulted probably in the immediate release of chlorhexidine from the sleeves after implant installation [21] and the residual chlorhexidine in the pin tract when the bacteria were inoculated to challenge the wounds. To clarify this effect, further research is needed either by achieving a sustained release of chlorhexidine or by frequently change of the sleeves. Finally, we should acknowledge that our study still has a limitation of lacking pin care routine. This lack of infection control routines together with our repeated inoculation with large amounts of bacteria may be the reason that a complete eradication of the bacteria was not achieved. Therefore, we would suggest that future animal model, like in the human clinical application, should include regular pin care routine.

5 Conclusion

A consistent rabbit tibial model with repeated bacterial challenge was constructed successfully to evaluate antibacterial strategies to prevent PTI. This PTI model was characterized by an 100% infection rate, while the implants maintained stably fixed in the tibial bone, and without the loss of animals. The evidence from inflammation symptoms, microbiological bacterial counting, and histomorphometrical analysis together proved that a silver loaded chitosan sleeve can reduce the inflammatory response of the pin tract compared to a control sleeve without silver. However, the addition of chlorhexidine into the sleeve did not contribute to further reduction of the inflammatory symptoms. Consequently, the silver loaded sleeve seems suitable for further (pre-) clinical exploration.
Chitosan-based sleeves loaded with silver and chlorhexidine in a percutaneous rabbit tibia model

References


Chapter 6

3D printed polycaprolactone scaffolds loaded with silver phosphate and lidocaine for antibacterial and analgesic applications

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1 Introduction

The restoration of the maxillofacial defects is a challenge because of the complicated pre-existing anatomy of the skull [1]. Recently, the advance in 3-Dimensional (3D) printing with computer-aided modeling techniques has enabled to create accurate scaffolds that contour to the patient's anatomical parts based on the 3D reconstruction of Computer Tomography (CT) scanning data [2,3]. Based on the baseline technology, type of machine architecture, and the material transformation physics, 3D printing can be classified into 5 categories, i.e. vat polymerization, powder bed fusion, material extrusion, material jetting and direct energy exposition [3]. Among them, the pneumatic extrusion method (Fig. 1), which is a sub-category of material extrusion, is attractive for biomedical application as it requires only a small amount of materials, i.e. in a few milligrams compared to a few hundred grams for other technologies [3]. After the 3D reconstruction file is loaded to the 3D printer, the object can be concisely printed via controlling the movement of the syringe (x-, y-, z- motion). Subsequently, a viscous or semisolid slurries, made by mixing a solvent and polymer, e.g. alginate, polycaprolactone (PCL), polyglycolic acid (PGA), polylactic acid (PLA) and their copolymer poly(lactic-co-glycolic acid) (PLGA), is loaded into the syringe [4]. The applied pneumatic pressure can continuously extrude a microfilament from the viscous slurry via a fine nozzle. The printability of the slurry is controlled by parameters such as the slurry viscosity, nozzle diameter, the speed of nozzle movement and the pneumatic pressure [5]. The final architecture of the scaffold can be characterized by the shape of the micro-filament diameter, pore size and shape, total porosity, and pore interconnectivity etc. [6].

Such 3D designed polymeric scaffolds can be used to restore and regenerate bone defects. However, maxillofacial defects can originate from or are associated with infection, accompanied by bacterial colonization and inflammation [7]. Despite radical surgical debridement, removal of the infected area is often incomplete [8]. As a consequence, the residual bacteria may attach to the restorative materials to form a biofilm, which are difficult to eradicate [9]. Over the past decades, attempts have been made to prevent and cure infections by incorporating antibiotics into the materials during either primary or revision surgery [10,11]. However, the long-term patient exposure to low doses of antibiotics has a high risk of inducing antibiotic resistance. Alternatively, chemical agents such as silver have been used due to the much lower tendency to give rise to resistance [12]. For instance, silver phosphate (Ag₃PO₄) has been incorporated into polyurethane composites through an in situ forming method and exhibited strong antibacterial effects with satisfactory cytocompatibility [13]. To eradicate the organisms from infected surrounding tissues usually requires the release of effective antibacterial drugs for 3 to 4 days including a high initial burst.
release during the first 2 days \[14\]. In addition to the infection, postoperative pain is an unavoidable symptom. During the first postoperative 24 hours, approximately 40% of the patients reported moderate to severe postoperative pain \[15\]. Although the pain decreased with time, the management of the postoperative pain can be important at home as it will last longer than 24 - 48 hours \[15\]. Currently, the patients usually are administered strong pain killers, like opioids, to relieve the pain \[16\]. Albeit being effective, a large intake of opioids can induce respiratory depression, nausea, and vomiting. Therefore, multimodal analgesic regimens, such as local anesthetic-opioid combinations, have been proposed to control postoperative pain \[17\]. In particular, continuous postoperative application of lidocaine can have a preventive effects on the postoperative pain and reduce opioid consumption \[18\]. The elongation of the duration of analgesia to longer than 24 h might prevent the need for continuous analgesic catheters in some clinical settings \[19\].

Figure 1 | Schematic representation of the direct digital manufacturing process using an air pressure extrusion based additive manufacturing system. (A) A digitally designed 3D object. (B) A slurry is transferred to the printing syringe, sealed with a piston, and then driven by air pressure to form a filament, while the movement of the syringe in various directions controls the printing progress. (C) The final 3D-printed object depicts the original design in A.
To achieve an antibacterial and analgesic effect, it can be advantageous to incorporate both drugs in a bone scaffold, which achieves the drug release rates above the therapeutic thresholds \[3\]. In a microscale polymeric drug delivery systems, the drug transport is primarily driven by diffusion \[20\]. Therefore, an important way to control the drug release is the alteration of the diffusion distance \[21,22\]. Since the diffusion distance can be easily adjusted by modulating the filament diameter of the printed scaffolds, we hypothesize that the drug release rate can be changed by varying the filament diameter of the 3D printed scaffolds. However, no research work on this topic can be found in literature until now.

In this study, we aimed to develop a polycaprolactone (PCL) scaffold loaded with Ag\textsubscript{3}PO\textsubscript{4} to prevent infection and lidocaine for pain relief by one-step 3D printing. PCL was selected due to its biocompatibility and printability by a pneumatic extrusion method \[5,23\]. 3D printed PCL scaffolds loaded with Ag\textsubscript{3}PO\textsubscript{4} and lidocaine were printed in a single step with extrusion nozzles of different sizes. The scaffold morphology, drug release profiles, antibacterial efficacy, and cytotoxicity were further evaluated.

2 Materials and Methods

2.1 Slurry preparation for printing

The slurries for printing were prepared by mixing PCL (M\textsubscript{n} = 80 000 g/mol, T\textsubscript{m} = 60 °C; Shenzhen Esun Industrial Co, Shenzhen, China), lidocaine (Sigma-Aldrich, St. Louis, MO) and Ag\textsubscript{3}PO\textsubscript{4} (Strem Chemicals, Newburyport, MA) in dichloromethane (Kelong Chemical Reagent Factory, Chengdu, China). All chemical reagents used in this research were of analytical quality. The sample groups were named as Drug0, L2, L4, Ag1, Ag3, and Ag3L4 based on the drug (lidocaine and Ag\textsubscript{3}PO\textsubscript{4}): PCL weight ratio (see Table 1). Materials were dissolved for 2 hours and stirred in a fume hood until a filament of around 20 cm in length could be extruded. The shear viscosity of the slurry was further determined by a viscometer (NDJ-9S digital viscometer, Vetus Electronic Technology, Hefei, China) at a rotation speed of 1.5 rpm at 25 °C. A proper shear viscosity of the slurry for the extrusion of filaments had to range from 0.25 to 0.35 Pa•s.
Table 1 | The composition of the slurry for printing in different groups

<table>
<thead>
<tr>
<th>Materials\Group</th>
<th>Drug0</th>
<th>L2</th>
<th>L4</th>
<th>Ag1</th>
<th>Ag3</th>
<th>Ag3L4</th>
</tr>
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<tbody>
<tr>
<td>PCL (g)</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Lidocaine (g)</td>
<td>-</td>
<td>0.2</td>
<td>0.4</td>
<td>-</td>
<td>-</td>
<td>0.4</td>
</tr>
<tr>
<td>Ag3PO4 (g)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.1</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Dichloromethane (mL)</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

2.2 Scaffold fabrication

The scaffolds were printed using a pneumatic extrusion-based 3D printer (Regenovo 3D Bioprinter, Hangzhou, China). The 3D printer was composed of a nozzle-connected printing head (syringe), pneumatic pressure controller and three-axis (x, y, z Cartesian coordinates) linear motion controllers. A digital file of a disc model (15 mm in diameter and 1.5 mm in thickness) was transferred to the printer, as the designed shape for the 3D printed samples. During the printing process, the slurries were delivered through a nozzle (0.21 mm or 0.41 mm in diameter) at a speed of 4 mm/s with a constant pressure of 450 kPa. The slurries were laid down in a layer-by-layer manner to form the scaffolds, which were further labeled with 0.21 and 0.41, respectively. A degree of 90° was set between the lower and upper layers to generate a latticed pattern. The 0.41 scaffolds were printed with an x- or y-motion step of 0.7 mm and a layer thickness of 0.2 mm (z-motion), while the 0.21 scaffolds were printed with an x- or y-motion step of 0.5 mm and a layer thickness of 0.1 mm. The printing was conducted under ambient conditions. After printing, all scaffolds were further dried in the fume hood overnight and then vacuum-dried for another 12 hours.

2.3 Morphological characterization

The morphology of the scaffolds was assessed by scanning electron microscopy (SEM, JEOL 6500LV; JEOL, Tokyo, Japan). All samples were sputter-coated with a thin layer of gold prior to observation. The diameter of the filaments was quantified from four different SEM images at 35× using Fiji software (NIH, Bethesda, MD). The scaffolds were cross-sectioned to further observe the drug status in the filaments. Energy Dispersive X-ray spectroscopy (EDX, Oxford Instruments, Abingdon, UK) was applied for analysis of elemental composition of the filaments. The 3D structure was reconstructed following micro-CT scanning (µCT40, Scanco Medical, Brüttisellen, Switzerland). The samples were scanned at 45 kV and 175 µA with a slice increment of 19 µm. The 3D images were reconstructed from a series of serial 2048 x 2048 bitmap images using Software CTAn and CT Vol Realistic 3D Visualization (Bruker microCT, Kontich, Belgium).
2.4 Drug release

Disk samples (n = 5 of each type) were weighed and placed into 15 mL Falcon™ conical centrifugal tubes containing 10 mL phosphate buffer saline (PBS, Gibco™, Invitrogen Corp., Paisley, Scotland). The tubes were incubated at 37 °C with gentle agitation. At each predetermined time point i.e. after 1, 2, 6, 24, 48, 96, and 168 hours, all supernatants were collected for further analysis and the tubes were refreshed with 10 mL PBS. The concentration of lidocaine was detected by reverse-phase high-performance liquid chromatography (HPLC) according to a previously reported method [24]. The HPLC system consisted of a L-2130 pump (Hitachi, Tokyo, Japan), a L-2400 UV detector (Hitachi), a L-2200 autosampler (Hitachi), and a LiChrospher RP-18 endcapped HPLC column (125 mm x 4 mm, particle size 5 µm, Sigma-Aldrich). For lidocaine detection, the used mobile phase was acetonitrile: ammonium acetate (0.0257 M), pH 4.85 (adjusted with acetic acid) in the ratio of 60/40 (v/v). The injection volume was 20 µL with a flow rate of 0.5 mL/min. The concentration of lidocaine was quantified at 254 nm using a standard calibration curve in the concentration range between 4 and 1000 µg/mL. The silver concentration in the collected supernatants was determined by atomic absorbance spectroscopy (AAS, SpectrAA 220FS, Varian, Palo Alto, CA). The collected supernatant was acidified using 65% nitric acid (Aladdin, Shanghai, China) to a 1% nitric acid solution before AAS analysis. A standard calibration curve was prepared with silver concentrations ranging from 8 to 1000 ng/mL.

2.5 Antibacterial property

Disk samples (n = 3 for each type) were immersed in 1.5 mL of PBS with gentle agitation at 37°C. The supernatants were collected daily for 7 days and stored respectively. Then 1.5 mL fresh PBS was added. Filter papers, 6-mm in diameter, were immersed into the collected supernatants for 30 seconds and subsequently air dried for further use. A zone of inhibition test (modified Kirby–Bauer test) was adopted to evaluate the antibacterial property of the filter papers against Staphylococcus aureus (S. aureus ATCC® 25923™, Manassas, VG) and Escherichia coli (ATCC® 25922™). Standard tablets containing 30 µg of cefoxitin were used as positive control. In brief, a bacterial suspension was prepared by mixing bacterial colonies with sterile saline (0.85% w/v NaCl in water) to an OD value of 0.090 and further diluted for 10 times. The bacterial suspension was smeared evenly on nutrient agar plates (Jiangmen Caring Trading Company Limited, Jiangmen, China). Subsequently, three filter papers from different disk samples were distributed on each agar plate. After incubation for 20 hours at 36°C, the diameters of the transparent zones of inhibition (ZOI) were measured.
The cytotoxicity of the membranes was evaluated by the Cell Counting Kit-8 (CCK-8) assay [25]. All experiments were done by following national guidelines for working with human materials (Dutch Federation of Biomedical Scientific Societies, human tissue and medical research: code of conduct for responsible use. Available at http://www.federa.org/). Before the evaluation, disk samples from each group (n = 3) were pre-immersed in 1.5 mL culture medium at 37 °C for 24 hours with gentle agitation to collect the supernatants. Human Foreskin Fibroblasts (HFFs, isolated from foreskin specimens from a healthy donor by following national guidelines for working with human materials) and MC3T3-E1 osteoblastic cells (ATCC) were cultured in αMEM cell culture medium (Gibco®) supplemented with 10% Fetal Bovine Serum (FBS, Sigma F7524, Taukirchen, Germany) and 1% Penicillin/ Streptomycin (Gibco®) under a 5% CO2 atmosphere at 37 °C. The cells were seeded onto a 24-well plate with a cell density of 20,000 cells per well. After 24 hours, the culture medium in the plate wells was replaced with the collected supernatants or 5% DMSO groups (positive control) to start the evaluation. Again, 20 hours later, the culture medium was refreshed with 1 mL culture medium containing 10 v/v% CCK-8 dye to measure the metabolic activity of the cells. After 2 hours of incubation, a 100 µl aliquot of the metabolized medium from each well was transferred into a 96-well plate in duplicate and the absorbance was measured at 450 nm in a Synergy HTX multimode reader (BioTek Instruments, Winooski, VT, USA).

2.7 Statistics

All data were reported as mean ± standard deviation (SD). The data were analyzed by a two-way ANOVA followed by a Tukey’s multiple comparisons test using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). Furthermore, an independent t-test was performed to compare the cytotoxicity of drug-loaded groups to negative controls. Differences were considered as statistically significant at p < 0.05.

3 Results and Discussion

3.1 The fabrication of the scaffolds

To illustrate the pneumatic extrusion-based 3D printing, a cylindrical plate provided with a pillar was designed and printed (Figure 1). All scaffolds, as used in the various assays, could be without any problems printed using a 0.21 or 0.41 mm-diameter nozzle at a speed of 4 mm/s and a constant pneumatic pressure of 450 kPa under ambient conditions.
3.2 Morphology of the scaffolds

Figure 2 | The structure of 3D printed scaffolds. (A) 0.21 scaffold, (B) 0.41 scaffold. Scale bar: 1 mm.

Figure 2 shows the 3D structure of the scaffolds after micro-CT reconstruction. The scaffolds displayed a porous interconnected structure formed by microfilaments. The scaffolds prepared with the 0.21 or 0.41 mm nozzle could easily be discerned from each other on basis of the filament diameter (Figure 2). Further quantification of micro-CT data demonstrated that the surface area-to-volume ratios of the scaffolds from 0.21 and 0.41 groups were identical, both around 0.15 /pixel. Since drug release is primarily driven by diffusion, the comparable surface areas of the scaffolds formed a sturdy basis for the further comparison on the effect of the filament diameter. The quantification of the filament diameter from SEM images (Table 2) confirmed that: (1) filament diameter of the 0.21 and 0.41 scaffold group was indeed different, i.e. ~ 73 µm and 144 µm, and (2) no significant differences existed in filament diameter among the different drug loaded groups printed with the nozzle of the same size. The observed discrepancy between filament diameter and the internal diameter of the nozzles may be due to the fluidal characteristics of the dissolved polymer. As soon as the polymer slurry leaves the tip of the nozzle, it will flow to a wider diameter till the solvent is completely vaporized and the polymer is stabilized again. This flow can even become increased by the constant pneumatic pressure in the delivery of the slurry.
Table 2 | The filament diameters of scaffolds from different groups.

<table>
<thead>
<tr>
<th></th>
<th>0.21</th>
<th>0.41</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug0</td>
<td>72.2 ± 6.8 µm</td>
<td>136.3 ± 13.2 µm</td>
</tr>
<tr>
<td>L2</td>
<td>74.5 ± 7.1 µm</td>
<td>133.2 ± 16.5 µm</td>
</tr>
<tr>
<td>L4</td>
<td>72.7 ± 8.3 µm</td>
<td>146.0 ± 16.7 µm</td>
</tr>
<tr>
<td>Ag1</td>
<td>73.6 ± 6.5 µm</td>
<td>150.6 ± 17.8 µm</td>
</tr>
<tr>
<td>Ag3</td>
<td>74.0 ± 6.1 µm</td>
<td>152.9 ± 17.7 µm</td>
</tr>
<tr>
<td>Ag3L4</td>
<td>72.8 ± 7.2 µm</td>
<td>143.8 ± 14.6 µm</td>
</tr>
</tbody>
</table>

Figure 3 | The morphology of the scaffold and the elemental distribution of lidocaine and Ag₃PO₄. The nitrogen was mapped in green to represent the lidocaine and the silver was mapped in red to represent Ag₃PO₄. Scale bar: 250 µm.
The distribution of the lidocaine and Ag₃PO₄ throughout the filaments was analyzed by SEM-EDX, which confirmed that filaments contained silver (in red) and nitrogen (in green, indication of lidocaine). Elemental mapping demonstrated an even, spot-like distribution of the silver and lidocaine over the filaments (Figure 3). The cross-section of the scaffolds (Figure 4) showed that Ag₃PO₄, confirmed by EDX, embedded in PCL as microparticles, which still existed after 7-day immersion in PBS. No lidocaine particles were found in the lidocaine groups (Figure 4B), indicating that lidocaine was dissolved by the organic solvent and dispersed in the filaments.

Figure 4 | The cross-section of filament from (A) Drug0 group, (B) L4 group, (C) Ag3 group, and (D) Ag3 group after 7-day immersion in PBS. (E) The energy dispersive spectrum from # area in C. Scale bar: 10 µm.
3.3 The drug release profiles

Figure 5A&B shows the release profile of the lidocaine from the various scaffolds. When the scaffolds were loaded only with lidocaine, the majority of the lidocaine was released in 6 hours and 24 hours from 0.21 groups (0.21L2 and 0.21L4) and 0.41 groups (0.41L2 and 0.41L4), respectively. These results corroborate with the principles of diffusion-driven drug release. A decrease in filament diameter reduces the diffusion distance of the drugs. Notably, the dual loading of lidocaine and Ag₃PO₄ resulted in a delayed release of lidocaine at 48 and 96 hours for the 0.21Ag3L4 group and the 0.41Ag3L4 group, respectively. Since the postoperative pain is greatest during the first 24-48 hours and will decrease with time [15] and the lidocaine-loaded scaffolds displayed a controlled release of lidocaine within 4-7 days, these scaffolds may well be a candidate to control postoperative pain. As the diameter of the nozzles, used in this study, did have the most commonly used nozzle diameters, i.e. 0.2 to 0.4 mm, for pneumatic extrusion [26], it is possible to further control and optimize the lidocaine release by using larger custom-made nozzles. Such larger tip diameters may also be beneficial to deliver other bioactive compounds for biomedical applications.

The silver release profiles are displayed in Figure 5C&D. Generally, the silver showed a sustained release pattern for all silver-loaded groups. The sustained silver release profiles indicate that the Ag₃PO₄ loaded scaffolds can work as a depot to release silver ions in a long-term, i.e. at least 7 days, which may be paramount to exert long-term antibacterial functions. Comparing the 0.21 with 0.41 groups, i.e. 0.21Ag1 vs 0.41Ag1 and 0.21Ag3 vs 0.41Ag3, no significant difference was found. This suggests that filament diameter did not play a key role in the silver release. Instead, comparison of the scaffolds with different Ag₃PO₄ amounts, the Ag1 groups (0.21Ag1 and 0.41Ag1) showed a significantly faster release rate than the Ag3 groups (0.21Ag3 and 0.41Ag3) but fewer silver amounts were released from the Ag1 groups than from Ag3 groups. Moreover, the Ag3 groups (0.21Ag3 and 0.41Ag3) showed a similar release profile with Ag3L4 groups (0.21Ag3L4 and 0.41Ag3L4). This result indicates that the further incorporation of lidocaine did not affect the silver release. Therefore, the data indicate that the silver release is correlated with the initial amount of Ag₃PO₄ as loaded in the scaffolds instead of the filament diameter. This discrepancy between lidocaine and silver release may be attributed to the different status of the drugs in the filaments, i.e. lidocaine was dispersed as molecules while Ag₃PO₄ was embedded as microparticles in the filaments (Figure 4). When the scaffolds were immersed in the release medium, lidocaine molecules are ready to be spread out, but the release was limited by the diffusion distance, which is related to the filament diameter. In contrast, the silver ions need to be firstly dissolved from Ag₃PO₄ particles and then diffused out of the filaments. The dissolution of silver ions from the Ag₃PO₄ particles is slow [13] and thus
dominated the silver release. As such, the silver release was mainly correlated with the Ag₃PO₄ loading amount instead of the filament diameter.

In this study, PBS was used as the release medium considering that PBS is the most simple and common medium for the release kinetics studies. However, the scaffolds will be subjected to completely different conditions when implanted into the body tissues, where the released drug is continuously diluted and undergoes protein binding. As a consequence, different drug release behavior and performance can occur under in vivo conditions than observed in the in vitro release studies [27]. Therefore, despite the promising drug release profiles, data must be interpreted with care and in vivo studies are necessary to confirm the obtained data and prove the clinical potential of the designed scaffolds.

Figure 5 | The drug release profiles of the lidocaine (A&B) and silver (C&D).

3.4 The antibacterial efficacy

*S. aureus* and *E. coli* are two representatives of common gram-positive and gram-negative bacteria in infected wounds. The results of the zone of inhibition test against these two bacteria are shown in Figure 6. Only the samples containing silver showed a transparent ZOI (Figure 6A). The ZOI against *S. aureus* was larger than against *E. coli*, which indicates that silver has a stronger effect against gram-positive bacteria than gram-negative bacteria (Figure 6B&C). This is confirmed by Taglietti et al., who reported that the extra thick peptidoglycan layer of the cell wall in the gram-positive bacteria prevents the penetration of silver [28]. No significant differences in antibacterial effect were found between the silver-loaded groups (Figure 6B&C). In addition, the
comparable antibacterial efficacy of Ag3 and Ag3L4 groups (Figure 6B&C) indicates that the loading of lidocaine did not affect the antibacterial efficacy of the silver-loaded scaffolds. Furthermore, the release media of Ag1 and Ag3 groups were further evaluated to 7 days (Figure 6D&E). No significant difference was found between 0.21 and 0.41 groups. As expected, the Ag3 groups showed a larger zone of inhibition than Ag1 groups. At day 7, the released medium from Ag1 groups lost its antibacterial efficacy, while the Ag3 groups remained effective. Evidently, the silver-loaded scaffolds proved that the clearance of organisms from infected tissues usually requires the sustained release of effective antibacterial drugs for 3 to 4 days [14]. Therefore, under the current experimental conditions, the antibacterial properties of the silver-loaded scaffolds seem suitable to prevent infections.

Figure 6 | The antibacterial results. (A) the macroscopic images of zone of inhibition of the released media collected at day 1. The diameter of the samples is 6 mm. (B&D) showed the ZOI against S. aureus, while (C&E) showed that against E. coli.
3.5 The cytotoxicity of the scaffolds

Since higher concentrations of silver can induce cytotoxicity [12], cytotoxicity of the samples was further tested. The cytotoxicity of the scaffolds was evaluated by the CCK-8 assay to evaluate the metabolic activity of HFFs and MC3T3 cells. Drug0 and 5% DMSO groups were set as negative and positive control, respectively. The results are shown in Figure 7. The 5% DMSO group resulted in a significant lower OD value for both cell types than the Drug0 group, which validated the test. No significant difference in cytotoxicity was found between 0.21 and 0.41 groups. Regarding the drug loading amounts, the lidocaine- or silver-loaded groups did not separately show any significant difference in cytotoxicity compared to the negative control, which indicates that the released lidocaine and silver ions after 24-hour immersion did not reach the toxic threshold to HFFs as well as MC3T3 cells. The therapeutic threshold of lidocaine is around 1.5 – 6.0 µg/mL [29,30]. Based on the release study, the released lidocaine from the L4 group was estimated to be around 800 µg/mL, which is far higher than the therapeutic threshold and thus implies the potential to control pain. In contrast, the Ag3L4 groups had a significant cytotoxic effect on the MC3T3 cells but not on HFFs. We hypothesize that this cytotoxic effect of Ag3L4 scaffolds to MC3T3 cells is because the released lidocaine and silver ions can increase the osmotic pressure of the culture medium and the accumulation of lidocaine and silver ions from Ag3L4 groups reached a tolerant hypertonic threshold to MC3T3 cells, but not to HFFs. This hypertonic condition is unlikely to happen in vivo due to the continuous flow and circulation of body fluids. Therefore, in vivo studies can now be designed to further elucidate the appropriate doses to achieve an appropriate antibacterial effect to prevent infections as well as analgesic efficacy to control the postoperative pain.

Figure 7 | The cell viability test of the scaffolds. (A) The results of human foreskin fibroblast. (B) The results of MC3T3 E1 clone 4 cell line. ** indicates \( P < 0.01 \) and *** indicates \( P < 0.001 \) compared to Drug0.
4 Conclusion

The technological breakthrough of computer and information technology facilitates the fabrication of custom-made products based on computer-assisted design. Ag$_3$PO$_4$ and lidocaine loaded PCL scaffolds were printed using a pneumatic extrusion-based 3D printing. The scaffolds showed a porous structure with high fidelity. The delivery of lidocaine could be controlled by adjusting the filament diameter while that of silver release is correlated to the Ag$_3$PO$_4$ loading amount. The lidocaine was released in a controlled manner during 4-7 days and resulted in a concentration far above the therapeutic level of lidocaine. Moreover, the Ag$_3$PO$_4$ incorporation showed a sustained silver release profile and enhanced the antibacterial properties of the scaffolds for at least 6 days. Although the Ag$_3$L$_4$ scaffolds showed a toxic effect to MC$_3$T$_3$ cells, which can be due to the hypertonic effects of the dual-released lidocaine and silver, the lidocaine or Ag$_3$PO$_4$ loaded scaffolds alone did not evoke cytotoxicity to human foreskin fibroblasts and MC$_3$T$_3$ cells. Therefore, pneumatic extrusion-based 3D printing can provide a practical way to fabricate drugs loaded scaffolds. In vivo explorations can now be performed to validate the clinical efficacy and safety of the scaffolds.
References


Chapter 7

Summary, closing remarks and future perspectives
1 Summary

The application of biomaterials in the medical field has contributed to the development of medical devices that can support the functional loss of organs and improve the quality-of-life of the patients. However, biomaterials also attract microorganisms and produce niches for infection, which may eventually lead to the failure of the device. To combat biomaterials-associated infections, antibacterial agents can be incorporated into biomaterials to enhance their antibacterial properties. Silver, as a common antibacterial agent, has attracted research interest due to its superior antibacterial property and little tendency to induce resistance. To date, only several studies are available that evaluate the antibacterial efficacy of silver incorporated biomaterials in vivo. Besides, to evaluate the antibacterial efficacy of biomaterials, valid and reliable experimental animal infection models have to be available for the dedicated testing of medical devices. To this end, this thesis aimed to explore the efficacy in the use of silver to enhance the antibacterial property of polymeric biomaterials for several biomedical applications including wound dressing, percutaneous pin, guided tissue engineering membranes, and scaffolds for repairing critical-sized bone defects. Chapter 1 provides a general introduction of biomaterials-associated infections and the current status of research in the use of silver to enhance the antibacterial properties of biomaterials. This summary briefly describes the main findings of the research questions that were addressed in this thesis.

1 Which animal models in literature are available to evaluate the antibacterial properties related to percutaneous devices?

A reliable animal model is a prerequisite for the further evaluation of biomaterials. Percutaneous devices are among the most complicated infection situations in the clinic. Chapter 1 focuses on the construction of animal models for percutaneous device-related infections, and specifically the role of inoculation of bacteria in such models. Infections around percutaneous devices, such as catheters, dental implants, and limb prostheses, are a recurrent and persistent clinical problem. To promote the research on this clinical problem, the establishment of a reliable and validated animal model would be of keen interest. In this review, literature related to percutaneous devices was evaluated, and special attention was paid to studies involving the use of bacteria. The design of percutaneous devices, the susceptibility of various animal species, bacteria strains, amounts of bacteria, and method of inoculation, as well as the methods for subsequent evaluation of the infection, are discussed in detail. Given that an ideal animal model for percutaneous device related infection study is still not existent, this article presents the basis for the construction of such a standardized animal model for percutaneous device-related infection studies. The inoculation of
bacteria is critical to obtain an animal model for standardized studies for percutaneous
device-related infections.

2 Do silver incorporated membranes hold potential for application in guided tissue/
bone regeneration?

The incorporation of silver nanoparticles into biomaterials is not an easy task and
whether the incorporated silver nanoparticles can enhance the antibacterial property
without compromising the biocompatibility of the biomaterial is questionable. Chapter
3 aimed to evaluate the antibacterial potential and biological performance of silver
nanoparticles in chitosan-based membranes. Electrospun chitosan/poly (ethylene
oxide) membranes with different amounts of silver nanoparticles were evaluated for
antibacterial properties and cytotoxicity *in vitro* and for tissue response in a rabbit
subcutaneous model. The nanoparticles displayed dose-dependent antibacterial
properties against *Porphyromonas gingivalis* and *Fusobacterium nucleatum*,
without showing noticeable cytotoxicity. The membranes with silver nanoparticles
evoked a similar inflammatory response compared to the membranes without silver
nanoparticles. The antibacterial effect, combined with the findings on cyto- and
biocompatibility warrants further investigation to the usefulness of chitosan/ poly
(ethylene oxide) membranes with silver nanoparticles, for clinical applications like
guided tissue regeneration.

3 Possess silver incorporated membranes antibacterial properties and wound
healing ability when used as wound dressing?

Silver nanoparticles are widely explored to enhance the antibacterial properties of
medical devices, such as wound dressings. However, no consensus has been reached
on the efficacy and safety of silver nanoparticle incorporation. Chapter 4 aimed (1)
to elucidate the effect of proteins and inorganic ions on the antibacterial properties,
(2) to confirm the antibacterial efficacy *in vivo*, and (3) to evaluate the wound healing
ability of silver nanoparticles incorporation into chitosan-based membranes. Three
membranes with different amounts of silver nanoparticles were prepared. The
antibacterial properties and silver release profile were evaluated after interacting with
phosphate buffered saline or with serum *in vitro*. The antibacterial efficacy and the
wound healing ability was explored in rats. The results indicated that the biological
environment had strong influences on the silver release: the inorganic ions resulted
in a slow release whereas the proteins formed a barrier to block the silver release.
Consequently, a high amount of silver nanoparticles incorporation was necessary to
achieve *in vivo* antibacterial effects. Moreover, the addition of silver nanoparticles did
not alter the wound healing rate and tissue response. We can conclude that the silver
Summary, closing remarks and future perspectives

Nanoparticle incorporation can enhance the antibacterial efficacy of biomaterials while having good wound healing ability. Therefore, the silver incorporated chitosan-based membranes are potential as antibacterial wound dressings.

4 Can silver nanoparticles incorporated in chitosan-based membranes effectively prevent infection as pin sleeves?

Various strategies have been explored to prevent pin tract infections (PTI), including the use of antibacterial sleeves. However, a validated and reliable animal model to evaluate the efficacy of antibacterial strategies is still lacking. Chapter 5 aimed to construct an animal model with a consistent induction of infection after bacterial challenge. Further, the efficacy of silver and chlorhexidine loaded chitosan sleeves was evaluated to prevent PTI around a percutaneous implant. Titanium pins wrapped with sleeves were implanted in the anterior lateral rabbit tibia. After two weeks, *Staphylococcus aureus* suspensions ($1 \times 10^6$ CFU) were injected weekly to the exit site, and the clinical infection status was recorded. After six weeks, all rabbits were euthanized to evaluate the bacterial colonization microbiologically and histomorphometrically. Results showed that the implant screw bilaterally penetrated the tibia and kept the implant stable. A rod length of twice the thickness of the soft-tissue layer was necessary to maintain the percutaneous penetration of the implants. A 100% infection rate was obtained by the bacterial inoculation. Silver loaded sleeves reduced significantly the bacterial density and reduced the inflammatory symptoms of the percutaneous pin tract. However, the addition of chlorhexidine to the sleeves had no added value in terms of further reduction of bacteria and inflammation. In conclusion, a consistent animal model was designed to evaluate strategies to prevent PTI. In addition, the use of silver loaded chitosan sleeves can be pursued for further (pre-)clinical exploration for the prevention of PTI.

5 Can silver and lidocaine be loaded into materials by 3D printing technique to prevent bacterial infection as well as provide pain relief (Chapter 6)?

Pneumatic extrusion-based 3D printing can be used to fabricate custom-made scaffolds to restore irregular bone defects. During the 3D printing process, therapeutic agents can be added to the scaffolds. This study aimed to develop a polycaprolactone (PCL) scaffold loaded with Ag₃PO₄ to prevent infections and lidocaine for pain relief by one-step 3D printing. We hypothesized that the drug release could be controlled by varying the filament diameter of the 3D printed scaffolds. To this end, polycaprolactone (PCL) slurry mixed with different amounts of silver phosphate and lidocaine was printed via differently sized nozzles. The obtained cylindric scaffolds displayed a porous interconnected microstructure with high fidelity. The Ag₃PO₄ and lidocaine were
distributed homogeneously. The lidocaine release could be controlled by adjusting the filament diameter while the silver release is correlated with the Ag₃PO₄ loading amount. The released medium from silver-loaded scaffolds exhibited an obvious inhibition zone against *Staphylococcus aureus* and *Escherichia coli* upon loading with 1% Ag₃PO₄ for up to 6 days and with 3% Ag₃PO₄ for at least 7 days. Cytotoxicity of all scaffolds was screened by cell assay. In conclusion, the pneumatic extrusion-based 3D printing provides a practical technique to fabricate drug-loaded scaffolds. The Ag₃PO₄ and lidocaine loaded PCL scaffolds showed the potential for infection prevention and pain relief.

### 2 Closing remarks and future perspectives

This thesis explored the use of silver to enhance the antibacterial efficacy of polymeric biomaterials both *in vitro* and *in vivo*. The silver loaded polymers displayed a sustained release of silver ions and correspondingly exhibited prolonged antibacterial properties against anaerobic gram-positive and gram-negative bacteria (Chapter 3) as well as aerobic gram-positive and gram-negative bacteria (Chapter 4, 5, and 6). The silver loaded chitosan-based membranes did not evoke a stronger subcutaneous inflammatory response compared to the membranes without silver (Chapter 3) and did not alter the cutaneous wound healing ability of skin wounds (Chapter 6). Nonetheless, since the design of medical devices is determined by their application site, the antibacterial efficacy of biomaterials has to be assessed for a specific application as well as situation in which the biomaterials are used [1]. Therefore, this thesis is an initial step towards the translation of the use of the silver to enhance the antibacterial effect of biomaterials for use in medical devices. More research has to be done to determine the final benefit of silver-based biomaterials and their human clinical application.

When biomaterials are placed or in contact with tissues of the body, a number of reactions to the materials may be seen over time. Some of these reactions may constitute the determinants of the functionality of the biomaterials. Regarding the silver incorporated polymers, the biological environment had a strong influence on the silver release, i.e. the inorganic ions resulted in a slow release, whereas the deposited proteins as present in the body fluids formed a barrier to block the silver release. Consequently, a higher amount of silver nanoparticles incorporation was necessary to achieve *in vivo* antibacterial effects (Chapter 4). To date, reliable methods to assess the antibacterial efficacy of the incorporated silver, as the possible the effect of biological factors, like the various ions and proteins present in the body fluids, are still unknown. Since the composition of body fluids varies, the proper characterization and quantification of silver in relevant medium and *in situ* (inside cells/organisms) is
of paramount importance for final medical application [2]. Lack of such assessments probably explains (at least partially) the differences between experimental results in literature and difficulties in the interpretation of the obtained data. We propose that a proper relation between analytical techniques and biological studies needs to be established. For instance, a balanced system that is suitable for the growth of both bacteria and cells has to be constructed to evaluate the antibacterial efficacy as well as cell viability of the modified biomaterials in similar conditions.

A major task of translational medicine is the “effective translation of the new knowledge, mechanisms, and techniques generated by advances in basic science research into new approaches for prevention, diagnosis, and treatment of disease is essential for improving health” [3]. In this process, animal models serve as a surrogate for patients to evaluate the effectiveness and safety of novel diagnostic and therapeutic advances. Currently, animal models remain a unique source of in vivo information and the irreplaceable link between in vitro studies and final application in patients [4]. Regarding silver-related research, there is the drive to develop novel silver forms such as silver nanoparticles or silver-based materials, but much less research has been done on the in vivo evaluation of the effectiveness and safety of these newly developed materials. A reliable animal model is of great value to the evaluation of the effectiveness and safety of the biomaterials. Since no ideal infection model related to percutaneous devices was available, a review was conducted to figure out the paramount factors of such an animal model (Chapter 2). Subsequently, a valid and reliable rabbit model was constructed based on this review and the applied silver loaded sleeve seems suitable for further (pre-) clinical exploration (Chapter 4). Certainly, the validation of infection-resistant biomaterials for effective downstream translation can only be obtained in human clinical trials, and unfortunately, this is often where the development of an antimicrobial biomaterial terminates due to the large, costly, and lengthy requirements in clinical trials [5]. For instance, to lower the incidence rate of infections for total hip arthroplasties from 2% to 1%, around 5000 patients are needed to demonstrate the 50% reduction of infection incidence based on a power calculation with \( P < 0.05 \) [5]. Moreover, when medical devices intend to enter the U.S. market through a Food and Drug Administration (FDA) regulatory, these devices will be first classified into three classes according to the Medical Device Amendments of 1976 to the Federal Food, Drug, and Cosmetic Act (the act). Class I and II devices only need to undergo review for substantial equivalence to devices that are already on the market, also called pre-amendment devices. In contrast, Class III devices have to undergo a more rigorous premarket approval (PMA), the only pathway that requires clinical data [6]. In this thesis, we explored the effect of the incorporation of silver for GTR membranes, wound dressings, and pin sleeves. Since the membranes will not be implanted permanently, these applications will probably be classified as class II
devices. Regarding the class I and II devices, animal experiments can be the last validation step before human application. Therefore, the decisions on the choice of a relevant animal model and the design, execution, and evaluation of the experiments are of pivotal importance for the effectiveness and safety. When the animal models related to percutaneous devices were reviewed, the animal species, the strain of bacteria, amount and method of inoculation, and the methods for subsequent evaluation of the infection were quite variable, which makes it difficult to compare the different studies. If the animal models can be standardized, the results will be better comparable and more valuable information may be extracted through systematic reviews. Finally, this will benefit the progress in the biomedical field.

Currently, silver has been applied as antibacterial agent in various forms such as silver compounds and silver nanoparticles [10]. The silver incorporated membranes displayed a dose-dependent antibacterial property and cytotoxicity (Chapter 3). Together with other studies [7,8], a therapeutic window, which can display antibacterial property without being toxic, is existing. While this therapeutic window provides a basis for the further exploration of the silver for biomedical applications, the risk of toxicity should not be neglected. The further exploration to control the delivery of silver by incorporating silver phosphate into polycaprolactone via 3D printing resulted in a controlled release of silver ions and sustained antibacterial effect for at least 6 days (Chapter 6). Therefore, the most advantageous property of silver agents, such as silver nanoparticles and silver phosphate particles, seems to be that the incorporated silver per se act as depots and show a sustained release of silver ions. To make full use of the silver, a precise control over the delivery of silver may be achieved through responsive controlled release techniques in the future. Varieties of drug release systems are constructed such as exogenously-triggered (thermo-responsive, light-responsive, and ultrasound-responsive), endogenously-triggered (redox-responsive, oxidation-responsive, and pH-responsive), and enzyme-triggered drug release [9]. For instance, when the silver can be incorporated into an ultrasound-responsive release system, the sustained release of silver can be triggered by ultrasound exposure upon the existence of infection symptoms. Overall, the use of silver to enhance the antibacterial properties of the biomaterials are quite promising. By precisely controlling the delivery of silver, antibacterial biomaterials can be of excellent benefit to prevent the occurrence of infections associated with medical devices.

In summary, the use of silver to enhance the antibacterial properties of biomaterials has been shown a promising potential for the prevention of infections. Although only several applications, including cutaneous wound dressing, percutaneous pin sleeves, and subcutaneous membranes and scaffolds, were explored in this thesis, the performed studies have laid down a firm basis for the further exploration of the use of silver incorporated biomaterials for antibacterial application.
References


Samenvatting, afsluitende opmerkingen en toekomstperspectief
Samenvatting

Biomaterialen worden in de medische sector gebruikt voor allerlei toepassingen om verloren weefsels en organen te vervangen, en daarmee de kwaliteit van leven van patiënten te verbeteren. Biomaterialen kunnen echter ook micro-organismen aantrekken en een haard van infectie vormen, waardoor een medisch hulpmiddel uiteindelijk kan falen. Om infecties rond biomaterialen te bestrijden, kunnen antibacteriële stoffen worden toegevoegd. Zilver wordt daarvoor bijvoorbeeld algemeen gebruikt. Zilver staat in de aandacht dankzij superieure antibacteriële eigenschappen, en de zeer lage waarschijnlijkheid van het optreden van resistentie. Op dit moment zijn er echter niet veel in vivo studies beschikbaar die de antibacteriële werking van zilver toegevoegd aan biomaterialen evalueren. Om de antibacteriële werking te evalueren, is het bovendien belangrijk dat er valide en betrouwbare diermodellen komen die beschikbaar specifiek gericht zijn op het testen van medische hulpmiddelen.

In dit proefschrift werd dan ook getracht om de effectiviteit van het gebruik van zilver te bestuderen, als antibacteriële stof toegevoegd aan polymere biomaterialen, in diverse toepassingen; zoals wondbedekkers, percutane fixatie pinnen, membranen voor weefselgeleiding, en dragermaterialen voor het repareren van kritische bot defecten. Hoofdstuk 1 geeft een algemene inleiding over infecties die geassocieerd zijn met biomaterialen, en de huidige stand van onderzoek op het gebied van het gebruik van zilver als antibacterieel middel. Deze samenvatting behandelt vervolgens de belangrijkste uitkomsten van de onderzoeksvragen zoals die in het inleidende hoofdstuk gesteld werden voor dit proefschrift.

1 Welke diermodellen zijn er in de wetenschappelijke literatuur beschikbaar om de antibacteriële eigenschappen te evalueren rond percutane implantaten?

Een betrouwbare diermodel is een voorwaarde om biomaterialen te kunnen evalueren. Percutane implantaten vormen daarbij de meest gecompliceerde infectueuze klinische situaties. Hoofdstuk 2 focuseert op het construeren van diermodellen voor infecties bij percutane implantaten, and specifiek op de rol die het inoculeren van bacteriën kan hebben in zulke modellen. Infecties rond percutane implantaten, zoals katheters, tandheelkundige implantaten, en ledemaatprothesen, vormen een continu en hardnekkig klinisch probleem. Het opzetten van betrouwbare en gevalideerde diermodellen is daarmee van groot belang. In dit overzichtsartikel, wordt de literatuur over percutane implantaten geëvalueerd, waarbij in het bijzonder aandacht werd gegeven aan studies die bacteriën gebruikten. Het ontwerp van de percutane implantaten, de vatbaarheid van verschillende species, de bacterie stammen,
de hoeveelheid bacteriën, en de inoculatie methode, zowel als de methode van uiteindelijke evaluatie van de infectie, worden allen in detail besproken. Gegeven dat een ideaal diermodel voor infecties gerelateerd aan percutane implantaten nog niet bestaat, geeft dit hoofdstuk de basis voor het opzetten van zo'n gestandaardiseerd diermodel. Het blijkt dat het inoculeren van bacteriën daarbij in elk geval van kritisch belang is.

2 Hebben membranen waarin zilver is opgenomen potentieel voor toepassingen in geleide weefsel / bot regeneratie

Het opnemen van zilver nanopartikels in biomaterialen is makkelijker gezegd dan gedaan, en het is nog maar de vraag of de zilver nanopartikels de antibacteriële eigenschappen kunnen verbeteren zonder dat daarbij de biocompatibiliteit in het geding komt. **Hoofdstuk 3** beoogde om het antibacteriële potentieel en het biologische gedrag van zilver nanopartikels te evalueren in membranen, die waren gebaseerd op chitosan. Chitosan/ poly (ethyleen oxide) membranen, vervaardigd met elektrospinnen, en voorzien van verschillende hoeveelheden zilver nanopartikels werden beschouwd op antibacteriële eigenschappen en cytotoxiciteit *in vitro*, en op weefsel response in een subcutaan konijnenmodel. De resultaten voor de nanopartikels lieten zien dat de antibacteriële eigenschappen afhankelijk waren van de dosering, werkzaam waren tegen *Porphyromonas gingivalis* en *Fusobacterium nucleatum*, en geen merkbare cytotoxiciteit vertoonden. De membranen met de zilver nanopartikels lieten een vergelijkbare ontstekingsreactie zien ten opzichte van membranen zonder zilver nanopartikels. In conclusie kan gezegd worden dat het antibacteriële effect, in combinatie met de bevindingen over cyto- en biocompatibiliteit zou moeten leiden tot verder onderzoek naar de bruikbaarheid van chitosan/ poly (ethyleen oxide) membranen met zilver nanopartikels, voor klinische toepassingen zoals geleide weefsel regeneratie.

3 Beschikken membranen met zilver over antibacteriële eigenschappen en stimuleren zij wondgenezing bij toepassing als een wondbedekker?

Zilver nanopartikels worden algemeen gebruikt om de antibacteriële eigenschappen van medische hulpmiddelen te verbeteren, bijvoorbeeld bij wondbedekkers. Er is echter zeker geen consensus over de werkzaamheid en veiligheid van de opgenomen nanopartikels. **Hoofdstuk 4** probeerde om (1) het effect te verduidelijken dat proteïnen en anorganische ionen hebben op de antibacteriële eigenschappen, (2) de antibacteriële werkzaamheid te bevestigen in een *in vivo* model, en (3) de mogelijkheid tot wondhealing rond de chitosan membranen met zilver nanopartikels te bestuderen. Er werden drie membranen gemaakt, met verschillende hoeveelheden
zilver nanopartikels. De antibacteriële eigenschappen en het profiel warmee het zilver vrijkwam, werden in vitro geëvalueerd, tijdens interactie met een fosfaatgebufferde zoutoplossing of met serum. De antibacteriële werkzaamheid en de wondheling werden ook onderzocht in ratten. De resultaten lieten zien dat de biologische omgeving het vrijkomen van zilver sterk beïnvloedde: de anorganische ionen zorgden namelijk voor een langzaam afgifteprofiel; terwijl de proteïnen een barrière vormden, die het vrijkomen in zijn geheel blokkeerde. Dientengevolge is een hoge hoeveelheid zilver noodzakelijk om ook in vivo antibacteriële effecten te kunnen bewerkstelligen. Het toevoegen van zilver nanopartikels veranderde de wondgenezing of het weefselgedrag niet. Daaruit kon geconcludeerd worden dat het toevoegen van zilver nanopartikels de antibacteriële werking van biomaterialen kan bevorderen, terwijl er nog steeds een adequate wondheling plaatsvindt. Chitosan membranen met toegevoegde zilver nanopartikels hebben potentieel als antibacteriële wondbedekkers.

4 Kunnen chitosan membranen met zilver nanopartikels infectie voorkomen, wanneer deze worden toegepast als een hoesje rond fixatie pinnen?

Voor het voorkomen van infectie rond fixatie pinnen (PTI) worden verschillende strategieën onderzocht, zoals het gebruik van antibacteriële hoesjes. Een gevalideerd betrouwbare diermodel, om de werkzaamheid van antibacteriële methoden te onderzoeken, ontbreekt echter. Hoofdstuk 5 probeerde een diermodel te ontwikkelen, met een consistente vorming van infectie na het toevoegen van bacteriën. Vervolgens werd de werkzaamheid van chitosan hoesjes met zilver en chloorhexidine bestudeerd, om PTI rond een percutaan implantaat te voorkomen. Titanium pinnen, bedekt met hoesjes, werden anterior lateraal geïmplanteerd in konijnten Tibia. Vanaf 2 weken, werden Staphylococcus aureus suspensies (1 × 10^6 CFU) wekelijks geïnjecteerd op de percutane passage, en werd de klinische infectie status vastgelegd. Na 6 weken, werden de konijnen geofferd om de bacteriële kolonisatie microbiologisch en histomorfometrisch te evalueren. De resultaten lieten zien dat de implantaatschroeven de tibia bilateraal penetreerden en het implantaat stabiel was. Om de percutane status te behouden moest het implantaat twee keer zo lang zijn, als de dikte van het zachte weefsel ter plekke. Door de inoculatie met bacteriën ontstond er in 100% van de gevallen een infectie. De hoesjes met zilver zorgden voor een significant verminderde dichtheid van bacteriën, en verlaagden de symptomen van de ontsteking rond de percutane pin. Het toevoegen van chloorhexidine aan de hoesjes had echter geen meerwaarde voor het verder terugdringen van bacteriën en ontsteking. In conclusie kan gezegd worden dat een consistent diermodel ontworpen is om strategieën gericht op PTI te testen. Daarnaast zouden chitosan hoesjes met zilver verder ontwikkeld moeten worden voor (pre-) klinische doeleinden ter voorkoming van PTI.
Kunnen zilver en lidocaïne in materialen geladen worden met behulp van een 3D print techniek, om bacteriële infectie te voorkomen en tegelijkertijd pijnverlichting te bewerkstelligen?

Een 3D techniek met pneumatische extrusie kan gebruikt worden om individuele dragermaterialen te produceren, waarmee onregelmatige botdefecten hersteld kunnen worden. Gedurende het proces van 3D printen, kunnen er ook therapeutica aan het dragermateriaal toegevoegd worden. Het doel van Hoofdstuk 6 was om door middel van een eenstaps 3D print proces een polycaprolacton (PCL) drager materiaal te maken, met Ag₃PO₄ om infecties te bestrijden en lidocaïne om pijn te bestrijden. De hypothese was dat het vrijkomen van deze medicijnen daarbij gecontroleerd kon worden door filament diameter van de 3D dragermaterialen te variëren. Voor dit doel werd een polycaprolacton (PCL) slurry gemengd met verschillende hoeveelheden zilverfosfaat en lidocaïne en geprint door mondstukjes van meerdere groottes. De zo verkregen cilindrische dragermaterialen lieten een zeer regelmatige poreuze en interconnectieve microstructuur zien. De Ag₃PO₄ en lidocaïne waren homogeen gedistribueerd. Door het aanpassen van de filament diameter, kon inderdaad de vrijgave van lidocaïne en zilver gecontroleerd worden. Het medium met daarin het vrijgekomen zilver, liet een duidelijke zone van inhibitie zien tegen Staphylococcus aureus en Escherichia coli, na het laden met 1% Ag₃PO₄ voor een periode van 6 dagen en met 3% Ag₃PO₄ voor een periode van tenminste 7 dagen. De cytotoxiciteit van alle dragermateriaal werd tenslotte nog beschreven met een celkweek proef. In conclusie kon gezegd worden dat 3D printen met pneumatische extrusie een praktische techniek is om dragermaterialen te produceren met daarin medicijnen. De PCL dragers geladen met Ag₃PO₄ en lidocaïne hebben potentieel ter bestrijding van infectie en pijn.

Afsluitende opmerkingen en toekomstperspectief

Deze thesis onderzocht het gebruik van zilver, om de antibacteriële werking van polymere biomaterialen te verbeteren, in zowel in vitro als in vivo modellen. De polymeren die met zilver geladen waren, vertoonden een langdurige vrijgave van zilver ionen, en waren daarmee langdurig antibacterieel werkzaam tegen anaerobe gram-positieve en gram-negatieve bacteriën (Hoofdstuk 3), zowel als tegen aerobe gram-positieve en gram-negatieve bacteriën (Hoofdstuk 4, 5, en 6). De chitosan membranen met zilver lieten geen sterkere subcutane ontstekingsreactie zien vergeleken met membranen zonder zilver (Hoofdstuk 3) en zorgden niet voor veranderingen in de helingscapaciteit van huidwonden (Hoofdstuk 6). Aangezien het ontwerp van medische hulpmiddelen grotendeels bepaald wordt door de toepassing, moet de antibacteriële werking echter altijd getest worden voor die ene specifieke
toepassing, en voor de locale situatie[1]. Dit proefschrift is dus alleen een initiële stap naar de translatie van het gebruik van zilver, en meer onderzoek zal nodig zijn om de uiteindelijke bijdrage van op zilver gebaseerde biomaterialen hard te maken in een humane klinische toepassing.

Wanneer biomaterialen in het lichaam worden geplaatst of in contact zijn met weefsels, dan kunnen een diverse reacties waargenomen worden. Een aantal van deze reacties is bepalend voor de functionaliteit van de biomaterialen. Ten aanzien van de polymeren met zilver, had de biologische omgeving een sterke invloed op het vrijkomen van het zilver, d.w.z. anorganische ionen resulteerden in een langzame vrijgave, terwijl eiwitten afkomstig uit lichaamsvloeistoffen afgezet werden en een barrière vormden die zilver afgifte verhinderde. Dientengevolge was er een hogere hoeveelheid zilver nanopartikels nodig om in vivo nog steeds antibacteriële effecten te bewerkstelligen (Hoofdstuk 4). Op dit moment zijn er nog geen betrouwbare methoden om het antibacteriële effect van zilver te bepalen, aangezien het mogelijke effect van biologische factoren, zoals de verschillende ionen en eiwitten aanwezig in lichaamsvloeistof, nog onbekend zijn. Aangezien de samenstelling van lichaamsvloeistof veranderlijk is, is de exacte karakterisatie en kwantificatie van zilver in relevante media en in situ (in cellen/organismen) uiterst belangrijk voor de uiteindelijke medische toepassing [2]. Het gebrek aan zulke bepalingen verklaart waarschijnlijk (tenminste gedeeltelijk) de verschillen tussen experimentele resultaten in de literatuur, en de problemen in de interpretatie van verkregen data. We stellen dan ook voor, dat een goede relatie tussen analytische technieken en biologische studies noodzakelijk is. Er zou bijvoorbeeld een gebalanceerd systeem gemaakt moeten worden dat geschikt is om tegelijkertijd bacteriën en cellen te kweken, om hierin zowel antibacteriële werking als de levensvatbaarheid van cellen onder gelijke condities simultaan te kunnen meten.

Een belangrijke taak van translationele geneeskunde is het “effectief vertalen van nieuwe kennis, mechanismen, en technieken uit voortschrijdend fundamenteel onderzoek, tot nieuwe aanpakken voor de preventie, diagnose, en behandeling van ziekten” [3]. In dit proces dienen diermodellen als een surrogaat voor patiënten om de effectiviteit en veiligheid van nieuwe diagnostische en therapeutische aanpakken te kunnen testen. Op dit moment blijven diermodellen nog steeds een unieke bron van in vivo informatie en vormen zij een onvervangbare brug tussen in vitro studies en de uiteindelijke toepassingen in patiënten [4]. Op het gebied van zilver onderzoek worden er continue nieuwe vormen van zilver ontwikkeld, zoals zilver nanopartikels of materialen gebaseerd op zilver. Maar er is veel minder onderzoek naar de in vivo evaluatie van de effectiviteit en veiligheid van deze nieuw ontwikkelde materialen. Een betrouwbare diermodel zou van grote waarde zijn om biomaterialen te kunnen
evalueren op basis van effectiviteit en veiligheid. Omdat een dergelijk ideaal model voor een infectie rond percutane implantaten nog niet bestond, is er eerst een literatuurstudie uitgevoerd om te kijken wat de belangrijkste factoren in een dergelijk model dienden te zijn (Hoofdstuk 2). Vervolgens werd een valide en betrouwbare model opgezet in het konijn, op basis van deze literatuurstudie. De hoesjes geladen met zilver, die in het model werden beschouwd, bleken geschikt voor verdere (pre-) klinische studies (Hoofdstuk 4). De eigenlijke validatie van biomaterialen die resistent zijn tegen infectie kan natuurlijk alleen gedaan worden in humane klinische experimenten. Helaas is dit echter het moment waarop de ontwikkeling vaak stopt, gezien de grootte, de kosten, en de lange tijd die klinische experimenten vereisen [5]. Om bijvoorbeeld de incidentie van infecties voor totale heup arthroplastie te verlagen van 2% naar 1%, zijn ongeveer 5000 patiënten nodig wanneer een 50% reductie aangetoond moet worden, gebaseerd op een statistische power calculatie met \( p < 0.05 \) [5]. Wanneer een medisch hulpmiddel bestemd is voor de Amerikaanse markt, dan zal de Food and Drug Administration (FDA) zulke hulpmiddelen eerst classificeren volgens de Medical Device Amendments of 1976 to the Federal Food, Drug, and Cosmetic Act. Klasse I en II hulpmiddelen moeten beschouwd worden op substantiële vergelijkbaarheid met hulpmiddelen die reeds toegelaten zijn, dit noemt men pre-amendment devices. In tegenstelling, moeten Klasse III hulpmiddelen door de stringente procedure van premarket approval (PMA), en deze route vereist ook klinische data [6]. In dit proefschrift werd het effect van de toevoeging van zilver voor GTR membranen, wondbedekkers, en hoesjes rond fixatie pinnen beschouwd. Aangezien membranen niet permanent geïmplanteerd worden, zal een dergelijke toepassing waarschijnlijk beschouwd worden als klasse II. Voor klasse I en II toepassingen kunnen dierexperimenten de laatste validatie stap vormen, voorafgaand aan toepassing in de mens. Daarom zijn keuzes over een relevant diermodel en de opzet, uitvoering, en evaluatie van die experimenten van wezenlijk belang voor het aantonen van de werkzaamheid en veiligheid. Wanneer diermodellen over percutane hulpmiddelen besproken worden, zijn de species, de bacterie stam, de hoeveelheid en methode van inoculatie, en de methode voor uiteindelijke evaluatie van de infectie steeds erg variabel, wat het onderling vergelijken van studies compliceert. Als diermodellen gestandaardiseerd kunnen worden, zijn resultaten onderling beter vergelijkbaar en kan veel waardevollere informatie gewonnen worden in systematisch literatuuronderzoek. Dit zal uiteindelijk ook meer vooruitgang opleveren op het medisch vlak.

Op dit moment wordt zilver in vele vormen toegepast als antibacterieel middel, zoals in zilversamenstellingen en zilver nanopartikels [10]. Het zilver wat opgenomen was in membranen liet een antibacteriële werking en cytotoxiciteit zien die afhankelijk was van de dosering (Hoofdstuk 3). Samen met andere studies [7,8], kan gezegd worden
dat er dus een therapeutische dosering moet bestaan die antibacterieel is, zonder tegelijkertijd toxisch te zijn. Dit schept een basis voor verder onderzoek naar zilver voor biomedische toepassingen, maar het risico op toxiciteit kan natuurlijk nooit geheel buiten beschouwing gelaten worden. Verder onderzoek naar gecontroleerde afgifte van zilver, door de zilverfosfaat op te nemen in polycaprolacton via 3D printen resulteerde in een dergelijke gecontroleerde afgifte van zilverionen en een aanhoudend antibacterieel effect gedurende tenminste 6 dagen (Hoofdstuk 6). De meest gunstige eigenschap van zilver samenstellingen zoals zilver nanopartikels en zilverfosfaat partikels lijkt dus de depot werking met de geleidelijke afgifte van zilverionen. Het zou verder optimaal zijn om echte exacte controle over de afgifte te verkrijgen, wat wellicht zou kunnen door gebruik te maken van nieuwe technieken. Er bestaan verschillende van zulke vernieuwende afgifte systemen die van buitenaf gestuurd kunnen worden (op basis van temperatuur, licht, en ultrasoon geluid), van binnen uit het lichaam (redox-, zuurstof, of pH- afhankelijk), of door enzymatisch gestuurde medicijnafgifte[9]. Zilver kan bijvoorbeeld ingesloten worden en vervolgens, bij het optreden van symptomen van infectie, vrijgegeven worden met behulp van blootstelling aan ultrasoon geluid. Over het geheel genomen is het gebruik van zilver veelbelovend. Door het verder controleren van de afgifte van zilver kunnen wellicht biomaterialen gemaakt worden die zelfs infectie kunnen voorkomen.

In samenvatting kan gesteld worden dat het gebruik van zilver een belovende techniek is. Alhoewel slechts enkele toepassingen zijn onderzocht, te weten huid wond bedekkers, hoesjes voor percutane implantaten, en subcutane membranen en dragermaterialen, hebben deze studies toch een duidelijke basis gelegd voor verder onderzoek naar het gebruik van zilver om antibacteriële eigenschappen voor biomaterialen te verkrijgen.
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Acknowledgements

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Dear Ting (Li) and Yan (Dr. Shi), I can imagine that life with children would be much harder. You continuously dedication and efforts to improve your life are admiring. Despite your busy family life, we appreciate a lot for your help with our moves. Those memories of nice talks and joyful dinners will never fade away.

Dear Wei (Zhou), thank you for being a very enthusiastic and responsible member of the ACSSNL-Nijmegen. I appreciate all your contributions to those successful events we held together. The thanks also go to the other members of ACSSNL-Nijmegen, we together make all these successful events happen. I wish you all have a bright future.

The company and support from the Chinese community in Nijmegen make my life more colorful. Thank you, Gaigai (Du), Baoxiu (Wang), Xinyu (Hu), Qiao (Chai), Chao (Guo), Xuan (Yan), Muqing (Li), Yingdi (Xie), Liping (Ye), Yanjun (Guo), Jiaying (Li), Jinshuo (Wang), Weiqiang (Dr. Dou), Yongjun (Dr. Men), Kaizheng (Liu) et al.

I would also like to acknowledge those friends who I met during different events. Dear Rongquan (Duan), Zhengchao (Guo), and Jia (Liang), from Twente University, we worked in the same fields, and those talks about research are inspiring. Let us keep in touch. Dear Min (Nie), thank you for the encouragement with the dental examination. Dear Liangliang (Yang) and Can (Wang), thank you for helping me out with the thesis delivery.

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thank you for always be ready to help me. Dear Changkun (Liu) and Na (Xu), thank you for your treats when I visit Jinan. Dear Ping (Qin), thank you for always be ready to meet me every time I traveled back. Dear Jianbo (Cao), thank you for your kind help with my dentist license and dental examination.

I would like to extend my acknowledgment to Prof. Ping Ji, who aid in my career development and always give me great advice with wisdom. I appreciate your encouragement to pursue my Ph.D. abroad, which broaden my horizon and enlighten my life. The encouragements offer me a great power. Those kind suggestions are shining light that guides me forward.

Dear Bing, we first met as classmates in 2007. Gradually, I was attracted by you since you are such a smart student who can almost achieve highest scores in all subjects, since you are such an easy-going person that can get along with everyone, since you are such a kind-hearted friend who can always be considerate, since you are such a beautiful, sweet, and lovely angel who caught my heart. Lots of time, we studied together and grew together. Later, when I made up my mind to pursue a Ph.D. position abroad, I appreciate your wholehearted support by joining to prepare the IELTS. The following spring, God played a joke on us that I got the scholarship while you did not. The first year was tough and it was you who always encouraged me and gave me the power to move forward. Luckily, the next year we reunited here in this beautiful city, Nijmegen. The mutual support between us makes the studies go more smoothly and
the life easier. Besides, we have traveled and enjoyed the splendid views and the
glorious culture all over Europe. All these experiences made our love deeper. Dear
Bing, you are the best! I am so grateful to have you always accompanying around!
Together, we can and will build a brighter future!
# PhD portfolio

**Name PhD candidate:** J. Shao  
**Department:** Biomaterials  
**Graduate School:** Radboud Institute for Molecular Life Sciences  
**PhD period:** 29-09-2014 – 05-09-2018  
**Promotor(s):** Prof. J.A. Jansen  
**Co-promotor(s):** Dr F. Yang, Dr X.F. Walboomers

<table>
<thead>
<tr>
<th>TRAINING ACTIVITIES</th>
<th>Year(s)</th>
<th>ECTS</th>
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<tbody>
<tr>
<td><strong>a) Courses &amp; Workshops</strong></td>
<td></td>
<td></td>
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<tr>
<td>Laboratory animal science</td>
<td>2015</td>
<td>3</td>
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<tr>
<td>Presentation skills</td>
<td>2015</td>
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<td>Advanced conversation</td>
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<td>Digital tools for scholarly information</td>
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<td>English pronunciation training</td>
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<td>Research data management for PhD's</td>
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<td>Cell-based bone regeneration</td>
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<td>Scientific integrity</td>
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<td>Perfecting your academic writing skills</td>
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<td>Introduction to R</td>
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<td>Molecules, Mice and Math: a statistical toolbox for the lab</td>
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<td>The art of presenting science</td>
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<td>Analytic storytelling</td>
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<td>Implant Dentistry from the University of Hong Kong</td>
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<td><strong>b) Seminars &amp; lectures</strong></td>
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<td>Program introduction day</td>
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<td>Graduate course for the PhD’s from RIMLS</td>
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<td><strong>c) Symposia &amp; congresses</strong></td>
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<td>RIMLS New Frontiers 2018</td>
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<td>International Training School on Advanced Characterization Techniques for Electrospun Nanofibers: Hands on Experience</td>
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<td>PhD Retreat ###*</td>
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<td>6th Thesinge Biofilm Conference</td>
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<td>The 6th Int'l Conference on Tissue Engineering in conjunction with the 3rd Int'l Conference on Regenerative Biomedical Materials *</td>
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<td>Netherlands Society for Biomaterials and Tissue Engineering 25th &amp; 26th Annual Meeting **</td>
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<td><strong>d) Other</strong></td>
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<tr>
<td>Organize the RIMLS Technical Forum</td>
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### TEACHING ACTIVITIES

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<th>e) Supervision of internships</th>
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<tr>
<td>Supervision of Bachelor Student - Marleen Reijnders</td>
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<tr>
<td>Supervision of Bachelor Student - Janniek Smit</td>
<td>2016</td>
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<tr>
<td>Supervision of Chinese Exchange Student - Shutian Dong</td>
<td>2016</td>
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<tr>
<td>Supervision of Chinese Exchange Student - Xiaoyu Luo</td>
<td>2017</td>
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<tr>
<td>Supervision of Master Student – Ingward Plugge</td>
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<td>1.5</td>
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</table>

**TOTAL** 44.80

Oral and poster presentations are indicated with a * and # after the name of the activity, respectively.
List of Publications

Related to this thesis:

List of Publications related to this thesis:


Other Publications:


Curriculum Vitae

Jinlong Shao was born on 02 June 1989 in Mengyin, Shandong Province, China. In September 2007, he was enrolled in School of Stomatology, Shandong University (Jinan, China), majoring in dentistry. During the next 7 years, he received basic medical education, dental education and further specialized as a prosthodontist under the supervision of Prof. Ping Ji. In 2014, he received both his bachelor's and master's degree in dentistry with honors from Shandong University.

From 29th September 2014 on, he worked as a Ph.D. candidate in the Department of Biomaterials, Radboud University Medical Center (Radboudumc, Nijmegen, the Netherlands) under the supervision of Prof. dr. John A. Jansen, Dr. Fang Yang, and Dr. X. Frank Walboomers. His project is focused on the incorporation of silver to enhance the antibacterial properties of biomaterials. In 2017, he went to Research Center for Nano Biomaterials, Analytical & Testing Center, Sichuan University (Chengdu, China) as a visiting researcher for two months under the supervision of Prof. Yi Zuo. The results of the performed studies are partly described in this thesis and are partly applying for patents.
The best time to plant a tree is twenty years ago; the second best time is now!

— An Ancient Proverb
The incorporation of silver to enhance the antibacterial properties of biomaterials