# DEVELOPMENT AND CHARACTERIZATION OF AN ANTIBACTERIAL ELECTROSPUN NANOFIBER WOUND DRESSING

**Stefan JA Remmers MSc[1], Fang Yang, Jiankang Song**

Corresponding Author: Stefan JA Remmers MSc (stefanremmers@live.nl)

[1] Affiliation: Fifth-year Biomedical Sciences student

**ABSTRACT:**

***Supplementary tables can be found on our website***

**BACKGROUND:** Wound dressings with non-antibiotic antibacterial properties are required to decrease the risk of wound infection, because the barrier-effect of standard dressings is insufficiently effective, and antibiotics lead to resistant bacterial strains
**OBJECTIVE**: To develop antibacterial electrospun membranes and determine their drug release kinetics and cytotoxicity.
**METHODS:** Chitosan-based nanofibrous membranes were electrospun with silver nanoparticles (AgNP's) from chitosan / polyethylene oxide / AgNO3 solutions, that were subsequently loaded with various amounts of chlorhexidine (CHX). Loading of CHX was expected to elicit a burst release while co-electrospinning AgNO3 together with chitosan was expected to result in AgNP's that provided a sustained release.
**RESULTS AND DISCUSSION:** Scanning electron microscopy showed chitosan-based membranes consisting of uniform and defect-free nanofibers. Transmission electron microscopy indicated the presence of AgNP's in the fibers with average diameters of 1.9 nm and 3.0 nm when adding 1% and 5% AgNO3, respectively. CHX was released within six hours while silver release was sustained, as determined by high performance liquid chromatography and inductively coupled plasma mass spectrometry respectively. Release of CHX and silver was in the same order of magnitude as many common minimal inhibitory concentrations (MIC's) in literature. Alamar Blue cytotoxicity assays showed that silver was not cytotoxic, whereas CHX was slightly cytotoxic at concentrations of 12.5 µg/ml and severely toxic above 25 µg/ml.
**CONCLUSION**: We were able to create chitosan-based membranes that elicited a strong CHX burst after loading and sustained release of silver over time. We created membranes that showed low or no cytotoxicity while the concentrations of released drugs were within range of various common MIC's. Future research should determine the synergetic antibacterial effect and the actual concentrations of antibacterials after application at the wound site.

**WHAT IS KNOWN**: Wounds are prone to infection. Current wound dressings are not effective enough to prevent wound infection. Antibacterial agents used in coatings or membranes could significantly reduce wound infections. None have succeeded in fabricating a wound dressing with three synergetic antibacterials.

**WHAT IS NEW**: We succeeded in developing a nanofiber wound dressing using three synergetic antibacterials. Different mechanisms of action of the antibacterials increase the effectiveness, yet limit the toxicity of the membranes.

**KEY WORDS:** Antibacterial Wound Dressing, Electrospinning, Chitosan, Silver, Chlorhexidine

**List of Abbreviations:** AA: Acetic acid, Ag: Silver, AgNP: silver nanoparticle, CHX: Chlorhexidine, DMSO: Dimethyl sulfoxide, FBS: Fetal bovine serum, HFF: human foreskin fibroblast, HPLC: High performance liquid chromatography, ICP-MS: Inductively coupled plasma mass spectrometry, MIC: minimal inhibitory concentration, PEO: Polyethylene oxide, PS: Pennicilin/streptomycin, SD: Standard deviation, SEM: Scanning electron microscopy, TEM: Transmission electron microscopy

## Introduction

Common bandages and wound dressings serve as a barrier between wounds and the external environment, yet wounds remain prone to infection[1]. Therefore, a wound dressing is needed that possesses intrinsic antibacterial properties via incorporated antibacterial agents. The dressing should ideally elicit a burst release of the antibacterial agents to clear the wound of microorganisms, and a sustained release for keeping the wound free of microorganisms until the dressing is changed[2,3] typically at least once every 48 hours.

Electrospinning, an inexpensive, simple, yet effective technique for creating nanofibrous membranes has attracted attention as a method to create wound dressings[4,5]. Using electrospinning, antibacterial biodegradable non-woven nanofiber meshes can be fabricated from a viscous polymer solution[4,6].

Chitosan was electrospun in many previous studies[7-9] It is a biocompatible and biodegradable polymer that is abundant, cheap and has haemostatic[10] and antibacterial properties[11,12]. However, chitosan's antibacterial properties are limited, and the minimal inhibitory concentrations (MIC) against microorganisms commonly exceed 1000 µg/ml[13]. Electrospun chitosan membranes are not capable of sufficient antibacterial inhibition alone[14,15] but due to the biocompatible and biodegradable nature, they are an excellent drug-carrier material for targeted and timed drug delivery[14,15].

To improve the antibacterial properties of electrospun chitosan-based membranes, compounds such as antibiotics[16] or antiseptics can be used. A known problem with antibiotics is the development of resistant bacterial strains, and antibiotics should therefore not be used. Ideal antibacterial compounds should have antibacterial properties against a broad spectrum of bacteria, have MICs that are obtainable and have no or low toxicity.

Silver is a relatively inexpensive antiseptic that has been used for its wide range of antibacterial properties and low toxicity[17-21] for thousands of years. It is bactericidal at low concentrations (MIC range against most common microorganisms: 1.69 – 13.5 µg/ml)[22-25], and acts in synergy with chitosan[26]. Silver nanoparticles (AgNP's) are regarded as the most effective method to incorporate silver for antibacterial purposes due to their high surface-area-to-volume ratio[17-21]. To produce AgNP's, Ag+ ions are chemically reduced and stabilized inside the nanofibers by a polymer such as chitosan[15]. AgNP's have

successfully been used in electrospun antibacterial membranes[15, 27-29] and have shown good antibacterial effects within several hours[15]. However, gram positive bacteria are less susceptible to silver than gram negative bacteria[17,23]. Silver can be toxic at higher concentrations, but study design and AgNP characteristics differ widely and make comparing results of different toxicity studies virtually impossible[30].

Chlorhexidine (CHX) is a relatively inexpensive antiseptic mostly used for dental applications. It is a potent and fast acting bactericidal compound (MIC range against most common microorganisms: 0.25 – 30 µg/ml)[31-33]. In contrast to silver, it is most effective against gram positive bacteria[31,32]. CHX has also shown synergy with chitosan[34]. It has been used both as a component of fibers[35] or loaded onto fibers[36] to elicit an antibacterial response where it has shown a fast release pattern. In contrast to silver and chitosan however, CHX can be toxic at concentrations of 20 µg/ml or higher[37-39].

Individually, silver and CHX possess decent antibacterial properties. Combined with chitosan, the different mechanisms of action, different susceptibility of bacteria and synergy with chitosan may lead to an antibacterial membrane that is effective against a wide range of bacteria yet is not toxic, acts fast, and also maintains a sustained release of antibacterials. Surprisingly however, these three compounds have not been used together before for the fabrication of antibacterial electrospun chitosan-based nanofiber wound dressings.

In this study, chitosan-based electrospun nanofiber membranes were created by electrospinning chitosan with polyethylene oxide (PEO) [40] and acetic acid (AA). AgNP's were introduced into the fibers by adding $AgNO_3$ to the electrospinning solution. CHX was loaded onto the membrane after electrospinning. The aim of this study was to determine the effectiveness of the electrospun membranes as wound dressing by answering the following questions:

1. What are the release kinetics of electrospun silver and loaded CHX from the electrospun membrane over two days?
2. How is the cytotoxicity level of the membrane related to the incorporated amount of silver and CHX?
3. In which way does the simultaneous use of both silver and CHX affect the release of both compounds and the cytotoxicity level compared to using only one compound?

## Materials and Methods

**Materials**

Chitosan (degree of deacetylation = 90%, molecular weight = 200-400 kDa, Heppe Medical Chitosan), Polyethylene oxide (Molecular weight = 900 kDa, Sigma-Aldrich®), chlorhexidine-digluconate (Sigma-Aldrich®) Acetic acid (99.9%, Boom BV, Netherlands), AgNO3 (Boom BV, Netherlands) and dimethyl sulfoxide (DMSO, Sigma-Aldrich®) were used as received. Donated Human Foreskin Fibroblasts were cultured in alpha Minimal Essential Medium (αMEM, Life Technologies, cat no. 22571) supplemented with 10% Fetal Bovine Serum (FBS, Sigma-Aldrich®) and 1% Penniciline/Streptomycin (PS, Sigma-Aldrich®).

*Solution preparation for electrospinning*

Four solutions were prepared for electrospinning. First, 2.25% (weight/volume, w/v) chitosan and 0.75% (w/v) PEO were added to a liquid phase of 25 vol% AA and 75 vol% ultrapure MilliQ grade water (MilliQ). To each solution, one of the following four $AgNO_3$ concentrations was added to create the final electrospinning solution. To create an 'empty', 'low silver', 'medium silver' and 'high silver' membrane, no $AgNO_3$, $AgNO_3$ concentrations of 0.1 wt%, 1.0 wt% and 5.0 wt% of the total polymer weight of chitosan and PEO were added to the solutions respectively. Solutions were stirred over night to ensure complete dissolution of the polymers and silver salts.

*Electrospinning*

Solutions were loaded in a 10 ml syringe (DB Plastipak). A silicon tube was connected to the syringe and the spinneret of a custom-made electrospinning apparatus, and the syringe was placed into a syringe pump (KD scientific). Aluminum foil was placed over a conducting drum (length: 16 cm, circumference: 25 cm) to facilitate fiber collection, and the distance between the spinneret and collector was set to 15 cm. Solutions were spun at a controlled flow rate of 4 ml/h at 27 kV on a drum rotating at 35 rpm while making a cyclic horizontal movement. The membranes were left to dry by air for at least one night, and were then stored at -20°C until further use. Samples were created by punching out discs with a surface area of 1 $cm^2$ from the membranes. The samples were weighed and samples with a similar weight were chosen for the following experiments. Average weights of the samples allocated to each group are displayed in Tables 1 and 2. In order to improve the mechanical properties of the membranes and reduce water solubility, fibers were cross-linked by treatment with glutaraldehyde in a vacuum vapor chamber for 40 hours[41]. This resulted in four groups with different membranes: a control group without silver, and three groups with different silver content. The silver content of all experimental groups containing silver and the control group is displayed in Table 1.

*Loading of Chlorhexidine*

From the four types of membrane, six more groups were created by loading CHX onto electrospun membranes. Chlorhexidine-digluconate stock solution was diluted to three concentrations: 4 mg/ml, 12 mg/ml and 20 mg/ml and samples were placed in 1.5 ml Eppendorf tubes (release kinetics experiment) or 24-well plates (cell toxicity experiment). Three groups containing a low, medium and high amount of CHX were created by loading 5 µl of the 4 mg/ml, 12 mg/ml or 20 mg/ml CHX solution onto control membranes respectively. Three more groups containing a fixed CHX content but different silver concentration were created by loading 5 µl of 12 mg/ml CHX onto 'low silver', 'medium silver' and 'high silver' membranes. This resulted in a total of ten sample groups: one control group without silver, three groups with different silver content, three groups with different CHX content, and three interference groups with different silver content and a fixed CHX content. All membranes were stored at 4°C over night to ensure proper absorption. The next day, the samples were freeze-dried for at least 24 hours. The CHX content of all experimental groups containing CHX and the control group is displayed in Table 2.

*Membrane characterization*

Fiber morphology was investigated using a scanning electron microscope (SEM, JEOL JSM-6340F). Nanofibers were deposited onto aluminum foil, cut and mounted on aluminum stubs using double-sided carbon tape and sputter-coated with gold for 60 seconds. Fiber diameter was determined by selecting 20 fibers randomly per image from five images, and measuring the average diameter using Image-J (NIH) software. Transmission electron microscopy (TEM, JEOL JEM-1010) was used to determine the presence and morphology of silver nanoparticles. Samples were collected by briefly placing copper sample support grids on the collector of the electrospinning device. Image-J software was used to determine the average diameter of the nanoparticles by measuring the diameter of 50 nanoparticles per sample.

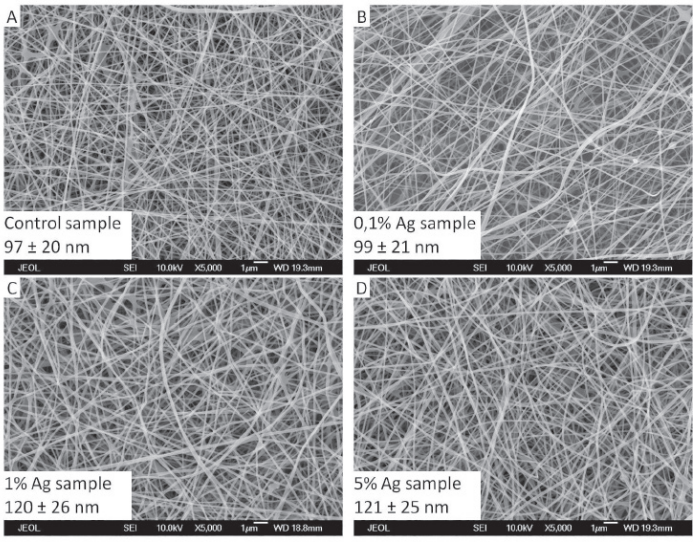


***Figure 1*** *Fiber morphology. SEM images of Electrospun chitosan / PEO nanofiber membranes illustrate the effect of AgNO3 addition on fiber diameter and morphology. (A) Control sample. (B) Sample with 0.1% AgNO3. (C) Sample with 1.0% AgNO3. (D) Sample with 5.0% AgNO3. All conditions produce uniform and defect-free fibers. With higher concentrations of AgNO3, fiber diameter tends to increase (but not significantly) and fibers seem to become more curly.*

*Release kinetics test*

Silver and CHX release from the membranes was measured at t = 6 hours, 1 day and 2 days (n = 5). Each sample was placed in an eppendorf tube (one sample per tube) and 1 ml of MilliQ was added to each tube. At each time point, 0.9 ml of liquid was removed and stored at 4°C for analysis and immediately replaced with 0.9 ml fresh MilliQ. The silver content was determined using inductively coupled plasma mass spectroscopy (ICP-MS, Thermo Electron Corporation, X series) using a sample matrix of 1.0% $HNO_3$. High Performance Liquid Chromatography (HPLC) was used to detect CHX release[42]. A mobile phase consisting of a 40% acetonitrile solution in MilliQ with 0.1% trifluoroacetic acid and 0.1% triethylamine was loaded into the HPLC device (Hitachi L-2130 pump, Hitachi L-2400 UV detector, Hitachi L-2200 auto sampler, Lichorspher RP-18 endcapped column). The flow rate was set to 1 ml/min, the injection value to 30 µl, and absorption was measured at λ = 260 nm. CHX retention time was approximately 3.1 minutes.

*Cytotoxicity*

Human Foreskin Fibroblasts (HFF) were thawed at passage 21 and cultured until passage 24 in culture medium aMEM supplemented with 10% FBS and 1% PS. Each of the 10 membrane sample groups contained three samples. First, an antibacterial medium was created by immersing each sample in 2.5 ml culture medium in 24-well plates for 24 hours (one sample per well). At the same time HFF cells in normal culture medium were placed into other 24-well plates (50000 cells/well) and were left to attach to the plate for 24 hours. After 24 hours, the medium was replaced with 1 ml of the new conditioned antibacterial medium (in duplo). Fresh medium and 5% DMSO were used as positive and negative control respectively. In addition, dilution series of CHX starting at 100 µg/ml and silver starting at 50 µg/ml was made in fresh medium and was tested for cytotoxicity simultaneously. The cells were incubated in the antibacterial medium or dilution series for 20 hours. Cytotoxicity was assessed with an Alamar Blue assay. The antibacterial medium was replaced by 1 ml culture medium containing 10% Alamar Blue dye. The cells were incubated for 4 hours wrapped in aluminum foil in an incubator. Clean medium with 10% Alamar Blue was used as control. After 4 hours, two 200 µl aliquots of metabolized medium from each well were placed in a transparent flat-bottom 96-well plate and fluorescence was measured at λ = 530/590 in a Bio-Tek® FL600 microplate fluorescence reader.

*Statistical analysis*

Sample groups were compared using a one-way ANOVA with Bonferroni correction for multiple testing. Means and standard deviation (SD) are presented in the following way: mean ± SD.

## Results

**Membrane characterization**

Figure 1 displays SEM images of the electrospun chitosan-based membranes with different silver content. All membranes consisted of uniform and defect-free nanofibers. The diameters of the fibers tended to increase as the silver concentration increased. The diameters of the control (no silver) and 0.1% silver fibers differed significantly from the 1% silver and 5% ($p < 0.001$) silver fibers. While the fibers in the control sample seemed to be straight, the fibers tended to become curlier as more silver was added to the solution.

The presence of silver nanoparticles was investigated using TEM (Figure 2). No AgNP's were detected in the control fibers or the fibers containing 0.1% of AgNO3 (Figure 2A and B). For the samples containing 1.0 and 5.0% AgNO3 (Figure 2C and D), AgNP's had an average diameter of 1.85 ± 0.50 nm and 2.97 ± 0.55 nm, respectively. Higher concentration of $AgNO_3$ resulted in higher amount of AgNP's presented in the fibers.

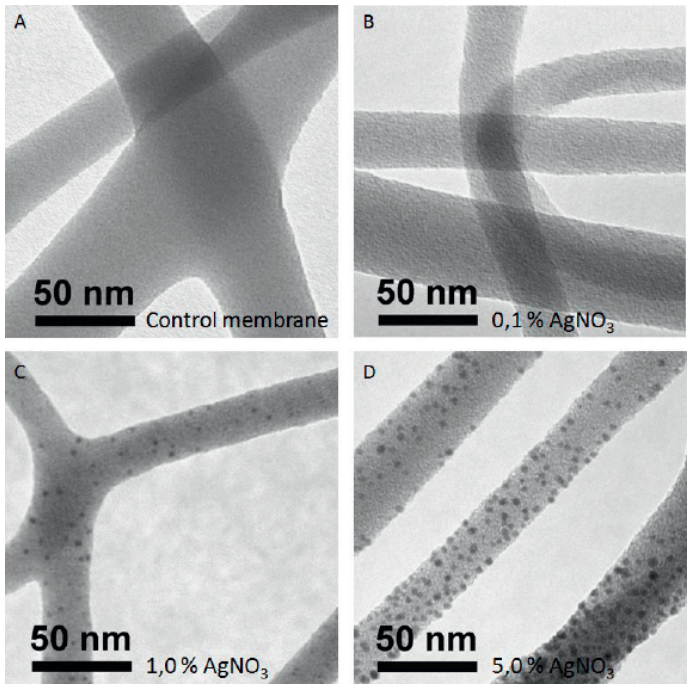


***Figure 2*** *Silver nanoparticle detection. TEM images of electrospun chitosan-based nanofibers with 0% (A), 0.1% (B), 1.0% (C) and 5.0% AgNO3 (D). No AgNP's were detected in the control fibers or the fibers containing 0.1% AgNO3. AgNP's in fibers with 1 and 5% AgNO3 had an average diameter of 1.85 ± 0.50 and 2.97 ± 0.55 respectively. Fibers with 5% AgNO3 contained more AgNP's than fibers with 1% AgNO3.*

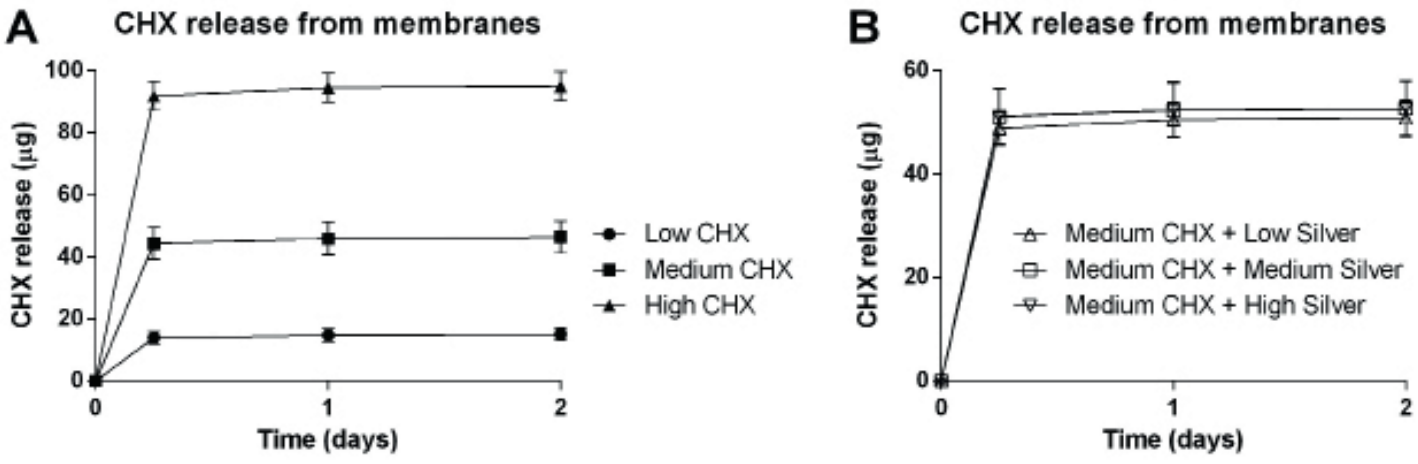


***Figure 3*** *Chlorhexidine release. These figures display the CHX release of the three membranes with different concentrations of CHX (A) and the CHX release of three membranes with identical CHX concentration but different silver content (B). All conditions show a burst release of CHX within the first 6 hours. Membranes with different CHX concentrations showed a burst at the same time, but reached a different cumulative release. The presence of silver in the membranes did not affect the release of CHX.*

**Release kinetics of antibacterial compounds**

CHX and silver release from the membranes in water was measured at different time points over a period of two days using HPLC (for CHX) and ICP-MS (for Ag). The results of the CHX release tests are presented in Figure 3. All six membrane groups that contained CHX gave a burst release of CHX within the first 6 hours, the height of which was dependent on the amount of CHX added to the membranes. The low CHX, med CHX and high CHX membranes released respectively 75%, 77% and 95% of the total CHX that was loaded onto the membranes. Figure 3B shows that the silver content of membranes did not influence the CHX release characteristics, although the percentage released for the three membranes with both silver and CHX is slightly higher than that of the membrane with the same CHX content but without silver (77% versus 87% on average). However, this difference was not significant ($p = 0.17$, $p = 0.61$ and $p = 0.60$ when comparing the membranes with only CHX to the ones with CHX and low, medium and high silver content respectively). The CHX content, 6 hour, 1 day and 2 day CHX release from the membranes are displayed in Table 2.

The silver release is presented in Figure 4 as detected by ICP-MS. The results were transformed to represent $AgNO_3$ release as opposed to silver ion release to make them comparable to the quantities incorporated into the membranes. This figure displays a strong sustained release that gradually weakens during the next two weeks. The membranes with different concentrations of silver showed corresponding release profiles; the membranes with the lowest concentration released the least amount of silver and the membranes with the highest concentration released the highest amount of silver. No significant difference in silver release was found between the membranes without CHX and corresponding membranes with CHX ($P < 0.05$). Cumulative silver release averaged over all membranes over 2 days was 0.91 µg, 12.1 µg and 40.6 µg for the membranes with low, medium and high silver content respectively. The silver content, 6 hour, 1 day, and 2 day cumulative release of silver from the membranes are displayed in Table 1. A logarithmic regression curve could be fitted to the individual groups ($R2 > 0.97$) using Microsoft Excel software, but no formula could be created that accurately predicted the sample data.

**Cytotoxicity**

Cytotoxicity of the membranes was measured by incubating them in fresh culture medium for 24 hours and culturing HFF cells in the conditioned medium. An Alamar Blue assay was used to quantify metabolic activity to determine the relative number of live cells. The results of the Alamar Blue assay are displayed in Figure 5. These results show no significant difference between the positive control and the metabolic activity of cells cultured in medium derived from the control membrane, 'low Ag', 'med Ag', 'high Ag' and 'low CHX' samples, indicating that these membranes were not cytotoxic. No significant difference was found between the negative control and the 'high CHX' group, indicating that these membranes are as cytotoxic as DMSO, the control sample. Samples cultured in medium derived from 'med CHX' containing membranes were significantly different from both the positive and negative control ($p < 0.001$) and hover

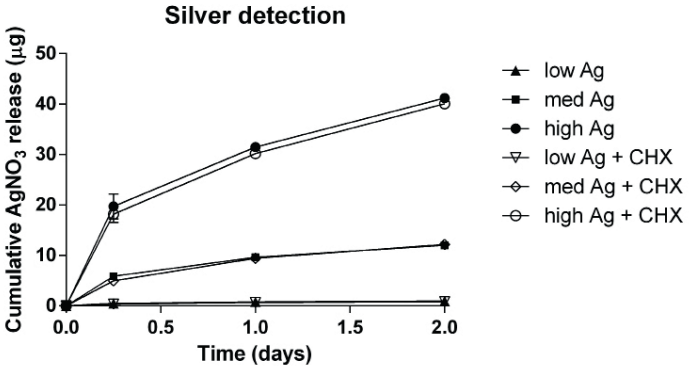


***Figure 4*** *Silver detection over time from membranes incubated in water for a period of 2 days. The figure clearly shows a strong burst release over the first six hours, and a steady but weaker gradual release over the next days. The presence of CHX on the membranes did not influence the release profile of Ag. The three used silver concentrations show three distinct release profiles, where the lowest concentration releases the least amount of silver and the highest concentration releases the highest amount of silver. Average cumulative silver release over 1 day was 0.76 µg, 9.53 µg and 30.84 µg and over 2 days was 0.91 µg, 12.1 µg and 40.6 µg for the membranes with low, medium and high silver content respectively.*

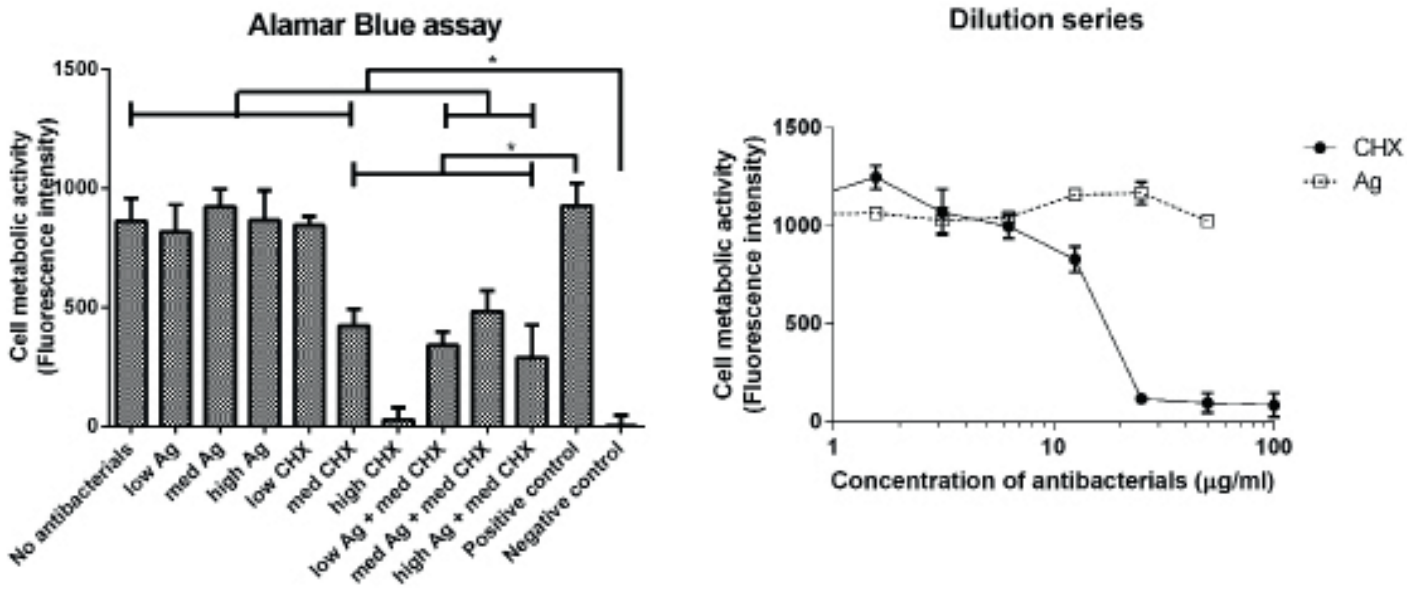


***Figure 5*** *Alamar Blue cytotoxicity assay (left). This figure displays the cell metabolic activity of cells after 24 hours of culture in antibacterial medium. Significance ($P < 0.05$) between a controlgroup and sample group is displayed as an asterisk above the figure. We found no significant difference between the positive control and the cell metabolic activity of cells cultured in medium derived from the control membrane, 'low Ag', 'med Ag', 'high Ag' and 'low CHX' samples. The negative control and the 'high CHX' group did not show a significant difference. Samples cultured in medium derived from 'med CHX' containing membranes showed a reduced number of cells and were significantly different from both the positive and negative control. Although they hover around the same values, some were shown to be significantly different from each other as well (right). The dilution series displays the cytotoxicity of a series of increasing silver or CHX concentrations. The silver dilution samples were not significantly different from the control sample. The first significant difference in the CHX dilution series with the positive control was found at 12.50 µg/ml. From 25 µg/ml and higher, no significant difference was found between the measurements and the negative control.*

around the same value, indicating they are cytotoxic, but there are still live cells present. The silver dilution series showed us that silver is not cytotoxic at the concentrations used in this study. When used against HFF cells, CHX has a mild cytotoxic effect at a concentration of 12.50 µg/ml. From 25 µg/ml and up, no significant difference was found between the measurements and the negative control, indicating the death of all cells.

## Discussion

The aim of this study was to develop and characterize antibacterial membranes utilizing chitosan, silver and CHX that could emit a quick burst of antibacterials and a sustained release of antibacterials over time. We were able to create membranes that were not toxic to human fibroblasts, and showed a strong burst release of CHX followed by a sustained release of silver over time that achieved concentrations around and above most common MIC's found in literature.

SEM images showed that uniform and defect-free nanofibers were created. The membranes with silver contents of 1% or higher contained silver nanoparticles whose size and number increased with increasing $AgNO_3$ concentrations. No AgNP's were detected in fibers with 0.1% $AgNO_3$, either because the detection method is not accurate enough to detect nanoparticles of such a small size, or because the amount of $AgNO_3$ was not sufficient to induce nanoparticle formation.

Release kinetics showed a burst release of CHX within six hours and a sustained but weakening release of silver over time. CHX was released quickly, because it was loaded onto the fibers and was absorbed without creating molecular bonds between the chitosan or PEO molecules. Submersion in liquid caused the CHX to be released from the fibers quickly.

Silver was incorporated into the fibers through electrospinning, and could be released in one of two ways. Firstly, silver ions (Ag+) that are not or no longer part of nanoparticles can diffuse from the nanofibers because silver ions are much smaller than chitosan or PEO molecules. Secondly, degradation of the chitosan/PEO fibers can release silver ions and particles trapped inside. The strong initial release of silver was likely caused by diffusion of silver ions on or near the surface of the fibers, showing a Fickian diffusion profile[43]. The sustained release was likely caused by a combination of Fickian diffusion and slow degradation. However, the role of degradation is expected to be minimal, as the membranes showed no visible degradation in structure after incubation for four weeks (data not shown). In addition, investigation of the interference groups with both silver and CHX showed that the presence of either compound in the membranes did not influence the release characteristics of the other compound.

The cytotoxicity test showed that both the silver dilution series and the conditioned medium derived from silver containing membranes were not cytotoxic at any of the tested concentrations. CHX became mildly toxic at concentrations of 12.5 µg/ml, and severely toxic at 25 µg/ml, as was detected with the CHX dilution series.

Combining the results from the release kinetics and cytotoxicity tests, the antibacterial medium used for the cytotoxicity tests contained an estimated concentration of CHX of approximately 6.0, 18.6, and 38 µg/ml for the low, medium and high CHX membranes respectively. Of these media, the 18.6 µg/ml (med CHX) medium was shown to be moderately toxic, while the 38 µg/ml (high CHX) was severely toxic. These results indicate that the cytotoxicity of CHX starts around

12.5 µg/ml, is moderately toxic around 18.6 µg/ml and severely toxic above 25 µg/ml. These results are in agreement with literature which indicated that CHX is cytotoxic at concentrations of 20 µg/ml[37-39] and higher. Our results showed cytotoxicity at a lower concentration which can be explained by the use of different cell types (HFFs versus chondrocytes or odontoblast-like cells in literature) and longer exposure times (24 hours versus up to 2 hours in literature)[37-39]. The toxicity of the interference groups containing both silver and CHX was compared with the groups containing only CHX. Toxicity of the interference group did not differ significantly from the corresponding CHX groups, indicating that the presence of silver does not affect the toxicity of CHX, or vice versa.

To investigate the antibacterial effectiveness of the membranes a hypothetical scenario was created: the membranes were applied to a wound for 24 hours, and antibacterials were released into an exudate volume of 1 ml. Comparing the 1 day release of CHX (Table 1) with the MIC's that were found in literature, both the low CHX and medium CHX membranes would fall within the range of common MIC's, and the high CHX membrane would exceed this range. The medium and high CHX membranes however are cytotoxic in this scenario. The low CHX membrane would not be toxic and would fall within the range of most common MIC's, indicating that it is possible to fabricate membranes loaded with CHX that have no cytotoxicity but are lethal to variety of microorganisms.

The incorporated silver was shown to be nontoxic to HFF cells at all tested concentrations. The MIC's of silver for most common microorganisms are in a range between 1.69 and 13.5 µg/ml. In contrast to CHX, which was released as a burst within six hours, silver was released gradually. Using the hypothetical scenario in which the membrane is placed on a wound for 1 day and the antibacterials are spread over 1 ml, the low Ag membrane would not reach the lower limit of MICs found in literature. The medium silver membranes would be well within the range of the MICs found in literature, while the high silver membranes would exceed the aforementioned range.

Translating the results to be accurate in in vivo circumstances will be a challenge. In the experiments, membranes were incubated in water to examine drug release, but the in vitro release in water does not necessarily correspond to the in vivo release in wound exudate[3]. However, silver ion detection required the use of MilliQ because the chloride salts in PBS, culture medium or simulated wound fluid would bind to the silver ions and precipitate[2]. This would lead to an inhomogeneous silver distribution in the tubes that would lead to false ICP-MS results if the 'supernatant' is analyzed.

In addition, toxicity of membranes was calculated by submersing the membrane in culture medium (2.5 ml / $cm^2$) to create the conditioned medium. The volume to surface area ratio over which released antibacterials are spread when the membrane would be used in vivo is not fixed. There are no standardized values to use, and this ratio is dependent on many variables such as wound type, size, location, tissue vascularization and exudation. To determine this ratio for a specific would type, in vivo tests should be conducted.

In addition to CHX and silver, chitosan has antibacterial properties as well, but chitosan release kinetics were not investigated in the present study. The antibacterial activity of all three compounds has been tested multiple times in literature, but never together. Concentrations of antibacterials were achieved that are, according to MIC's found in literature, capable of inhibiting the growth of a variety of microorganisms. However, the MIC's found in literature differ widely among authors, especially for AgNP's, as the effect of AgNP's is dependent on the preparation method, size, shape and environment[17]. We hypothesize that these antibacterials can complement each other and have a synergic effect against a variety of microorganisms when used simultaneously, because all three antibacterials act through different mechanisms[26,33]. However, the release profiles of the tested compounds are considerably different, limiting the period in which the synergetic effect takes place to the first few hours. To investigate the net effect of the synergetic use of these compounds, additional in vitro tests are required that focus on testing the antibacterial properties of membranes containing various combinations and concentrations of the antibacterial compounds.

## Conclusion

In this study, nanofibrous membranes were created from three synergetic antibacterial compounds. The membranes elicited a strong burst release as well as a sustained gradual release of antibacterials, effective against a wide range of bacteria at concentrations that are not toxic to human fibroblasts. However, because obtained results were compared with MIC's found in literature, additional in vitro tests need to investigate the antibacterial effectiveness of all three antibacterial compounds and their synergetic effect, and in vivo studies are required to investigate the actual released concentrations of antibacterials that the membranes can reach after application to a wound. Further investigation and development of these membranes could lead to affordable antibacterial wound dressings that are more effective than regular dressings in preventing wound infections.

## Acknowledgements

This work was funded by the NutsOhra Foundation (project no.: 1303-024). The authors would like to thank Martijn Martens for his help with in obtaining SEM images, Monique Kersten for her help doing cell culture, and Wanxun Yang for her help with the Alamar Blue assay. Finally, the authors would like to thank the department of Biomaterials for providing a challenging and friendly environment that made working there a pleasure.

## References

1. Dumville JC, Gray TA, Walter CJ, Sharp CA, Page T. Dressings for the prevention of surgical site infection. The Cochrane database of systematic reviews 2014; 9: CD003091.
2. Canada TA, Wiencek KM, Cowan ME, Lindsay BJ. Challenging Silver – Influence of Extraction Medium on the Release of Silver from Commercial Silver Dressings. In: LLC MHP, editor. Spartanburg: Milliken Healthcare Products LLC; 2007.
3. Jakobsen C. Relation of silver release and antimicrobial effect in-vitro of silver containing wound dressings: Linköpings universitet; 2010.
4. Bhardwaj N, Kundu SC. Electrospinning: a fascinating fiber fabrication technique. Biotechnology advances 2010; 28(3): 325-47.
5. Li CW, Fu RQ, Yu CP, et al. Silver nanoparticle/chitosan oligosaccharide/poly(vinyl alcohol) nanofibers as wound dressings: a preclinical study. Int J Nanomed 2013; 8: 4131-45.
6. Li D, Xia YN. Electrospinning of nanofibers: Reinventing the wheel? Adv Mater 2004; 16(14): 1151-70.
7. Klossner RR, Queen HA, Coughlin AJ, Krause WE. Correlation of chitosan's rheological properties and its ability to electrospin. Bio-

macromolecules 2008; 9(10): 2947-53.
8. Desai K, Kit K, Li J, Zivanovic S. Morphological and surface properties of electrospun chitosan nanofibers. Biomacromolecules 2008; 9(3): 1000-6.
9. Zhang YZ, Su B, Ramakrishna S, Lim CT. Chitosan nanofibers from an easily electrospinnable UHMWPEO-doped chitosan solution system. Biomacromolecules 2008; 9(1): 136-41.
10. Pusateri AE, McCarthy SJ, Gregory KW, et al. Effect of a chitosan-based hemostatic dressing on blood loss and survival in a model of severe venous hemorrhage and hepatic injury in swine. The Journal of trauma 2003; 54(1): 177-82.
11. Rabea EI, Badawy ME, Stevens CV, Smagghe G, Steurbaut W. Chitosan as antimicrobial agent: applications and mode of action. Biomacromolecules 2003; 4(6): 1457-65.
12. Koburger T, Hubner NO, Braun M, Siebert J, Kramer A. Standardized comparison of antiseptic efficacy of triclosan, PVP-iodine, octenidine dihydrochloride, polyhexanide and chlorhexidine digluconate. The Journal of antimicrobial chemotherapy 2010; 65(8): 1712-9.
13. Goy RC, Britto D, Assis OBG. A Review of the Antimicrobial Activity of Chitosan. Polímeros: Ciência e Tecnologia 2009; 19(3): 7.
14. Dilamian M, Montazer M, Masoumi J. Antimicrobial electrospun membranes of chitosan/poly(ethylene oxide) incorporating poly(hexamethylene biguanide) hydrochloride. Carbohyd Polym 2013; 94(1): 364-71.
15. An J, Zhang H, Zhang JT, Zhao YH, Yuan XY. Preparation and antibacterial activity of electrospun chitosan/poly(ethylene oxide) membranes containing silver nanoparticles. Colloid Polym Sci 2009; 287(12): 1425-34.
16. Genuit T, Bochicchio G, Napolitano LM, McCarter RJ, Roghman MC. Prophylactic chlorhexidine oral rinse decreases ventilator-associated pneumonia in surgical ICU patients. Surgical infections 2001; 2(1): 5-18.
17. Rai MK, Deshmukh SD, Ingle AP, Gade AK. Silver nanoparticles: the powerful nanoweapon against multidrug-resistant bacteria. Journal of applied microbiology 2012; 112(5): 841-52.
18. Kumar R, Munstedt H. Silver ion release from antimicrobial polyamide/silver composites. Biomaterials 2005; 26(14): 2081-8.
19. Sondi I, Salopek-Sondi B. Silver nanoparticles as antimicrobial agent: a case study on E. coli as a model for Gram-negative bacteria. Journal of colloid and interface science 2004; 275(1): 177-82.
20. Maaskant JM, De Boer JP, Dalesio O, Holtkamp MJ, Lucas C. The effectiveness of chlorhexidine-silver sulfadiazine impregnated central venous catheters in patients receiving high-dose chemotherapy followed by peripheral stem cell transplantation. European journal of cancer care 2009; 18(5): 477-82.
21. Damm C, Munstedt H, Rosch A. The antimicrobial efficacy of polyamide 6/silver-nano- and microcomposites. Mater Chem Phys 2008; 108(1): 61-6.
22. Panacek A, Kvitek L, Prucek R, et al. Silver colloid nanoparticles: Synthesis, characterization, and their antibacterial activity. J Phys Chem B 2006; 110(33): 16248-53.
23. Jain J, Arora S, Rajwade JM, Omray P, Khandelwal S, Paknikar KM. Silver Nanoparticles in Therapeutics: Development of an Antimicrobial Gel Formulation for Topical Use. Mol Pharmaceut 2009; 6(5): 1388-401.
24. Cho KH, Park JE, Osaka T, Park SG. The study of antimicrobial activity and preservative effects of nanosilver ingredient. Electrochim Acta 2005; 51(5): 956-60.
25. Lkhagvajav N, Yasa I, Celik E, Koizhaiganova M, Sari O. Antimicrobial Activity of Colloidal Silver Nanoparticles Prepared by Sol-Gel Method. Dig J Nanomater Bios 2011; 6(1): 149-54.
26. Norowski PA, Courtney HS, Babu J, Haggard WO, Bumgardner JD. Chitosan coatings deliver antimicrobials from titanium implants: a preliminary study. Implant dentistry 2011; 20(1): 56-67.
27. Son WK, Youk JH, Lee TS, Park WH. Preparation of antimicrobial ultrafine cellulose acetate fibers with silver nanoparticles. Macromol Rapid Comm 2004; 25(18): 1632-7.
28. Penchev H, Paneva D, Manolova N, Rashkov I. Electrospun hybrid nanofibers based on chitosan or N-carboxyethylchitosan and silver nanoparticles. Macromolecular bioscience 2009; 9(9): 884-94.
29. Jin WJ, Lee HK, Jeong EH, Park WH, Youk JH. Preparation of polymer nanofibers containing silver nanoparticles by using poly (N-vinylpyrrolidone). Macromol Rapid Comm 2005; 26(24): 1903-7.
30. Beer C, Foldbjerg R, Hayashi Y, Sutherland DS, Autrup H. Toxicity of silver nanoparticles - nanoparticle or silver ion? Toxicol Lett 2012; 208(3): 286-92.
31. Block C, Furman M. Association between intensity of chlorhexidine use and micro-organisms of reduced susceptibility in a hospital environment. J Hosp Infect 2002; 51(3): 201-6.
32. Buxbaum A, Kratzer C, Graninger W, Georgopoulos A. Antimicrobial and toxicological profile of the new biocide Akacid plus((R)). J Antimicrob Chemoth 2006; 58(1): 193-7.
33. Rolla G, Melsen B. On the mechanism of the plaque inhibition by chlorhexidine. Journal of dental research 1975; 54 Spec No B: B57-62.
34. Decker EM, von Ohle C, Weiger R, Wiech I, Brecx M. A synergistic chlorhexidine/chitosan combination for improved antiplaque strategies. Journal of periodontal research 2005; 40(5): 373-7.
35. Lin YN, Chang KM, Jeng SC, Lin PY, Hsu RQ. Study of release speeds and bacteria inhibiting capabilities of drug delivery membranes fabricated via electrospinning by observing bacteria growth curves. Journal of materials science Materials in medicine 2011; 22(3): 571-7.
36. Chen L, Bromberg L, Hatton TA, Rutledge GC. Electrospun cellulose acetate fibers containing chlorhexidine as a bactericide. Polymer 2008; 49(5): 1266-75.
37. Campbell J, Filardo G, Bruce B, et al. Salvage of contaminated osteochondral allografts: the effects of chlorhexidine on human articular chondrocyte viability. The American journal of sports medicine 2014; 42(4): 973-8.
38. de Souza LB, de Aquino SG, de Souza PP, Hebling J, Costa CA. Cytotoxic effects of different concentrations of chlorhexidine. American journal of dentistry 2007; 20(6): 400-4.
39. Lessa FC, Aranha AM, Nogueira I, Giro EM, Hebling J, Costa CA. Toxicity of chlorhexidine on odontoblast-like cells. Journal of applied oral science : revista FOB 2010; 18(1): 50-8.
40. Zivanovic S, Li J, Davidson PM, Kit K. Physical, mechanical, and antibacterial properties of chitosan/PEO blend films. Biomacromolecules 2007; 8(5): 1505-10.
41. Schiffman JD, Schauer CL. Cross-linking chitosan nanofibers. Biomacromolecules 2007; 8(2): 594-601.
42. Yue IC, Poff J, Cortes ME, et al. A novel polymeric chlorhexidine delivery device for the treatment of periodontal disease. Biomaterials 2004; 25(17): 3743-50.
43. Fu Y, Kao WJ. Drug release kinetics and transport mechanisms of non-degradable and degradable polymeric delivery systems. Expert opinion on drug delivery 2010; 7(4): 429-44.