Journal of Materials Science: Materials in Medicine

Alginate membranes loaded with hyaluronic acid and silver nanoparticles to foster tissue healing and to control bacterial contamination of non-healing wounds

--Manuscript Draft--

Manuscript Number:	JMSM-D-17-00556R1
Full Title:	Alginate membranes loaded with hyaluronic acid and silver nanoparticles to foster tissue healing and to control bacterial contamination of non-healing wounds
Article Type:	Original Research
Corresponding Author:	Eleonora Marsich, Ph.D University of Trieste Trieste, ITALY
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	University of Trieste
Corresponding Author's Secondary Institution:	
First Author:	Lorena Tarusha
First Author Secondary Information:	
Order of Authors:	Lorena Tarusha
	Sergio Paoletti
	Andrea Travan
	Eleonora Marsich, Ph.D
Order of Authors Secondary Information:	
Funding Information:	EU-FP7 Project “AnastomoSEAL” (Contract Number 280929) — Prof Sergio Paoletti
Abstract:	Chronic non-healing wounds are a clinically important problem in terms of number of patients and costs. Wound dressings such as hydrogels, hydrocolloids, polyurethane films and foams are commonly used to manage these wounds since they tend to maintain a moist environment which is shown to accelerate re-epithelialization. The use of antibacterial compounds is important in the management of wound infections. A novel wound-dressing material based on a blended matrix of the polysaccharides alginate, hyaluronic acid and Chitlac-silver nanoparticles is here proposed and its application for wound healing is examined. The manufacturing approach to obtain membranes is based on gelling, foaming and freeze-casting of alginate, hyaluronic acid and Chitlac-silver nanoparticles mixtures using calcium ions as the cross-linking agent. Comprehensive evaluations of the morphology, swelling kinetics, permeability, mechanical characteristics, cytotoxicity, capability to inhibit metalloproteinases and of antibacterial property were conducted. Biological in vitro studies demonstrated that hyaluronic acid released by the membrane is able to stimulate the wound healing meanwhile the metal silver exploits an efficient antibacterial activity against both planktonic bacteria and biofilms. Overall, the experimental data evidence that the studied material could be used as antibacterial wound dressing for wound healing promotion.
Response to Reviewers:	Answers to Reviewer #1 Reviewer: Abstract - End of first paragraph - antibacterial compounds are not essential in the management of wounds. Author: We changed in the text the sentence “The use of antibacterial compounds is essential in the management of the wounds to avoid infections” with the more correct “The use of antibacterial compounds is important in the management of wound infections”.

Reviewer: P3 line 20 change "to chronic" to "with chronic".
Author: Changed in the text as suggested

Reviewer: P3 line 20 drug-resistant infection
Author: We changed in the text the sentence “A key problem associated with chronic non-healing wounds is bacterial infection because the skin barrier functionality has been compromised” with the one “A key problem associated with chronic non-healing wounds is drug-resistant infection because the skin barrier functionality has been compromised”

Reviewer: P3 line 52 elaborate on biofilms - they are not necessarily composed of antibiotic resistant bacteria per se.
Author: We agree with the Reviewer. In a biofilm the bacteria are not necessarily antibiotic resistant and in the same way antibiotic-resistant bacteria are not always organized in a biofilm. For this reason, we replaced the sentence “the rising antibiotic-resistance of bacteria requires the use of new broad-spectrum antibacterial agents active against biofilms” with the more generic one “the rising antibiotic-resistance of bacteria requires the use of new broad-spectrum antibacterial agents”

Reviewer: P8 line 40 change "were added" to "was added". Same for line 44 and 52. P9 line 2 "was used" P9 line 32 change to "bacterial growth"
Author: The changes were done in the text

Reviewer: P10 Was any attempt made to evaluate the number of bacteria on the material? A biofilm could have formed which the experimental protocol would have missed. This is also true of the CLSM protocol - as well as examining the TC plate, the material should also have been viewed. Please consider dong this experiment or countering my concern.
Author: We understand the concern of the reviewer but some considerations can be done about this. The scope of the experiment described in paragraphs 2. 9 and 3.4 was to verify the capability of the membrane to eradicate a preformed biofilm. For this reason, the biofilm formation was induced on a TC plate surface and following on the surface was applied the membrane. The aim of the MTT assay and of the CLSM test was to point out the capability of the material to break apart mature pre-existing biofilms. Moreover, we understand that the term “biofilm formation inhibition assay” used at the beginning of the paragraph 2.9 to describe the experiment can be misunderstood, so we described the experiment with the term “biofilm eradication assay”. Indeed, the capability of the material to prevent the bacterial growth and proliferation is demonstrated by the bacterial growth inhibition assay described in the same paragraphs.

Reviewer: P12 line 47. Please define extract medium - is it that described in the paragraph above?
Author: We have described and defined the “extract medium” in the manuscript.

Reviewer: P14 line 57. Suggest change "pointed out" to "revealed".
Author: We changed as suggested

Reviewer: P17 line 49. Suggest expand to describe the values obtained.
Author: We expanded in the manuscript the description of the experimental data.

Reviewer: P19 line 4. Explanation for mechanism should go in the discussion.
Author: We moved the explanation in the discussion part.

Reviewer : P20 line 52. Suggest to re-word sentence - "membranes and non-treated cells" part unclear
Author: We re-worded the sentence more clearly

Reviewer #2
Reviewer: Why silver and not iodine? As iodine is used against a broad-spectrum of bacteria
The authors agree with the Reviewer that iodine is a good antiseptic compound still not diffusely used in wound dressing materials. Literature data report a spectrum of action

	against microorganisms comparable to that of silver ions. Moreover, reports are conflicting as the adverse effects of both iodine and silver in the treatment of wounds when the compounds are systemically absorbed. One of the main advantages of the material described in the present paper is the capability of the polymeric matrix to firmly entrap the antibacterial component, so avoiding systemic toxic side effects due to its massive diffusion in the body, without impairing the topic bactericidal activity. Reviewer: For mechanical characterization, was the sample dry or preswollen? Clarify this in the Methods part Author: The material is in its dry state and we clarified this in the Methods part Reviewer: 2.7 this set-up is unclear for me. Would it perhaps be possible to visualize this in a sketched figure? Author: The experimental set-up was designed following the guidelines reported in the ASTM E96 standard test document as cited in the text. So, we considered sufficient to describe in words the set-up without making heavier the manuscript with an additional figure. Reviewer: Quality figures need to be increase. Try to save them as .Tiff files. Especially Figure 1, Figure 2, Figure 5, Figure 8, … Author: The figures have been resubmitted in TIFF and eps format with the resolution specified by the guidelines of the journal Reviewer: "The hydrogel was formed by the use of Ca2+ ions (derived from CaCO3 dissociation by acidification of the solution) which homogeneously cross-link the alginate chains" -> why was CaCl2 not used to ionically cross-link? Author: The alginate-gelation method used in this paper is based on the slowly acid hydrolyzing of D-gluconic acid delta-lactone (GDL) that induces the slow release of cross-linker calcium ions from CaC03 insoluble particles. This internal controlled gelation process is commonly used to prepare alginate gels (Biomaterials. 2001 Mar;22(6):511-21). It guarantees the formation of a higher homogeneous gel in comparison with gels formed by the dropping of a CaCl2 solution into an alginate solution. Reviewer: 3.2 Reswelling (writing mistake) Author: Corrected Reviewer: Figure 4a: is the first point at 0 minutes relevant? This is evident and unneeded here I would say. Author: We agree with the reviewer and we removed the point at 0 minutes from the graph. Reviewer: Figure 5: "long-term slow release of the antimicrobial compound": is this necessary? Is the application for longer than a week? The release is too slow for the intended application. What percentage should be released and at what time span? Author: The aim of the experiment was to demonstrate that free silver ions, cytotoxic when present at high concentrations, are released very slowly and then are present at low concentrations in the surrounding environment. The developed membranes are aimed to exploit a bactericidal activity both by direct contact of the bacteria and by the release of the bactericidal compound. Reviewer: Conclusion: indeed, the membranes are non-cytotoxic, but for the antibacterial properties, is the slow release of silver sufficient? Author: We understand the concern of the Reviewer, but in vitro experimental data confirm that the material is active against both planktonic bacteria and biofilm.

Alginate membranes loaded with hyaluronic acid and silver nanoparticles to foster tissue healing and to control bacterial contamination of non-healing wounds

Lorena Tarusha[1], Sergio Paoletti[1], Andrea Travan[1], and Eleonora Marsich[2*]

[1] Department of Life Sciences, University of Trieste, Via Licio Giorgieri 5, I-34127 Trieste, Italy

[2]Department of Medical, Surgical and Health Sciences, University of Trieste, Piazza dell'Ospitale 1, I-34129 Trieste, Italy

*Corresponding author: Eleonora Marsich, Department of Medical, Surgical and Health Sciences, University of Trieste, Piazza dell'Ospitale 1, I-34129 Trieste, Italy, phone +39 040 558 8733 email: emarsich@units.it

Abstract

Chronic non-healing wounds are a clinically important problem in terms of number of patients and costs. Wound dressings such as hydrogels, hydrocolloids, polyurethane films and foams are commonly used to manage these wounds since they tend to maintain a moist environment which is shown to accelerate re-epithelialization. The use of antibacterial compounds is important in the management of wound infections.

A novel wound-dressing material based on a blended matrix of the polysaccharides alginate, hyaluronic acid and Chitlac-silver nanoparticles is here proposed and its application for wound healing is examined. The manufacturing approach to obtain membranes is based on gelling, foaming and freeze-casting of alginate, hyaluronic acid and Chitlac-silver nanoparticles mixtures using calcium ions as the cross-linking agent. Comprehensive evaluations of the morphology, swelling kinetics, permeability, mechanical characteristics, cytotoxicity, capability to inhibit metalloproteinases and of antibacterial property were conducted. Biological in vitro studies demonstrated that hyaluronic acid released by the membrane is able to stimulate the wound healing meanwhile the metal silver exploits an efficient antibacterial activity against both planktonic bacteria and biofilms. Overall, the experimental data evidence that the studied material could be used as antibacterial wound dressing for wound healing promotion.

Keywords: alginate, hyaluronic acid, chitlac, silver nanoparticles, wound healing

1.Introduction

The cascade of events leading to a non-healing wound is not well defined, although several features of the impaired physiological process have been discovered [1]. These wounds are characterized by a prolonged inflammation [2, 3], an inhibited proliferative activity [4, 5], a defective migration of keratinocytes and a modified fibroblasts function with passage from a synthetic phenotype to a degrading and senescent phenotype [6]. Senescent fibroblasts cause defects on ExtraCellular Matrix (ECM) integrity because the production of high levels of proteolytic enzymes and of decreased levels of MMPs' inhibitors [7-9].

A key problem associated with chronic non-healing wounds is drug-resistant infection because the skin barrier functionality has been compromised. The bacteria strains isolated from chronic wounds are mainly *Staphylococcus*, *Enterococcus* and *Pseudomonas* [10]. Co-existence of aerobic and anaerobic bacteria and biofilm formation can contribute to create a drug-resistance infection that escapes the host immune response [11, 12].

Although treatments of chronic wounds can differ slightly, the most common ones consist in removing damaged and necrotic tissue, reducing tissue exudates and minimizing pain [13-15]. Wound dressings such as hydrogels, hydrocolloids, polyurethane films and foams are commonly used since they tend to maintain a moist environment that was shown to accelerate re-epithelialization and create a physical barrier that reduces bacterial contamination [16, 17]. Alginate dressings appear to be particular effective in the management of highly exuding wounds. The topical and systemic administration of antibiotics constitutes the main clinical practice to prevent and treat bacterial infections in these wounds.

However, the rising antibiotic-resistance of bacteria requires the use of new broad-spectrum antibacterial agents [18]. Silver has been proposed as a bactericidal agent in wound management. Wound dressing containing silver in different forms like ions, nanocrystalline silver, inorganic complexes and silver sulfadiazine are described in the scientific literature [19-

21] and some products are available on the market (*e.g.* Aquacel® Ag from Convatech, Acticoat™ from Smith and Nephew, UrgoTul® Ag/Silver from Urgo Medical). Their effectiveness has been ascribed to silver release [22].

Travan *et al.* [23] reported the production of well-dispersed silver nanoparticles (AgNPs) by chemical reduction of silver ions in the presence of a lactose-modified chitosan (Chitlac). Embedding the nanocomposite system Chitlac-AgNPs into alginate and chitosan gels results in anti-bacterial and non-cytotoxic biomaterials [23-26].

Hyaluronic Acid (HA) is a bioactive molecule with a wide range of biological functions such as stimulation of cell proliferation, differentiation, migration and angiogenesis [27, 28], modulation of inflammation and of the immune cells function [29]. The beneficial effects of HA released by alginate-based membranes on viability and migration of human primary fibroblasts and keratinocytes have been already demonstrated by Travan *et al.*[30].

In this work, we describe a pliable and porous alginate-based membrane in which Chitlac-AgNPs and HA are combined together to endow the material with biological properties exploitable in the treatment of non-healing wounds. The membrane is produced by foaming, gelling and freeze-casting a ternary mixture of alginate, HA and Chitlac-AgNPs; the material is characterized in terms of physical-chemical, mechanical and biological properties. This material is designed to combine the bioactive properties of HA and the bactericidal activity of silver nanoparticles in a foamed alginate matrix.

2.Materials and methods

2.1Materials

Chitlac (lactose-modified chitosan, CAS number 85941-43-1) was synthesized starting from commercial chitosan from Sigma-Aldrich (degree of acetylation 18%) as reported by Donati *et*

al.. [31]. The composition of Chitlac was determined by means of ^{1}H-NMR and resulted to be: glucosamine residues 20%, N-acetyl-glucosamine 18% and 2-(lactit-1-yl)-glucosamine 62%. The relative Molecular Weight (MW) of Chitlac is around $1.5x10^{6}$.

Alginate from *Laminaria hyperborea* (Alginate Pronova UP LVG; MW= 120 000; fraction of guluronic residues, FG = 0.69; fraction of guluronic diads, F_{GG} = 0.59; number average of G residues in G-blocks, $N_G>1$ = 16.3) was kindly provided by Novamatrix/FMC Biopolymer (Sandvika, Norway). The sodium hyaluronate (Phylcare Sodium Hyaluronate extra LW) was purchased from Biophil Italia Spa (MW ≈ 100 - 400 kDa).

Hydroxy-methyl-2-propyl cellulose (HMPC, CAS number 9004-65-3, Pharmacoat 603, substitution type 2910) was purchased from Shin-Etsu (Tokyo, Japan).

Calcium carbonate ($CaCO_3$), D-Gluconic acid δ-lactone (GDL), glycerol (ReagentPlus® ≥ 99.0%), silver nitrate ($AgNO_3$), ascorbic acid, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), EDTA, Mitomycin C, 2-(N-Morpholino)ethanesulfonic acid (MES), sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$), hydrochloric acid (HCl), MTT formazan powder [1-(4, 5-dimethylthiazol-2-yl)-3,5-diphenylformazan], glucose, ethanol, Hanks' Balanced Salt solution (HBSS, product code H8264), phosphate buffered saline (PBS), Luria Bertani (LB) broth, LB Agar and Brain Heart Infusion (BHI) broth were purchased from Sigma-Aldrich (Chemical Co. U.S.A).

Dulbecco's Modified Eagle's Medium high glucose and fetal bovine serum were from Euroclone (Italy). AlamarBlue® Cell Viability Reagent, Medium106 and Low Serum Growth supplement was purchased from Life Technologies. MMP activity assay Kit (Fluorimetric-Green), MMP-9 and MMP-2 recombinant enzymes were from Abcam®.

2.2Synthesis of silver nanoparticles

Silver nanoparticles were prepared using Chitlac as a stabilizing agent as reported by Travan *et al.* [23]; briefly, Chitlac 4 g/L was dissolved in deionized water and the solution was added with $AgNO_3$ 2 mM and ascorbic acid 1 mM (final concentrations), incubated overnight at room temperature in the dark and subsequently stored at 4°C.

2.3Manufacturing of membranes

The membranes were prepared according to the following procedure: alginate (8 g/L) and HA (4 g/L) were completely dissolved in water and then HEPES buffer pH 7 to final concentration 0.01 M was added. Chitlac-AgNPs solution (2 g/L of polymer final concentration), HPMC (4 g/L) and glycerol (5% V/V) were then added. An *in situ* gelation of the mixture was obtained by the addition of $CaCO_3$ (20 mM) and GDL (40 mM). Immediately after GDL addition, the solution was foamed for 30 seconds and its aliquots were cured in tissue culture plates for 24 h at room temperature to allow complete gelification. The hydrogels were then step-wise cooled by immersion in a liquid cryostat. Temperature was decreased stepwise from 20 to -20 °C by 5 °C steps with 30 minutes' intervals; the samples were then freeze-dried.

2.4Mechanical characterization

Mechanical characterization was done according to ASTM D638 standards – by using a Universal Testing Machine (Multitest Mecmesine 2.5-i) with load cell of 100 N. The membranes were carved with a cutter into dog-bone shape and gripped with metallic clamps. Tensile tests were performed on dry membranes at a crosshead speed of 5 mm/min. Tensile stress was calculated dividing the load by the average original cross sectional area in the gage length segment of the specimen. Young's Modulus was calculated as the slope of the linear portion in the stress-strain curve, considering the deformation range of 1%-3%. For each

formulation, five replicates were used, the data were averaged and standard deviations calculated.

2.5Scanning electron microscopy (SEM) analysis

Morphological analysis of the freeze-casted membranes was performed with a Leica-Stereoscan 430i Scanning Electron Microscope. In environmental conditions, membrane specimens were sputter-coated with gold prior to observation.

2.6Swelling tests

Freeze-casted membranes were cut into pieces of 20 mm of diameter, weighed and immersed in 10 mL of HBSS. After 1, 2, 10, 30, 45 and 60 minutes, materials were blotted for 1 minute on a filter paper and weighted. The swelling ratio was calculated as the ratio between the weight at time X and the initial dry weight at time 0, following the equation:

$$Swelling\ ratio = \frac{Weight\ at\ time_x}{Weight\ at\ time_0}$$

2.7Water-vapor transmission rate

For determining the ability of the membranes to transmit vapor, ASTM E96 standard test method for Water Vapor Transmission of materials was used.

Glass bottles with a top closure of 16 mm of diameter were filled with mQ water in order to have 2 cm of distance between the water and the sample, closed with a round shaped piece of membrane sample of 17 mm of diameter and sealed laterally with Parafilm®. The bottle was weighted and incubated for 24 and 48 hours at 32 ± 0.4 °C and 45 ± 2 % humidity. At the end of the incubation time the bottle was weighed.

Commercially available dressings were used as control samples: *Connettivina plus*® (Fidia farmaceutici S.p.A.) and *Chitoderm*™ (Pietrasanta Pharma S.p.A.). Furthermore, uncapped

bottles and bottles capped with Parafilm were used as control of free evaporation and no evaporation, respectively.

Water vapor transmission rate was calculated according to the formula:

$$WVTR \quad (g/m^2h) = \frac{W_{24} - W_0}{A * 24h}$$

where:

W_{24} = weight at 24 hours (in grams),

W_0 = initial weight of the filled and capped bottle (in grams),

A = area of the top closure of the bottle (in m^2).

Four replicates were performed per each sample and the analysis was done by calculating average and standard deviation of samples both at 24 and 48 hours.

2.8Silver release

For the quantification of released silver, 60 mg of membrane composed of alginate 8 g/L, HA 4 g/L, Chitlac-AgNPs 2 g/L, HPMC 4 g/L, glycerol 5 %, $CaCO_3$ 20 mM, and GDL 40 mM was immersed in 3 mL of HBSS for 2, 24, 72 hours and 7 days at 37 °C. At the defined time the membrane was removed from HBSS solution to which 0.5 mL NH_4OH 1 N solution was added in order to solubilize silver precipitates (release solution). The membrane was washed in 3 mL of HBSS for 2 minutes and, after membrane removing, 0.5 mL NH_4OH 1 N was added to the solution. Both release solution and washing solution were used for the measurement. Membranes without Chitlac-AgNPs (45 mg) and composed of alginate 8 g/L, HA 4 g/L, HPMC 4 g/L, glycerol 5%, $CaCO_3$ 20 mM, GDL 40 mM) were used as control samples.

Measurements were performed by Electro-Thermal Atomic Absorption Spectrometry (ETAAS) with Zeeman background correction. A Thermo M series AA spectrometer equipped

with a GF95Z Zeeman Graphite Furnace and a FS95 Furnace Autosampler (Thermo Electron Corporation, Cambridge, UK) was used for analysis.

The obtained values have been compared with a calibration curve calculated with a standard solution of silver (Silver ICP/DCP-Sigma Aldrich). The limit of detection (LOD) at the analytical wavelength of 328.1 nm was 0.5 µg/L and the precision of the measurements as repeatability (RSD %) for the analysis was always less than 5%.

Data analysis was performed by summing the silver found in the release solution and the washing solution. The values were express as percentage of silver released in comparison with the total amount of silver contained in the membrane (148 µg/60 mg of membrane).

2.9Antimicrobial tests

To assess the antimicrobial activity three different bacterial strains were used: *Staphylococcus aureus* (ATCC® 25923™), *Staphylococcus epidermidis* (ATCC® 12228™) and *Pseudomonas aeruginosa* (ATCC® 27853™). Two different protocols were employed: bacterial growth inhibition assay and biofilm eradication assay.

Bacterial growth inhibition assay:

Membrane samples were cut into pieces of 40 mg and sterilized under UV rays. Bacteria were inoculated in Luria Broth medium (LB) and incubated for 16 hours at 37 °C. After 16 h, 500 µL of bacterial suspension was diluted in 10 mL of LB and grown up for 120 min at 37 °C in order to restore an exponential growth phase. The optical density at 600 nm was measured in order to assess bacterial concentration. Bacteria were diluted to $5x10^6$ CFU/mL in 10% (V/V) LB in PBS and 1 mL of bacteria was added to each sample and incubated for 24 hours at 37°C. Tests were carried out in shaking condition (140 rpm) to optimize the contact between bacteria and membranes. At the end of the incubation time, bacterial suspensions were diluted in PBS and each suspension was spread on LB agar. After overnight incubation at 37 °C, the colony

forming units (CFU) were counted. A suspension of bacteria grown in liquid medium was used as control.

Biofilm eradication assay:

S. aureus and *P. aeruginosa* were inoculated in Brain Heart Infusion (BHI) broth plus 3 % w/V sucrose and incubated for 16 hours at 37 °C. Bacteria were then diluted 1:100 in the same broth and seeded (300 µL/well) into 24-well plates for 48 hours in static conditions at 37 °C. Broth was then removed and biofilm was carefully rinsed twice with 100 µL of sterile PBS in order to remove non-adherent cells. 300 µL of PBS were added to each well and circular specimens of membranes (40 mg) were deposited on the bacterial layer and incubated for 24 hours. MTT assay was then performed according to Brambilla *et al.* (Brambilla *et al.*, 2012). Briefly, MTT stock solution was prepared by dissolving 5 mg/mL of MTT powder in PBS and sterilized by filtration. Membranes and PBS were gently removed from the plates and each well was carefully rinsed three times with 100 µL of PBS. 200 µL of MTT solution (prepared by mixing 0.5 mL of MTT stock solution and 4.5 mL of sterile PBS) were placed into each well and the plates were incubated for 3 h under lightproof conditions at 37 °C. The MTT solution was then gently removed and formazan crystals were dissolved by adding 200 µL of DMSO to each well. Plates were stored for 1 h under lightproof conditions at room temperature, then 80 µL of the solution were transferred into the wells of a 96-well plate and, absorbance was measured using a spectrophotometer (Infinite M200 PRO NanoQuant, Tecan) at a wavelength of 550 nm. Outcomes were expressed as optical density (O.D.) units.

For confocal laser scanning microscopy (LSCM) analyses of biofilm, bacteria were seeded on sterile 13 mm tissue culture coverslips (Sarstedt, U.S.A.) placed inside a 24-well plate. After biofilm growth and its treatment with membranes as described above, FilmTracer Live/Dead biofilm viability kit (Invitrogen™) was used for biofilm staining following the manufacture's protocol. Images were acquired on a Nikon Eclipse C1si confocal laser-scanning microscope

with a Nikon Plan Fluor 20X as objective. Resulting stacks of images were analyzed using ImageJ software. The ratio between red and green signals was calculated as:

$$Normalized \cdot rate = \frac{Sample\left(\frac{\mathrm{Re}\, d}{Green}\right)}{Non-treated\left(\frac{\mathrm{Re}\, d}{Green}\right)}$$

where:

Red = means grey value of the red channel

Green = means grey value of the green channel

2.10Cell culture

Primary human dermal fibroblasts (HDFa) were purchased from Gibco™. Cells were grown in Medium 106 supplemented with Low Serum Growth supplement, 100 U/mL penicillin, and 100 µg/mL streptomycin.

Human keratinocyte cell line HaCaT (kindly gifted by Dr. Chiara Florio, University of Trieste) was grown in Dulbecco's Modified Eagle's Medium high glucose, 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine. Cells were maintained at 37 °C in a humidified, 5 % CO_2 environment.

2.11Cytotoxicity test

Evaluation of cellular toxicity of membranes was performed according to ISO10993-5 specifications by direct-contact test.

Membranes were cut using biopsy punches into cylinders of 4 mm of diameter and sterilized under UV-rays.

HDFa and HaCaT cells were seeded in 24-well plates at a density of $2x10^4$ cells per well and 16 hours later, test samples were placed directly on the cell layer for 24 and 72 hours. Samples were then removed, cells were washed in PBS and 250µL of Alamar Blue dye diluted 1:10 in culture medium was added and incubated for 4 hours. 100 µL of Alamar solution were transferred onto a 96-well microtiter plate and fluorescence was measured with a microplate reader (Tecan Infinite® M100 Pro) at λ_{ex} 544nm / λ_{em} 616 nm. As positive control material, polyurethane films (PU) containing 0.25% zinc dibuthyldithiocarbamate (ZDBC) (6 mm disks) were used (Hatano Research Institute / Food and Drug Safety Center Reference Material Office - Japan). As a negative control material, polystyrene sheets (PS) (6 mm disks) were used (Wako Pure Chemical Industries).

2.12Scratch wound healing assay

UV-sterilized membrane samples were placed in Dulbecco's modified Eagle's medium, FBS 10%, penicillin 100 U mL-1, streptomycin 100 µg mL-1 and L-glutamine 2 mM for 24 h at 37 °C (extract medium). The ratio between sample weight and medium volume was 15 mg/mL for the membrane without Chitlac-AgNPs and 20 mg/mL for the membrane with Chitlac (ChM membranes) and with Chitlac-AgNPs (SM membranes).

HDFa and HaCaT cells were seeded in 6-well plates at a density of $2.5x10^5$ and $3.5\ x10^5$ cells, respectively, and incubated at 37 °C until reaching a confluent monolayer. Cells were then treated with extract medium. Each treatment was performed in duplicate. 24 hours after treatment cell layers were wounded by scratching with a 200 µl pipette tip and the scratch closure was followed over time through an Olympus CK2 Inverted Microscope (Phase Condenser ULWCD 0.3) equipped with a camera Canon PowerShot A630. The images of the scratch were acquired over time to monitor the wound closure. The analysis was performed

using software Image J: the region of interest (ROI) was outlined per each scratch and the percentage of closure over time was plotted. For each treatment, eight images were analyzed.

2.13Matrix Metalloproteinases activity assay

MMPs activity was determined by using the MMP Activity Assay Kit following the manufacture's protocol. Recombinant MMP-2 and MMP-9 enzymes were activated by treatment with 1 mM 4-Aminophenylmecuric Acetate (APMA) for 1 h and 2 h, respectively and used in the reaction mixture at the concentration 1 U/μL.

For MMPs extraction, HDFa and HaCaT were lysed in PBS by freeze-thaw cycles using a dry ice/ethanol bath and a water bath at 37°C. MMP enzymes present in HDFa and HaCaT lysates were activated with 1 mM APMA for 3 hours at 37 °C.

Chitlac and Chitlac-AgNPs were pre-incubated 20 minutes at RT with recombinant MMPs or cell lysates before addition of the substrate solution supplied by the manufacturer of the kit. After 1 h of incubation with substrate at RT, endpoint measurement was performed at λ_{ex} 490 nm and λ_{em} 525 nm with a Tecan Infinite® M100 Pro plate reader.

3. Results

3.1Material preparation and characterization

The employed method for the manufacturing of the membranes consists of gelling a foamed solution composed of alginate, HA, a colloidal suspension of antimicrobial silver nanoparticles coordinated by the polymer Chitlac (Chitlac-AgNPs), HPMC and glycerol (as the plasticizer), followed by freeze casting the resulting hydrogel (Figure 1). The experimental conditions to obtain the miscibility of the oppositely charged polysaccharides (namely Chitlac, alginate and

HA), avoiding polymer phase separation, have been already explored in previous studies of this group [32].

The hydrogel was formed by the use of Ca^{2+} ions (derived from $CaCO_3$ dissociation by acidification of the solution) which homogeneously cross-link the alginate chains.

After freeze-drying, the gel became a soft, flexible and spongy membrane (Figure 1). The orange-brown color is produced by the combination of the specific size (*i.e.* 35 nm) and of the spectroscopic properties of the silver nanoparticles. It should be noticed that, owing to the procecure devised, such material can be easily produced with the desired dimensions or cut in different shapes to match the wound shape and size.

For the sake of clarity, the resulting membranes will be named in the manuscript as "SM" when containing silver (in the form of the Chitlac-AgNPs system), as "ChM" when containing Chitlac (without silver nanoparticles), and as "CM" when containing neither Chitlac nor silver nanoparticles.

The foamed membrane was analyzed by electron microscopy in the dry state both as top view and as cross-section. Figure 2 reports the SEM images of SM and CM membranes. In both cases, the cross section shows a highly porous structure while the top view shows a smooth surface.

An ideal wound dressing material must have good tensile strength because the dressing should not be damaged by handling procedures and must withstand the stress resulting from skin movements without rupturing.

The stiffness (Young's Modulus), the resistance (stress at break) and the deformation (strain at break) of the material were then studied by uniaxial tensile tests; the results are reported in Figure 3.

The results revealed that, in the presence of Chitlac-AgNPs (SM), the material displays lower strength and deformation at break as compared with the membrane without Chitlac-AgNPs

(CM): this could be reasonably ascribed to the destabilizing role of the polycation Chitlac with respect to the alginate cross-linked matrix. However, in the range of small (elastic) deformations, the Young's modulus of SM and CM membranes are comparable.

3.2Reswelling and water vapor transmission rate studies

The reswelling behavior of SM was studied in HBSS buffer. Figure 4a reports the liquid uptake expressed as a variation over time of the normalized weight (weight of the membrane at t_x over weight of the dry membrane). It can be observed that within the first minute of soaking the material weight underwent a six-fold increase.

After the initial liquids uptake, the slow decrease of membrane weight can be ascribed to the slow release of HA, in line with the data previously reported by Travan *et al.* (2016) [30].

WVTR (Water Vapor Transmission Rate) was measured for the SM membrane, as well as for the ChM and CM membranes. Their thickness was 3 mm. WVTR is defined as the transmission of the water vapor per unit time through a unit area of the tested material. Two commercially available membranes were used as control samples: Connettivina plus® (Fidia farmaceutici S.p.A.) is a highly permeable gauze soaked with hyaluronic acid while Chitoderm™ (Pietrasanta Pharma S.p.A.) is a chitosan-containing pad with a polyurethane layer which limits water permeability. The results are reported in Figure 4b.

The SM membrane has a WVTR (78.5 ± 2.6 g/m^2h) comparable with that of ChM and CM membranes. Moreover, the WVTR of these membranes are comparable with the WVTR of *Connettivina plus*®. As expected, the WVTR of *Chitoderm*™ is much lower (20.8 ± 1.2 g/m^2h) than that of the other samples.

3.3Silver release studies

The amount of silver released from the membranes was quantified by Electro-Thermal Atomic Absorption Spectrometry (ETAAS).

A progressive, albeit very low, release of silver can be seen over time (Figure 5). The silver released from membranes is only a minimal fraction of the total content of silver: after one week, only 0.9% of the total silver is leaked, indicating that most of the metal is firmly entrapped inside the material, which represents a potential reservoir for a long-term slow release of the antimicrobial compound. Similar results were described also by Travan *et al.* for alginate hydrogels containing Chitlac-AgNPs [23]. It is noteworthy to observe that the capability of the polymeric matrix to stabilize silver particles is an important feature to avoid an excessively fast release of silver in the physiological environment, thus limiting potential toxic effects towards eukaryotic cells [23].

3.4 Antibacterial activity against planktonic bacteria and mature biofilms

The antimicrobial activity on planktonic bacteria was tested on *Staphylococcus aureus, Staphylococcus epidermidis* and *Pseudomonas aeruginosa* by incubating the bacterial suspension with the membranes for 24 hours. The Colony Forming Units per milliliter (Log CFU mL^{-1}) calculated for each sample after 24 hours are reported in Figure 6.

In Figure 6 it can be observed that the SM membrane induces a significant decrease of CFUs for all the bacterial strains tested, in comparison with the growth of bacteria treated with the CM membrane. More specifically, there is a decrease of 3.5, 5.6 and 4.8 Log CFU mL^{-1} for *S. aureus, S. epidermidis, P. aeruginosa,* respectively.

There is a significant difference also between the bacteria in plain broth and the bacteria treated with the SM membrane. In detail, there is a decrease of 3.1, 3.5 and 4.8 Log CFU mL^{-1} for *S. aureus, S. epidermidis, and P. aeruginosa,* respectively.

Bacteria growing into biofilms are metabolically very different from planktonic bacteria and often display a high antibiotic resistance [11]. Antibacterial tests on mature biofilms have been then performed: Figure 7 pointed out the results obtained after 24 h of treatment of *P.*

aeruginosa and *S. aureus* biofilms with the antibacterial membrane. In both cases, the antibacterial activity is preserved even when bacteria grow into biofilms. The optical density after MTT assay is twice lower for biofilms treated with the membrane containing silver compared with the control membrane.

To confirm the data obtained *via* MTT assay, bacterial viability in the biofilms was visualized by fluorescence confocal microscope after treatment with the Live/Dead staining assay. Biofilms were grown into glass coverslips and treated for 24 hours with the membranes. After Live/Dead staining, images were acquired and analyzed calculating the ratio between the red signals (dead cells) and the green signal (living cells) both for the treated sample and the non-treated sample as shown in Figure 8.

The Live/Dead assay confirmed data from MTT assay: in the biofilm treated with the silver-containing membrane, there is a significant increase of the ratio between red and green signal compared to the non-treated biofilms. The difference is definitely higher for *S. aureus* biofilms (ratio value = 3.2) than for *P. aeruginosa* (ratio value = 1.85).

3.5 Viability of cells treated with membranes

In order to assess the in vitro biocompatibility of SM membranes for dermatological applications, their toxicity was tested by the Alamar Blue assay using human primary fibroblasts (HDFa) and a keratinocyte cell line (HaCaT). Sterilized membranes were applied by direct contact on the cells and the analysis were performed after 24 and 72 hours. As a positive control of cell death, cells treated with a toxic material (Pb/Zn) were considered. Figure 9 shows the results on HDFa and HaCaT cells, respectively.

The result pointed out that 24 and 72 h after treatment, the products released from SM sterilized membranes did not affect cell viability. The growth rate of the cells treated with the SM membranes is not significantly different from that of the cells in contact with the membranes

without silver (ChM) and in contact with the negative control material (PS). On the contrary, a dramatic and significant decrease of growth rate is observed for the cells treated with the positive control material (Pb/Zn).

3.6Scratch test on cells treated with components released from membranes

Travan *et al.* demonstrated that HA released from the alginate matrix during the first hours of soaking in physiological solutions promotes *in vitro* fibroblast proliferation and migration [30]. To confirm also for the SM membranes the wound healing effect *in vitro*, scratch tests were performed on both keratinocytes (HaCaT) and fibroblasts (HDFa) treated with the components released from membranes in culture medium. Confluent monolayers of cells were scratched as described in the Methods and then allowed to re-epithelialize for 48 h at 37 °C. The percentage of scratch closure over time is shown in Figure 10 for both HDFa and HaCaT cells. The behavior of the cells treated with the extracts from SM and ChM membranes can be considered as comparable, indicating that the presence of the metal does not impair the positive biological response. For HaCaT cells, after 24 hours from the treatment, there is a significant difference between cells treated with the membrane containing Ag and the non-treated cells (75% *vs.* 56% of gap closure, respectively, with p-value < 0.05). Also HDFa cells, exposed to SM membranes, showed significantly improved scratch closure (p-value < 0.01) after 24 and 30 hours in comparison with the control. In former case the gap closure was 60% after 24 hours and almost 100% after 32 hours, while in the control the gap closure was 40% and 87% at 24 and 32 hours, respectively.

Overall, the data indicates that HA released from the SM membrane can support the physiological healing process.

3.7 MMPs activity assay

The influence of Chitlac-AgNPs on MMPs activity was then studied both on total MMPs extracted from cells and on purified MMP-2 and MMP-9 enzymes (Figure 11).

Chitlac-AgNPs are more efficient to influence MMP-2 activity rather than MMP-9 and they display an inhibition efficacy more pronounced than that exerted by Chitlac alone, used as control.

4.Discussion

The biomaterial proposed in this work is based on the modification and implementation of a protocol used for the preparation of alginate/hyaluronic acid membranes recently published by some authors of this paper [30]; that material was designed to release HA, as a bioactive polysaccharide, from the 3D solid-like architecture of the calcium-alginate structure to promote the healing of tissues.

Based on the above mentioned paper, the present study is aimed at implementing such membranes with additional bioactive components for the specific management of infected non-healing wounds**.** The resulting reticulated matrix was meant to provide the physical structure for the entrapment of silver nanoparticles coordinated by Chitlac and the release of the bioactive component HA upon contact with the target tissue.

The process of foaming aims at creating a spongy structure to enable a high uptake of water and to increase the extension of the bactericidal surface.

HPMC was chosen as foaming agent because of its biocompatibility and its use in the preparation of porous alginate-based matrices [33]. The concentration of HPMC was selected by testing cell viability at different concentrations of HPMC inside the membranes (data not shown). The HA here used (MW 240 000) was previously selected between a range of HA with

different molecular weights. HA 240 000 was shown to stimulate proliferation of fibroblasts and keratinocytes and migration of fibroblasts [30].

All the three polysaccharides (alginate, hyaluronic acid and Chitlac) used for membrane preparation are very hydrophilic polymers because of the presence of a large number of hydrophilic groups such as hydroxyl, carboxyl and amino groups. It was so not unexpected to observe that in water solution the polymeric structure rapidly absorbed a high amount of liquid (Figure 4a**).** Hydrophilic dressings able to efficiently absorb large amount of fluids have been shown to possess a key role in the treatment of highly exudative wounds. A wound requires a moist but not wet environment for proper functioning of the cells responsible for wound healing and to avoid wound margin maceration [34]. Moreover, the absorption of the wound fluids by the dressing promotes the entrapment of colonizing bacteria inside the membrane matrix where antibacterial agents like silver nanoparticles can exert their bactericidal activity**.**

Besides the capability of swelling in contact with fluids, an important requisite of a wound dressing is a suitable water vapor transmission rate (WVTR), adequate to maintain a moist environment, without risking dehydration or exudates accumulation. The biomaterial should keep a balance between presence of liquid and water evaporation [35].

Commercial dressings have been shown to cover a very large spectrum of WVTR, ranging from 90 (Dermiflex®, Johnson&Johnson) to 3350 g/m^2 /day (Beschitin®, Unitika). Clearly, the WVTR is related to the structural properties (thickness, porosity) of the dressing as well as to the chemical composition of the material. However, WVTR within the range 2000 – 2500 g/m^2day have been claimed by Queen et al. as the ideal rate (half of that of a granulating wound) that a wound dressing should possess to provide adequate level of moisture without risking wound dehydration [36]. The SM membrane described in this paper displays an average WVTR of 1920 g/m^2/day (Figure 4b), which matches with reasonable approximation the WVTR value range indicated by Queen et al.[36].

The silver-release test (Figure 5) points out the successful entrapment of the nanoparticles inside the membrane and the low release of free silver ions, evidenced also by the lack of cytotoxicity of the material (Figure 9). The potential cytotoxicity of silver, both as ions or nanoparticles, is a big concern to be given much attention when this metal is used for its antibacterial features. Despite the large use of this compound in silver dressing, delivering of the metal into tissues can result in toxic effects on growing keratinocytes and fibroblasts. Paddle-Ledinek et al. reported cytotoxicity of extracts of some commercial silver-containing dressings (Acticoat, Aquacel-Ag, Contreet-H, and Avance) when exposed to keratinocyte cultures [37].

Besides chronic infections, excess of proteolytic enzymes is one of the major issues of the chronic non-healing wound since it shifts the balance of matrix synthesis towards its degradation, so impairing the granulation tissue formation. MMPs activity in chronic wounds is thirty times higher than the MMPs activity in acute wounds [38]. In particular, elevated levels of MMP-2 and MMP-9 have been found in chronic wounds [38-40]. Selective inhibition of MMPs at the wound site by a dressing is considered an efficient way in achieving a fast tissue healing [41]. The, albeit weak, inhibiting effects that Chitlac exerts on MMPs is not surprising since also chitosan is described to reduce peptidolytic activity of these enzymes, in particular of MMP2. A possible mechanism for the reduction of the activity of MMPs by chitosan is a direct molecular interaction between the enzyme and the polysaccharide [42, 43].

It should be stressed that our findings show a quantifiable higher inhibitory effect of Chitlac-AgNPs on MMPs with respect to Chitlac alone (see Figure 11): in fact, in the former case, the presence of the metal seems to play a leading role to reduce the proteinase activity. Indeed, the use of silver-containing wound care products has been already reported to reduce MMP activity both in vitro and in vivo, although the mechanism for remains unclear. One explanation may

be the displacement of zinc ions by silver (ions) from the proteolytic enzyme, [44, 45] the Ag+ ions deriving from the nanoparticle reservoir.

5. Conclusions

Chronic non-healing wounds are a great clinical problem that is expected to become more serious due to the increasing duration of life and thus the increasing number of people affected by diseases correlated with chronic non-healing wounds. Despite a number of products in the form of membranes, gels and pomades are available in the market [46], there is continuous need of biocompatible biomaterials with a wide spectrum of antimicrobial efficacy and with the capability to prevent biofilm formation, while at the same time stimulating wound closure.

In this work, a wound dressing material based on alginate, hyaluronan and Chitlac-nAg was successfully designed and manufactured in the form of a pliable membrane by using a technical procedure that could be easily scaled-up.

The membrane was shown to support *in vitro* the healing process and to be effective against both planktonic bacteria and bacterial biofilms. This feature makes them appealing for the prevention of bacteria colonization as well as for the treatment of infected sites. Moreover, the component Chitlac-AgNPs was demonstrated to inhibit *in vitro* the proteolytic activity of MMPs, whose overexpression is shown to impair the recommencement of healing in recalcitrant wounds.

In vitro biological studies on the biocompatibility of the membrane on a keratinocyte cell line and on primary fibroblasts showed that the membranes are non-cytotoxic. This behavior can be ascribed to the very low rate of silver release over time due to the effective nanoparticles stabilization within the polysaccharide network.

The reswelling kinetic studies demonstrated that the biomaterial is highly hygroscopic, an important feature in the perspective to remove excessive exudates containing bacteria nutrients

from the wound beds. At the same time, the membranes possess a good water-vapor transmission rate (WVTR) value, which could ensure a moist environment on the wound beds, without risking dehydration or exudates accumulation. The membranes are flexible and can be easily cut into the desired shape. Overall, this novel biomaterial appears particularly suited to contrast the drawbacks of chronic non-healing wounds and so to support tissue regeneration.

References

[1] P. G. Bowler, *Ann. Med.* **2002**, *34*, 419.

[2] S. A. Eming, T. Krieg, J. M. Davidson, *J. Invest Dermatol.* **2007**, *127*, 514.

[3] M. J. Portou, D. Baker, D. Abraham, J. Tsui, *Vascul. Pharmacol.* **2015**, *71*, 31.

[4] M. H. Katz, A. F. Alvarez, R. S. Kirsner, W. H. Eaglstein, V. Falanga, *J. Am. Acad. Dermatol.* **1991**, *25*, 1054.

[5] O. C. Thamm, P. Koenen, N. Bader, A. Schneider, S. Wutzler, E. A. Neugebauer, T. A. Spanholtz, *Int. Wound. J.* **2015**, *12*, 143.

[6] K. G. Harding, K. Moore, T. J. Phillips, *Int. Wound. J.* **2005**, *2*, 364.

[7] S. M. McCarty, C. A. Cochrane, P. D. Clegg, S. L. Percival, *Wound. Repair Regen.* **2012**, *20*, 125.

[8] A. Medina, P. G. Scott, A. Ghahary, E. E. Tredget, *J. Burn Care Rehabil.* **2005**, *26*, 306.

[9] D. R. Yager, B. C. Nwomeh, *Wound. Repair Regen.* **1999**, *7*, 433.

[10] G. A. James, E. Swogger, R. Wolcott, E. Pulcini, P. Secor, J. Sestrich, J. W. Costerton, P. S. Stewart, *Wound. Repair Regen.* **2008**, *16*, 37.

[11] N. Hoiby, O. Ciofu, H. K. Johansen, Z. J. Song, C. Moser, P. O. Jensen, S. Molin, M. Givskov, T. Tolker-Nielsen, T. Bjarnsholt, *Int. J. Oral Sci.* **2011**, *3*, 55.

[12] G. Zhao, M. L. Usui, S. I. Lippman, G. A. James, P. S. Stewart, P. Fleckman, J. E. Olerud, *Adv. Wound. Care (New Rochelle.)* **2013**, *2*, 389.

[13] J. G. Powers, C. Higham, K. Broussard, T. J. Phillips, *J. Am. Acad. Dermatol.* **2016**, *74*, 607.

[14] A. Stojadinovic, J. W. Carlson, G. S. Schultz, T. A. Davis, E. A. Elster, *Gynecol. Oncol.* **2008**, *111*, S70-S80.

[15] F. Werdin, M. Tenenhaus, H. O. Rennekampff, *Lancet* **2008**, *372*, 1860.

[16] J. Boateng, O. Catanzano, *J. Pharm. Sci.* **2015**, *104*, 3653.

[17] C. D. HINMAN, H. MAIBACH, *Nature* **1963**, *200*, 377.

[18] L. R. Peterson, *Clin. Microbiol. Infect.* **2005**, *11 Suppl 5*, 4.

[19] Y. Barnea, J. Weiss, E. Gur, *Ther. Clin. Risk Manag.* **2010**, *6*, 21.

[20] H. Carsin, D. Wassermann, M. Pannier, R. Dumas, S. Bohbot, *J. Wound. Care* **2004**, *13*, 145.

[21] K. Dunn, V. Edwards-Jones, *Burns* **2004**, *30 Suppl 1*, S1-S9.

[22] M. Ip, S. L. Lui, V. K. Poon, I. Lung, A. Burd, *J. Med. Microbiol.* **2006**, *55*, 59.

[23] A. Travan, C. Pelillo, I. Donati, E. Marsich, M. Benincasa, T. Scarpa, S. Semeraro, G. Turco, R. Gennaro, S. Paoletti, *Biomacromolecules.* **2009**, *10*, 1429.

[24] E. Marsich, A. Travan, I. Donati, L. A. Di, M. Benincasa, M. Crosera, S. Paoletti, *Colloids Surf. B Biointerfaces.* **2011**, *83*, 331.

[25] E. Marsich, F. Bellomo, G. Turco, A. Travan, I. Donati, S. Paoletti, *J. Mater. Sci. Mater. Med.* **2013**, *24*, 1799.

[26] P. Sacco, A. Travan, M. Borgogna, S. Paoletti, E. Marsich, *J. Mater. Sci. Mater. Med.* **2015**, *26*, 128.

[27] K. Nakagawa, C. Takahashi, Y. Nishi, H. Jyuen, R. Sugiyama, Y. Kuribayashi, R. Sugiyama, *J. Assist. Reprod. Genet.* **2012**, *29*, 679.

[28] R. Zhu, S. C. Wang, C. Sun, Y. Tao, H. L. Piao, X. Q. Wang, M. R. Du, L. Da-Jin, *PLoS. One.* **2013**, *8*, e74812.

[29] D. Jiang, J. Liang, P. W. Noble, *Physiol Rev.* **2011**, *91*, 221.

[30] A. Travan, F. Scognamiglio, M. Borgogna, E. Marsich, I. Donati, L. Tarusha, M. Grassi, S. Paoletti, *Carbohydr. Polym.* **2016**, *150*, 408.

[31] I. Donati, S. Stredanska, G. Silvestrini, A. Vetere, P. Marcon, E. Marsich, P. Mozetic, A. Gamini, S. Paoletti, F. Vittur, *Biomaterials* **2005**, *26*, 987.

[32] I. Donati, M. Feresini, A. Travan, E. Marsich, R. Lapasin, S. Paoletti, *Biomacromolecules.* **2011**, *12*, 4044.

[33] T. Andersen, J. E. Melvik, O. Gaserod, E. Alsberg, B. E. Christensen, *Carbohydr. Polym.* **2014**, *99*, 249.

[34] K. Ousey, K. F. Cutting, A. A. Rogers, M. G. Rippon, *J. Wound. Care* **2016**, *25*, 122, 124, 130.

[35] D. McColl, B. Cartlidge, P. Connolly, *Int. J. Surg.* **2007**, *5*, 316.

[36] D. Queen, J. D. Gaylor, J. H. Evans, J. M. Courtney, W. H. Reid, *Burns Incl. Therm. Inj.* **1987**, *13*, 357.

[37] J. E. Paddle-Ledinek, Z. Nasa, H. J. Cleland, *Plast. Reconstr. Surg.* **2006**, *117*, 110S.

[38] E. A. Rayment, Z. Upton, G. K. Shooter, *Br. J. Dermatol.* **2008**, *158*, 951.

[39] N. Lopez-Lopez, I. Gonzalez-Curiel, M. B. Trevino-Santa Cruz, B. Rivas-Santiago, V. Trujillo-Paez, J. A. Enciso-Moreno, C. J. Serrano, *Arch. Dermatol. Res.* **2014**, *306*, 809.

[40] N. J. Trengove, M. C. Stacey, S. MacAuley, N. Bennett, J. Gibson, F. Burslem, G. Murphy, G. Schultz, *Wound. Repair Regen.* **1999**, *7*, 442.

[41] C. Wiegand, R. J. White, *J. Wound. Care* **2013**, *22*, 221.

[42] C. Gorzelanny, B. Poppelmann, E. Strozyk, B. M. Moerschbacher, S. W. Schneider, *Biomacromolecules.* **2007**, *8*, 3035.

[43] M. M. Kim, S. K. Kim, *FEBS Lett.* **2006**, *580*, 2661.

[44] M. Walker, P. G. Bowler, C. A. Cochrane, *Ostomy. Wound. Manage.* **2007**, *53*, 18.

[45] J. B. Wright, K. Lam, A. G. Buret, M. E. Olson, R. E. Burrell, *Wound. Repair Regen.* **2002**, *10*, 141.

[46] J. Dissemond, M. Augustin, S. A. Eming, T. Goerge, T. Horn, S. Karrer, H. Schumann, M. Stucker, *J. Dtsch. Dermatol. Ges.* **2014**, *12*, 541.

Acknowledgements: The authors would like to acknowledge Dr. Gianluca Turco for providing assistance in SEM analysis and Dr. Matteo Crosera for ETAAS analysis.

Funding: This study was supported by the EU-FP7 Project "AnastomoSEAL" (Contract Number 280929)

Conflict of Interest: The authors declare that they have no conflict of interest.

Figure captions

Fig.1 Sketch of the procedure to obtain foamed membranes containing Chitlac-AgNPs (left) and image of a membrane with cut edge where the internal spongy structure and the flexibility of the material can be observed (right)

Fig. 2 SEM images of SM (lower figures) and CM membranes (upper figures)

Fig. 3 Mechanical properties of the foamed membranes (SM and CM membranes): Young's modulus (left), stress at break (center) and strain at break (right). Data are expressed as mean ± st.dv. with n = 5

Fig. 4 a) Reswelling behavior of SM membranes. Data are expressed as mean ± st.dv. with n = 4. b) WVTR of membranes and of commercial samples (Connettivina plus® and Chitoderm™). No capped bottles and bottles capped with parafilm were used as control for free evaporation and no evaporation. Data are expressed as mean ± st.dv. with n = 6

Fig. 5 Silver release from the SM membrane over time. Data are expressed as percentage of the total silver contained into the membrane. Data are mean ± st.dv. with n = 3

Fig. 6 Growth rate expressed as Log CFU mL-1 of *S. aureus* (a), *S.epidermidis* (b) and *P. aeruginosa* (c) following 24 h of treatment with SM membranes, ChM membrane and grown in plain culture medium. Data are expressed as mean ± st.dv. with n = 3. For statistical analysis, samples treated with SM membrane were compared with samples treated with ChM membrane. In the Student's test *** indicate $p < 0.001$

Fig. 7 Viable biomass MTT assay expressed as O.D. at 550 nm of *P. aeruginosa* (a) and *S. aureus* (b) following 24 h of treatment with SM and ChM membranes. For statistical analysis, samples treated with SM membrane were compared to samples treated with ChM sample. In the Student's test * indicate $p < 0.1$; ** indicate $p < 0.05$

Fig. 8 Live/Dead assay for *P. aeruginosa* (a) and *S. aureus* (b) biofilms treated with SM membranes: the graphs show the ratio between the red (propidium iodide, indicating dead cells) and the green signal (Syto9, indicating living cells) normalized over the non-treated sample

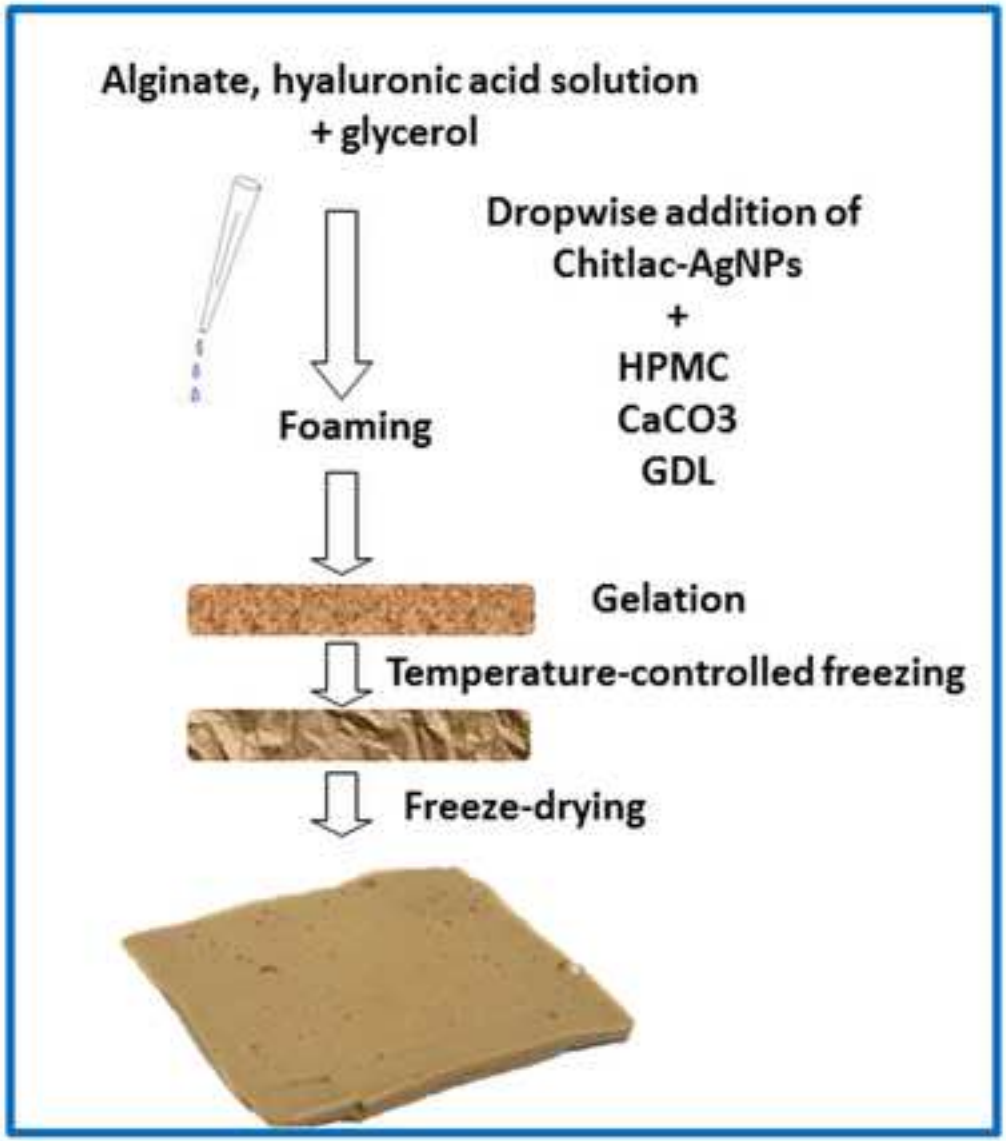
Fig. 9 In vitro cytoxicity tests of HDFa (left) and HaCaT cells (right) treated with membranes by direct contact. PS represents the negative control and ZDBC the positive control. Data are expressed as mean ± st.dv. with n = 4. For statistical analysis (Student's test), samples treated with SM and ChM membrane were compared to samples treated with negative control (PS)

Fig. 10 Scratch assay using human dermal fibroblasts (HDFa) and keratinocytes cells (HaCaT) cultured in absence and presence of extracts from ChM and SM membranes. Data represent means ± st.dv. Statistically significant differences were calculated with a paired student t-test with n=8; *$p<0.05$, **$p<0.01$

Fig. 11 MMPs activity in presence of Chitlac-AgNPs (ChAg) or Chitlac (Ch): (a) effects on MMP-2; (b) Effects on MMP-9; (c) Effects on MMPs extracted from fibroblasts (HDFa cells); and (d) effects on MMPs extracted from keratinocytes (HaCaT cells). Data are expressed as mean ± st.dv. with n=4. Ch = Chitlac, ChAg = Chitlac-AgNPs. The percentage of enzyme inhibition is calculated in comparison with the enzyme activity in the absence of polymers. Statistically significant differences were calculated with a paired student t-test with *$p<0.05$, **$p<0.01$

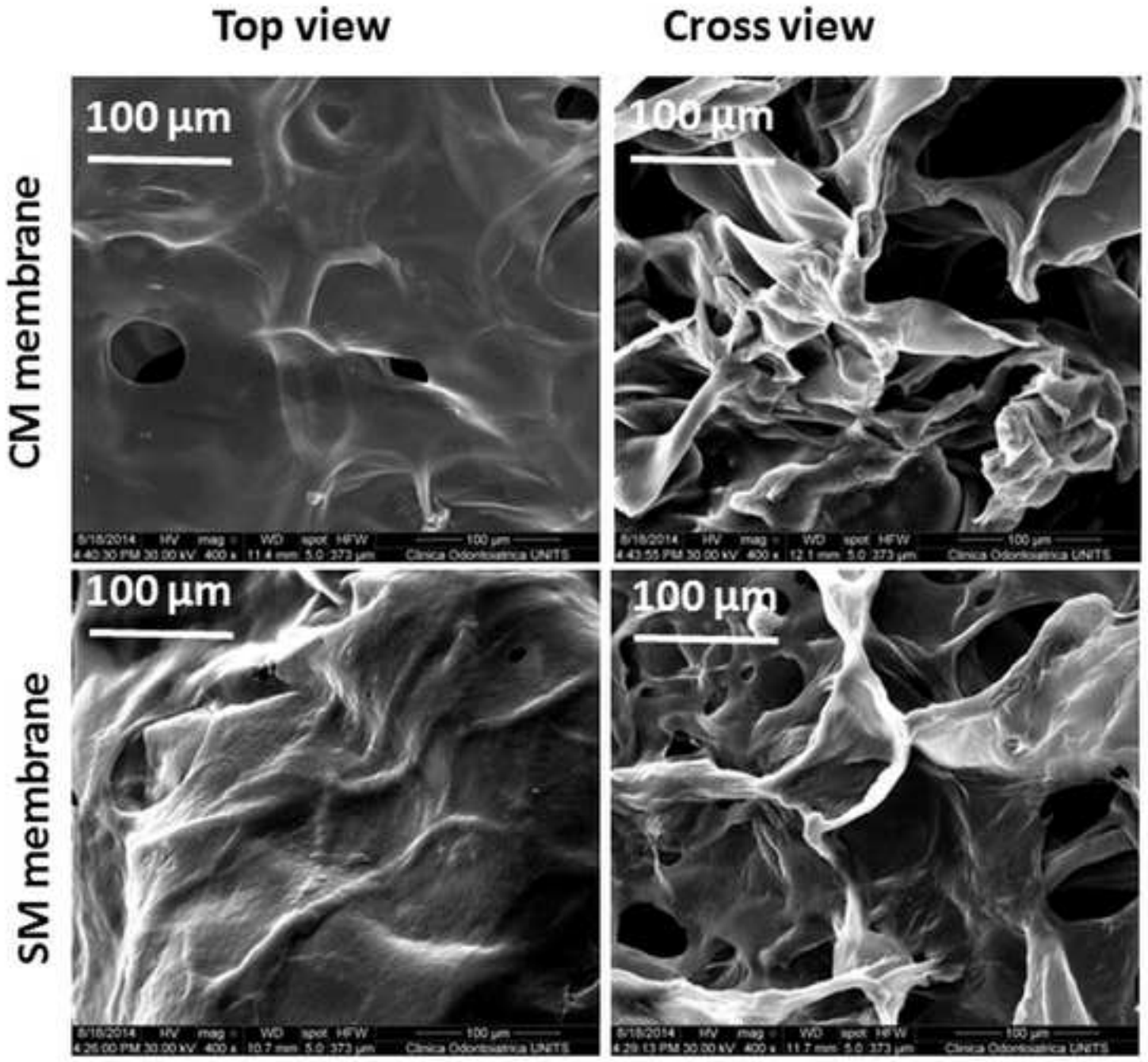
Figure

Click here to download Figure Fig 1.tif



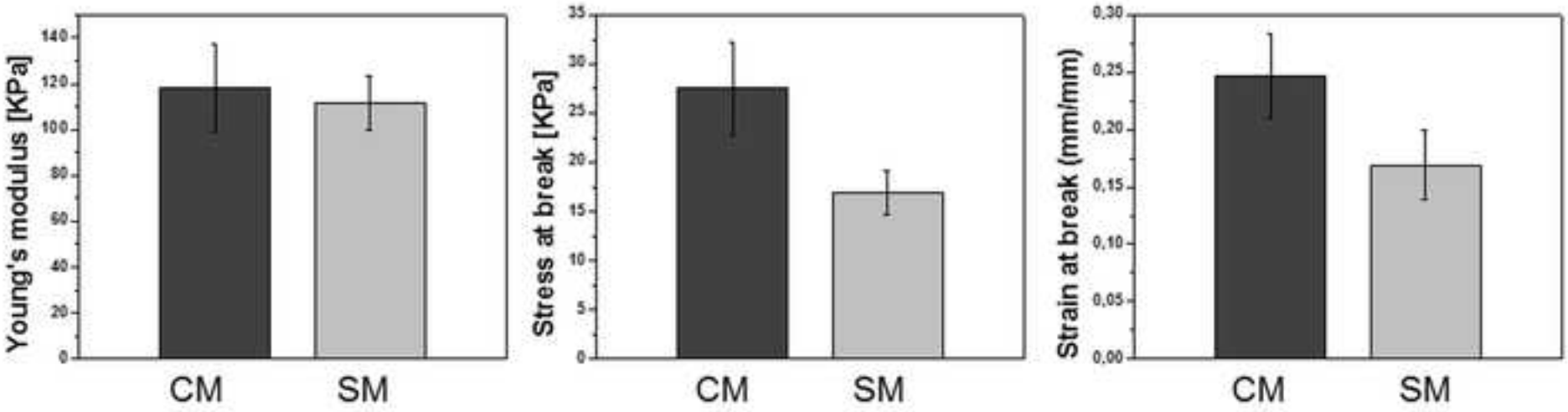
Figure

Click here to download Figure Fig 2.tif



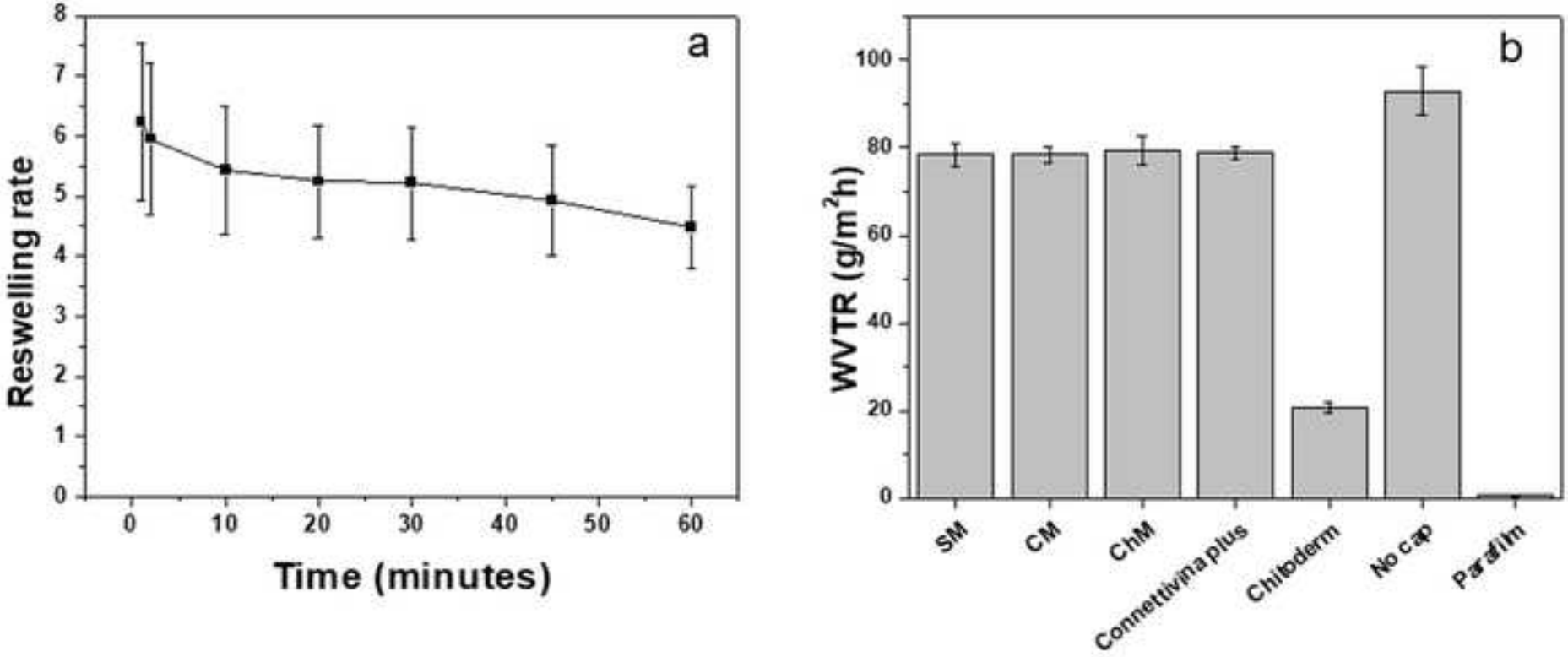
Figure

Click here to download Figure Fig 3.tif



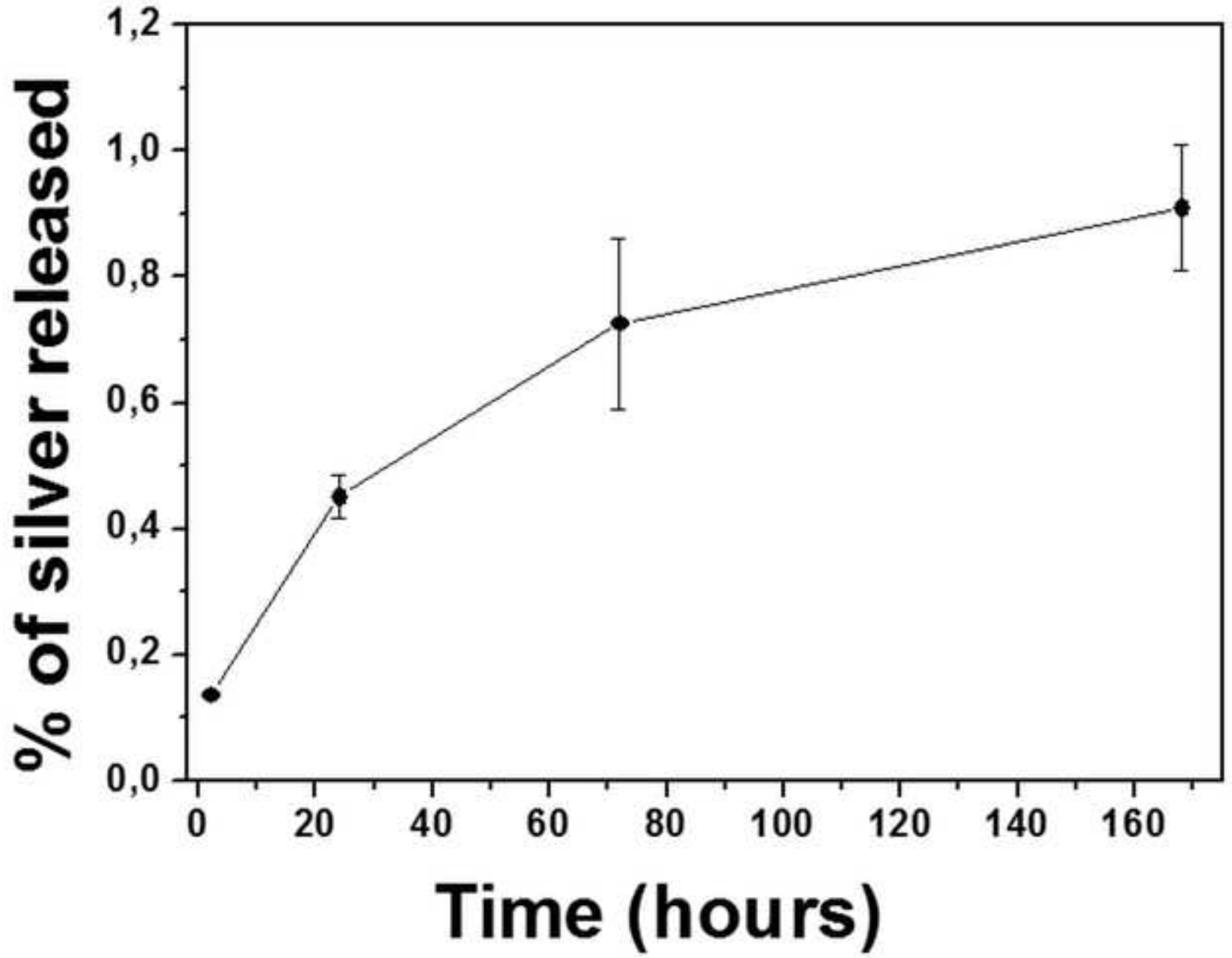
Figure

Click here to download Figure Fig.4.tif



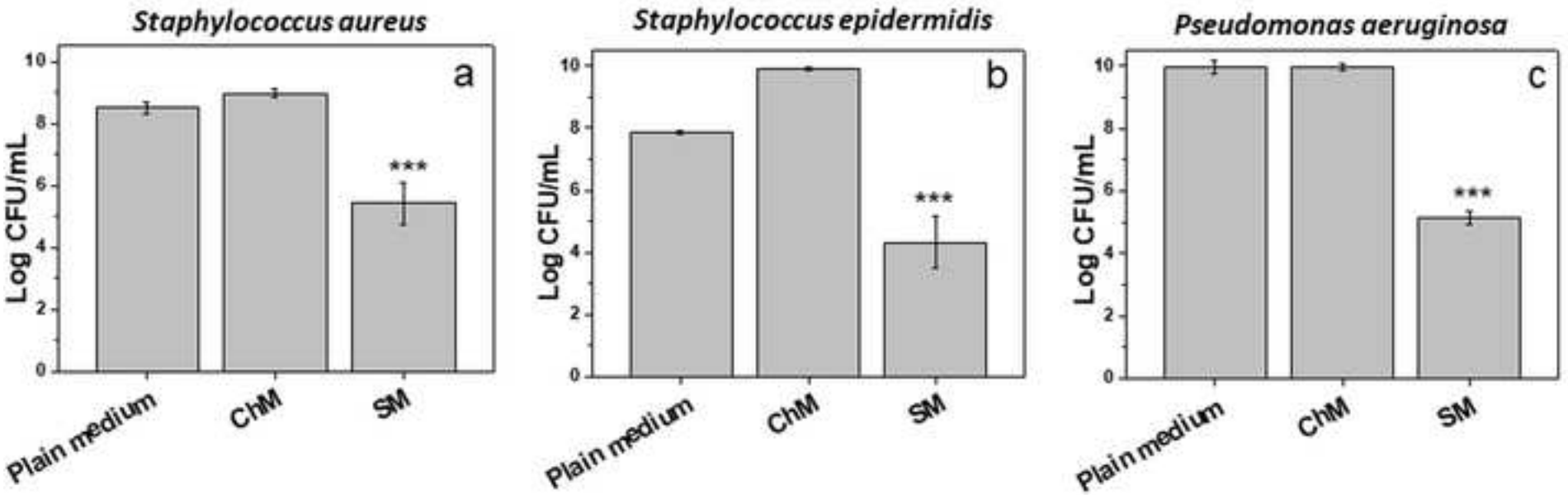
Figure

Click here to download Figure Fig 5.tif



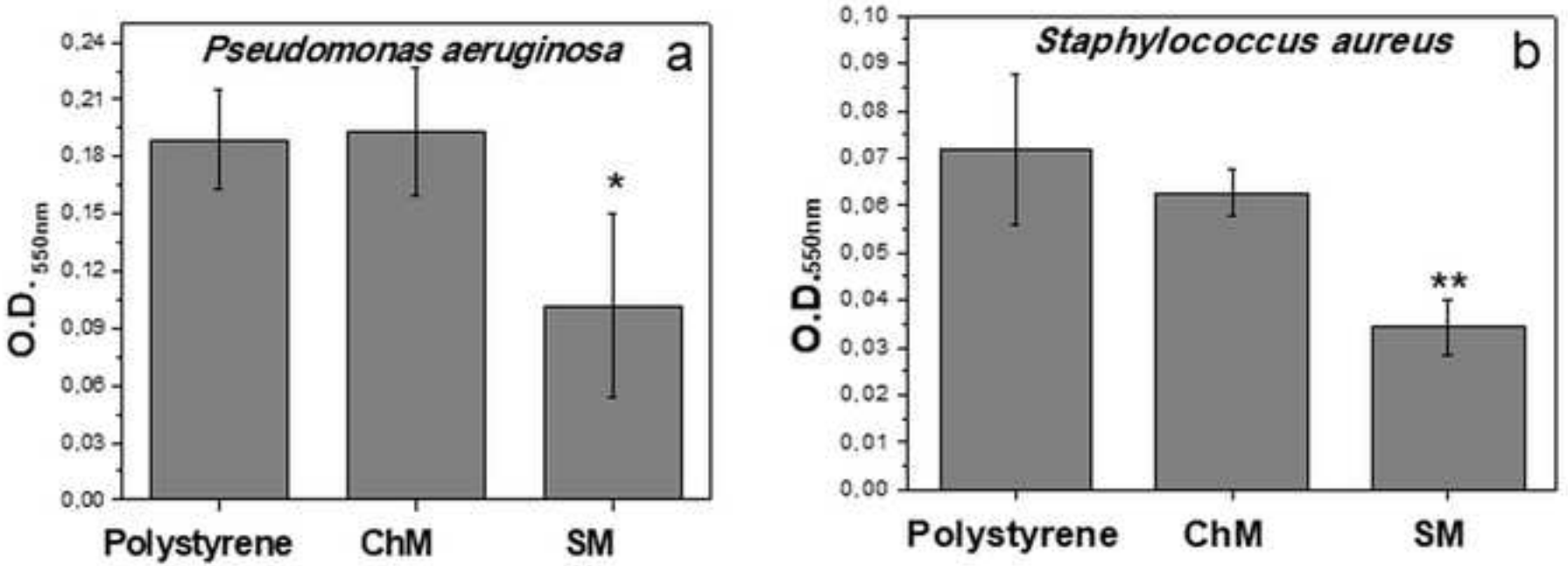
Figure

Click here to download Figure Fig 6.tif



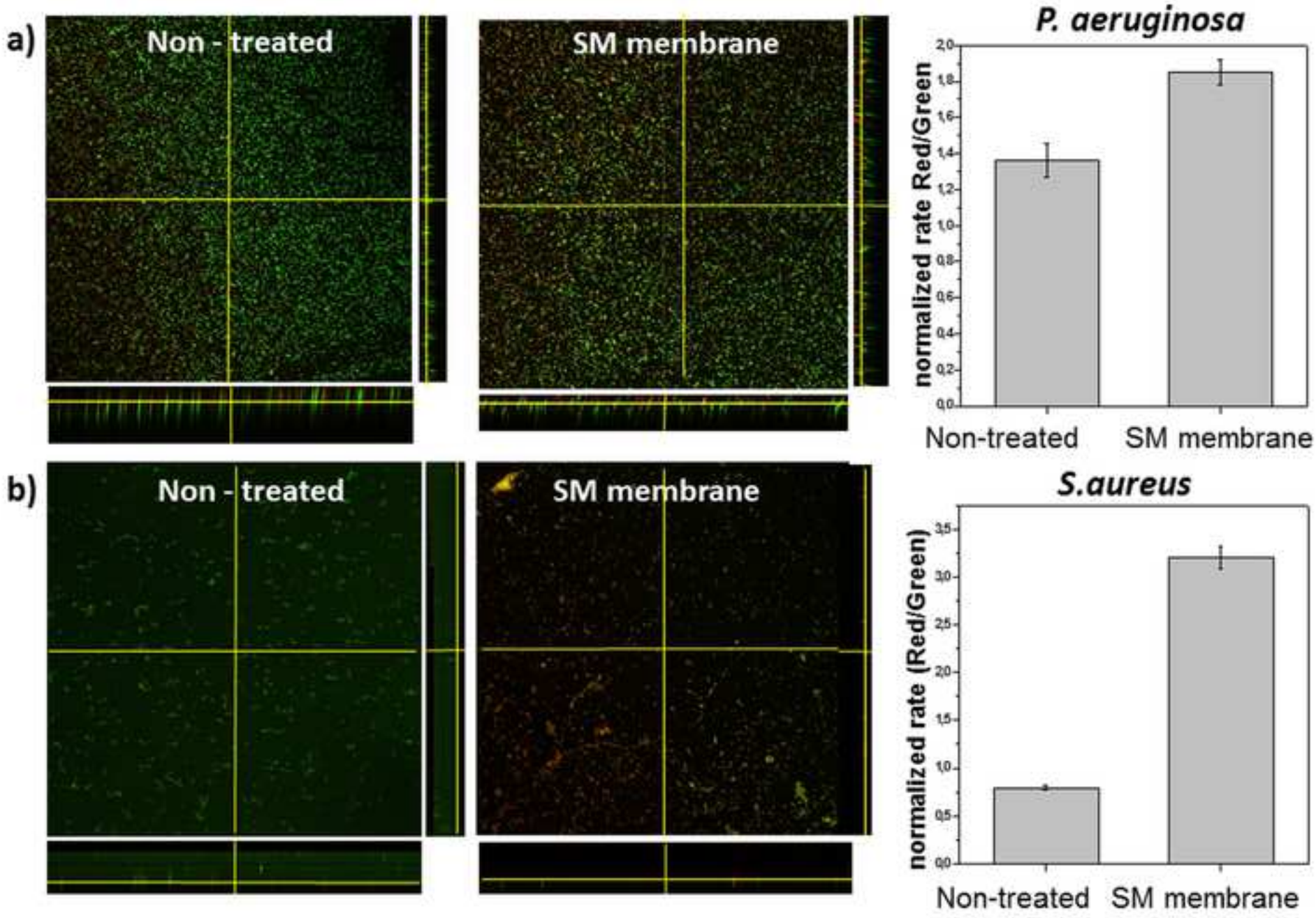
Figure

Click here to download Figure Fig 7.tif



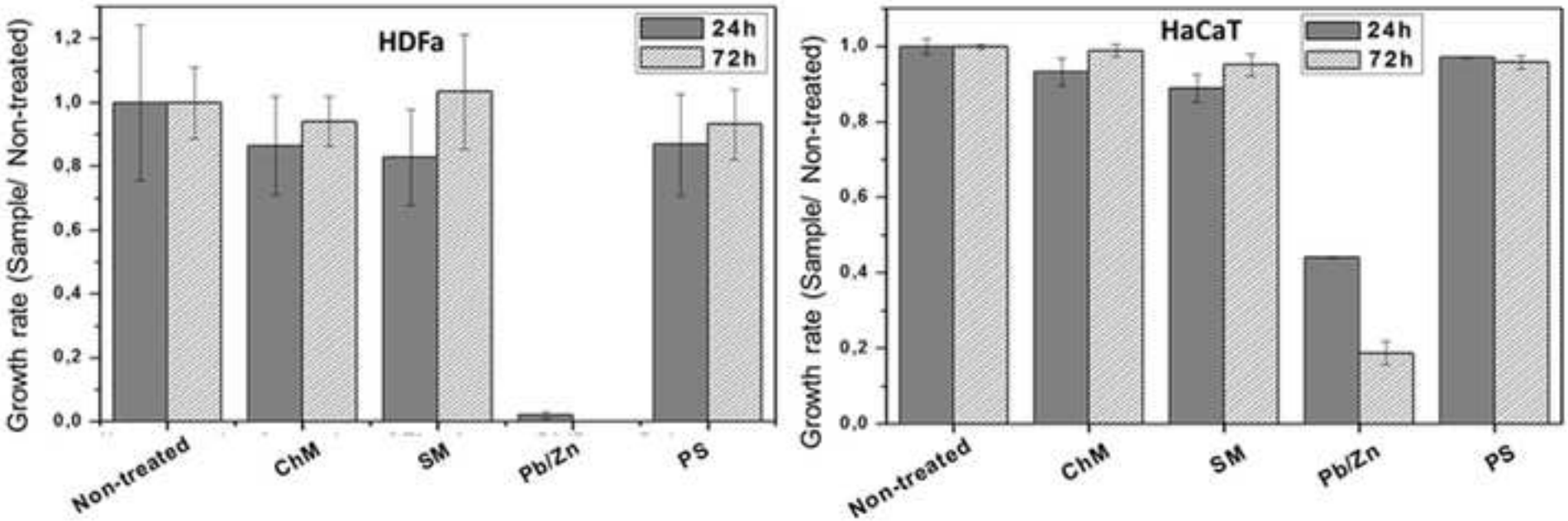
Figure

Click here to download Figure Fig 8.tif



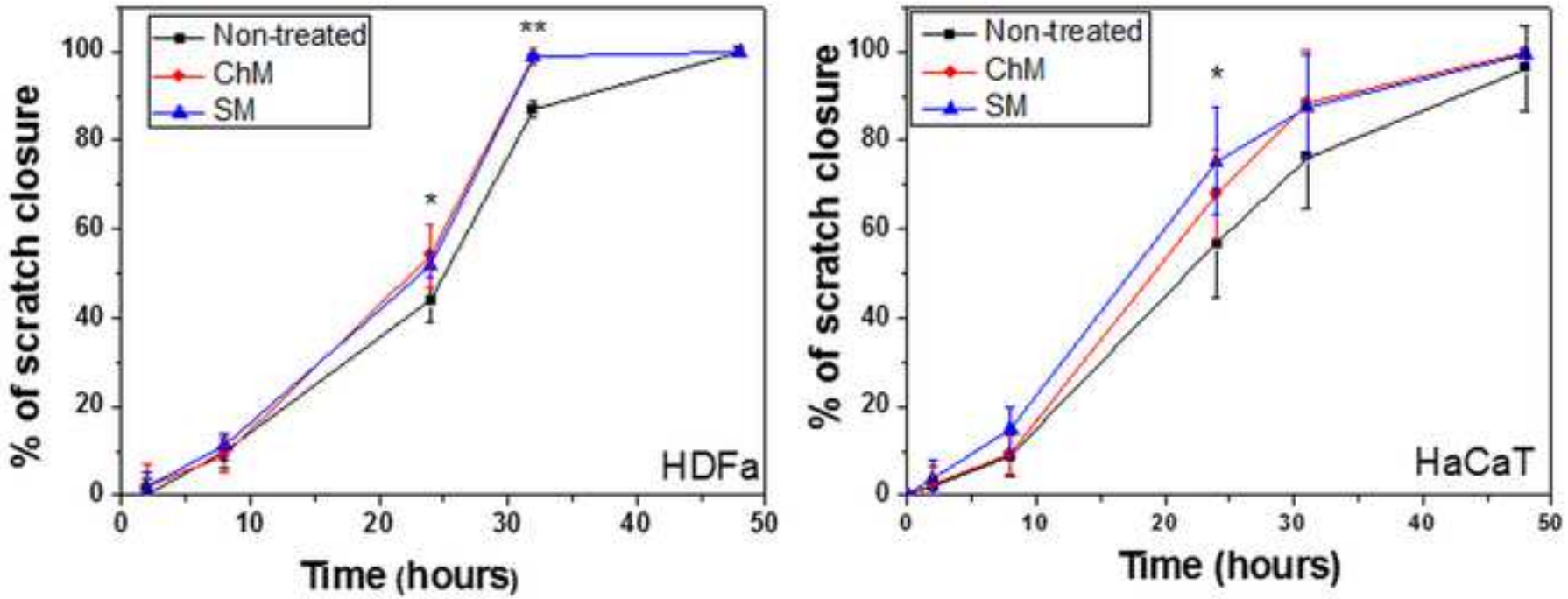
Figure

Click here to download Figure Fig 9.tif



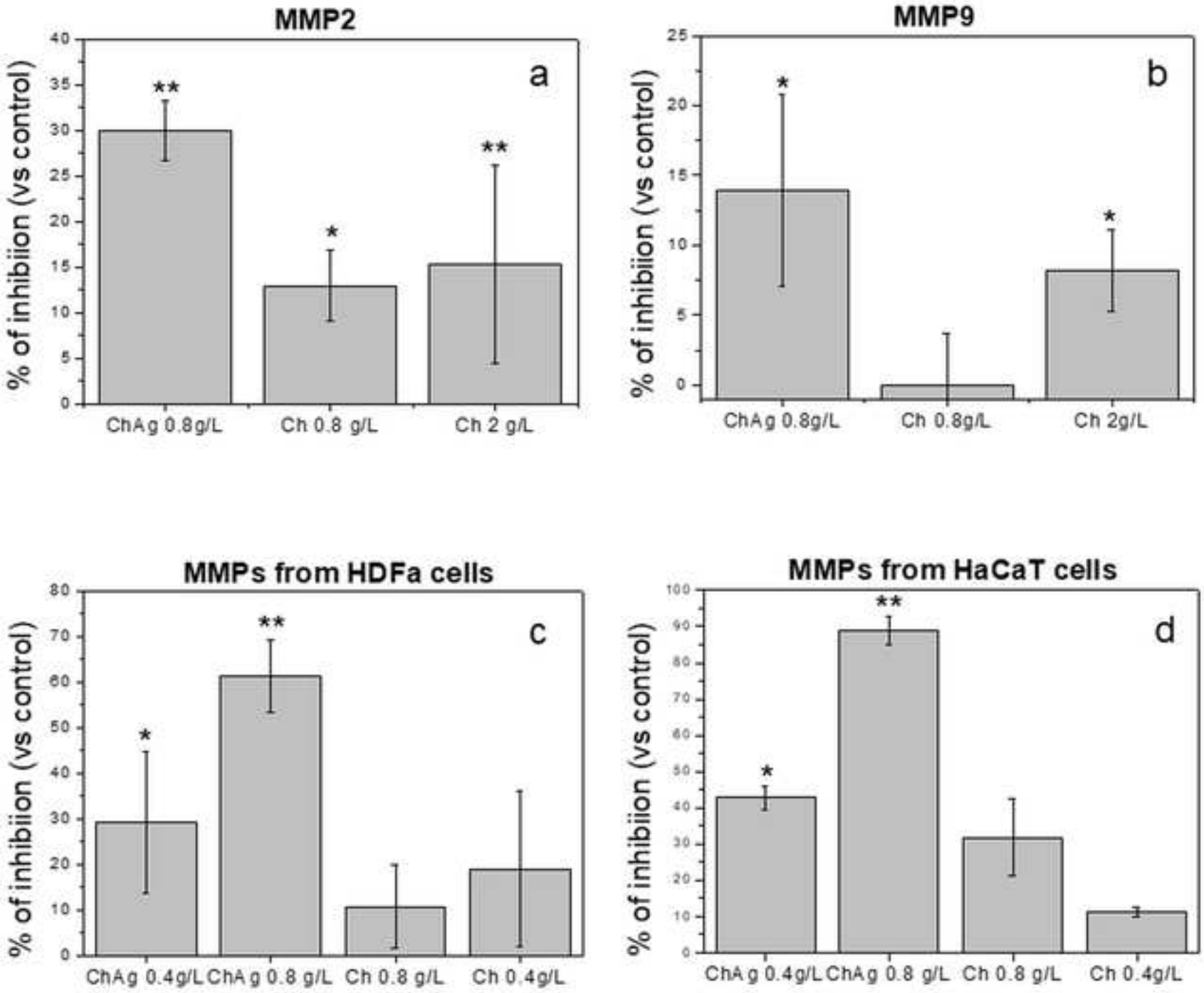
Figure

Click here to download Figure Fig 10.tif



Figure

Click here to download Figure Fig 11.tif



Graphical Abstract