Journal of Applied Polymer Science

# Comparison of the Antibacterial Activity of Modified-Cotton with Magainin I and LL-37 with Potential as Wound-Dressings

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**ABSTRACT:** Wounds are the ideal setting for the development of micro-organisms, so it is often necessary to apply a dressing to control bacterial colonization. Cotton is commonly used in dressings, as it exhibits important hydrophilic characteristics such as high moisture and fluid retention properties, but it may provide a sustainable media for the development of micro-organisms. In this way, the development of new strategies to provide cotton materials with lasting and effective antimicrobial properties is of the utmost importance. Consequently, here we described two processes to develop cotton-dressings functionalized with antimicrobial peptides (AMPs) magainin I (MagI) and LL-37, in order to give cotton-dressings an antibacterial effect. The AMPs showed no cytotoxic effect against human fibroblasts so they are safe to contact with skin. In addition, the functionalized materials with either LL-37 or MagI present an antimicrobial effect exhibiting inhibition ratios of 89% against *Klebsiella pneumoniae* and 58% against *Staphylococcus aureus*, respectively. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 40997.

**KEYWORDS:** biomedical applications; biocompatibility; functionalization of polymers

Received 28 August 2013; accepted 7 May 2014
**DOI: 10.1002/app.40997**

## INTRODUCTION

Skin is a large barrier organ that protects against injuries, helps maintaining fluid homeostasis and aids in sensory detection.[1] Because skin protects against the environment, any break in it must be rapidly and efficiently repaired. Furthermore, wounds are an ideal environment for bacterial development, as the wound bed provides a surface and plenty supply of nutrients.[2] Among the most common micro-organisms that cause wound infection are *Staphylococcus aureus* which is considered "transient flora" of the skin.[3] Often skin wounds need coverage in order to control bacterial colonization, absorb exudate, provide an optimum moisture balance at the wound surface and prevent maceration of surrounding tissue. Hence, it is important to choose an appropriate dressing.[2] Cotton is a standard dressing for the management of wounds, as it presents considerable gas permeability comparatively to occlusive dressings.[4] Wounds can be highly exudative and require frequent dressing changes and cotton gauze is still successfully employed in hospitals and nursing homes for long-term wound care.[4] Nevertheless, cotton textiles have long been recognized as media to support the development of micro-organisms that can grow rapidly in the presence of moisture, nutrients, and temperature. Carbohydrates in cotton can act as energy sources under certain conditions.[5] Also soil, dust, solutes from sweat and some textile finishes can also serve as energy sources for micro-organisms.[5] Therefore, the growth of micro-organisms on textiles may lead to increased likelihood of infection.[5] Consequently, it is highly desirable a reduction of the development and growth of micro-organisms on fabrics.[5] This can be done through the application of an antimicrobial treatment to textiles.

Depending on the bioactive agent and fiber type, several methods have been developed or are under development to confer antimicrobial activity to textiles.[5] For synthetic fibers, the antimicrobial active agents can be incorporated into the polymer prior to extrusion or blended into the fibers during their formation.[5] The conventional exhaust and pad–dry–cure processes have been used to establish an antimicrobial finishing of mainly natural fibers.[5] After the incorporation of the antimicrobial finishing in the textile it is necessary to determine the efficacy of the functionalization process.[5] To do so, a number of test methods have been developed and they generally fall into two categories: the agar diffusion test and suspension test.[5] *Staphylococcus aureus*, a Gram-positive bacterium and *Klebsiella pneumoniae*, a Gram-negative micro-organism, are recommended in these methods since they are potentially pathogenic.[5]

Antimicrobial agents in cotton textiles to be applied as wound dressings need to satisfy a number of requirements: low toxicity, be effective against a broad spectrum of micro-organisms and

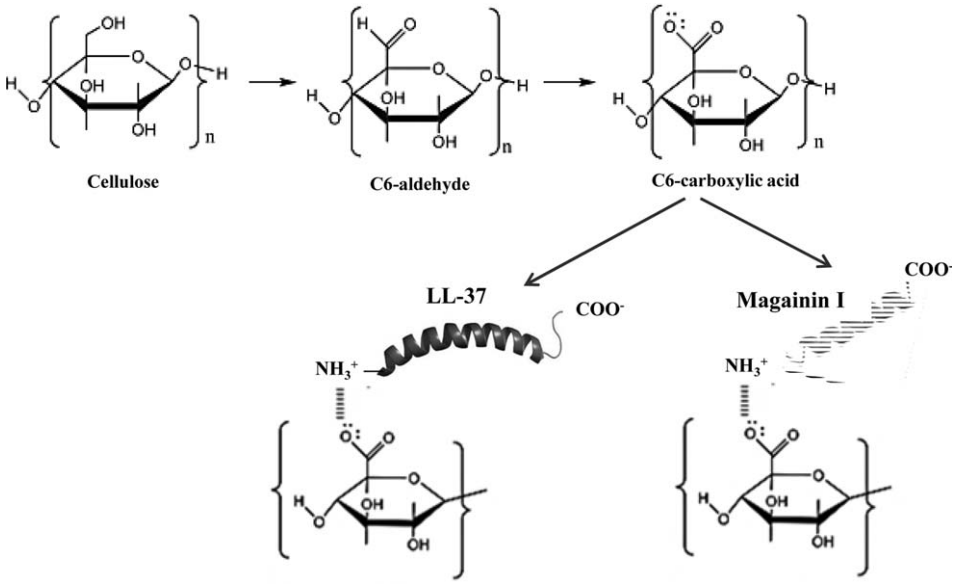


**Figure 1.** Schematic representation of the proposed mechanism of linkage of the antimicrobial peptides onto TEMPO activated cotton samples.

be compatible with textile chemical processes. Conventional antimicrobial agents for textiles are metal and metal salts, quaternary ammonium compounds, polyhexamethylene biguanides, bis-phenols, among others. However, these agents can present side effects, they also can act on non-target micro-organisms and lead to severe environmental impact.[6] Now a days, there is a variety of commercially available dressings from different materials and antimicrobial agents. Specifically, there are several dressings using silver as the antimicrobial agent like, Acticoat™ Absorbent with nanocrystalline silver on a polyethylene mesh dressing; Aquacel® Ag with ionic silver on a sodium caboxymethyl cellulose dressing; Tegaderm™ Ag with silver sulfate on an absorbent mesh dressing.[7] The treatment of natural fibers, like cotton, with metals can only be undertaken at the finishing stage and various strategies have been devised to enhance the uptake and durability.[5] Nakashima et al. (2001)[8] pretreated cotton with succinic acid anhydride to make adsorption of metallic salts more effective. This pretreatment is very effective at increasing the amount of metal ions adsorbed and thus improving antimicrobial activity. However, some concerns have been expressed about the emergence of bacterial resistance due to the overuse of silver, especially in the clinical environment.[5,9]

A Promising alternative to the traditional antimicrobial agents are antimicrobial peptides (AMPs), which are natural molecules produced by many tissues and cell types in a variety of invertebrate, plant, and animal species.[10] Their amino acid composition, amphipathicity, net charge, and size allow them to attach to and create pores in the membranes of micro-organisms.[10]

Magainin I (MagI) is a 23-amino acid AMP obtained from the African frog *Xenopus laevis*.[11] This peptide, has an α-helical structure and is amphipathic.[11] MagI reveals multiple functions related to membrane interactions, being active toward multiple pathogens.[11]

LL-37 is the only member of the cathelicidin family of host defense peptides expressed in humans and it is a linear 37 amino acid peptide produced from the C-terminus of the hCAP18 precursor protein by a proteolytic cleavage.[12]

Taking into account the emergency of AMPs as new antimicrobial agents and the importance of developing more effective and non-cytotoxic antimicrobial cotton gauzes with potential as wound-dressings, this study proposes the production of antimicrobial textiles, by immobilizing magainin I and LL-37 onto cotton. Electrostatic binding of the AMPs was achieved by oxidation of the cotton surface using 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO), which converts hydroxyl groups of carbon-6 of cellulose into carboxylate one's. This step provided the negative net charge at the surface of cotton enabling the binding of the cationic antimicrobial peptides during the functionalization process (Figure 1). The bioactive textiles proved to be able to decrease *Klebsiella pneumoniae* of 15 and 89%, after 18 hours of incubation with the cotton functionalized with magainin I and LL-37, respectively. The *Staphylococcus aureus* reduction percentage after 18 hours of incubation with the textile functionalized with MagI and LL-37 was 58 and 59 %, respectively. Cotton functionalized with LL-37 appears to be the best choice for the development of wound-dressings as it presented a higher bacterial reduction percentage against *Klebsiella pneumoniae* and *Staphylococcus aureus*, after 18 of incubation. In addition, the AMPs showed no cytotoxic effect against human fibroblasts, making them safe to contact with the human skin.

## EXPERIMENTAL

### Organisms

The bacterial strains used were the Gram-positive strain *S. aureus* (ATCC 6538) and the Gram-negative strain *K. pneumoniae* (ATCC 4352), both from the American Type Culture Collection.

### Chemicals

Magainin I and LL-37 were purchased from Eurogentec (Seraing, Belgium).

Nutrient agar and Nutrient broth were from Cultimed (Barcelona, Spain). Sodium hypochlorite and methanol were from Panreac (Barcelona, Spain) and bovine fetal serum (FBS) was from Biochrom (Portugal). All other reagents were obtained from Sigma-Aldrich (St. Louis).

### Minimal Inhibitory Concentration of the AMPs against *Klebsiella pneumoniae* and *Staphylococcus aureus*

The minimal inhibitory concentration (MIC) of magainin I and LL-37 against *S. aureus* (ATCC 6538) *and K. pneumoniae* (ATCC 4352) was determined using the broth microdilution method, adapted from National Clinical and Laboratory Standard, NCLS M7-A6.[13] MagI and LL-37 stock solutions were prepared in sterile deionised water (pH of 5.5) to a concentration of 20 μg/mL. Serial dilutions of the AMPs stock solutions were made in Mueller–Hinton Broth (MHB) with concentrations ranging from 10 to 0.156 μg/mL.

The inoculums were prepared from fresh overnight liquid cultures that were incubated for 24 hours (h) and the bacterial turbidity was adjusted to 0.5 McFarland with sterile 0.85% (w/v) NaCl solution. Afterward, bacterial work suspensions were made by diluting 500 μL of the 0.5 McFarland suspensions in 4500 μL of MHB. Total of 50 μL of the bacterial work suspensions and 50 μL of the AMPs dilutions were added to the wells in a 96 multi-well plate. The multi-well plates incubated for 24 h at 37°C. For each AMP concentration, the turbidimetry of bacterial growth was compared with the controls and all determinations were performed in triplicate.

### Cytotoxicity Assay

Cytotoxicity of the AMPs was evaluated by an MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide) viability assay[14] using normal human dermal fibroblasts (NHDF), since the textile material is intended to be in contact with the human skin. Cells were routinely maintained at 37°C in a humidified atmosphere containing 5% $CO_2$ and cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS), HEPES (0.01*M*), L-glutamine (0.02*M*) and sodium pyruvate (0.001*M*), and 1% antibiotic/antimycotic (10,000 units/mL penicillin, 10 mg/mL streptomycin, and 25 μg/mL amphotericin B). Experiments were performed in 24-well tissue culture plates with $2 \times 10^4$ cells/well. Cells were used on the 20th passage.

Briefly, cells were seeded in 24-well plates ($2 \times 10^4$ cells/well) in culture medium containing FBS and after 48 hours adherence, some wells were treated with two concentrations of MagI (0.20 and 4.17 μg/mL), other wells treated with two concentrations of LL-37 (4.17 and 5.00 μg/mL) and incubated at 37°C, in a 5% $CO_2$ atmosphere, for 48 hours. The concentrations tested were the MIC values against *K. pneumoniae* and *S. aureus*. Untreated cells were used as control. Afterward the liquid content of the wells was removed and it was replaced with 200 μL of MTT solution of 1 mg/mL in PBS. The multi-well plates were incubated for 4 hours, at 37°C, with a 5% $CO_2$ atmosphere, in the dark. Next, the content of the wells was removed and it was added 200 μL of DMSO and 20 μL of Glicil–Glicin buffer to dissolve the formazan crystals and to stabilize the color, respectively. The absorbance of each well was measured at 570 nm using a Biochrom Anthos 2020 microplate reader from Biochrom Ltd. The extent of cell viability was expressed as the percentage of viable treated cells in comparison with control cells.

The cytotoxicity results were submitted to a Student's *t*-test in 95% confidence interval, using the computer software, IBM SPSS Statistics for Windows (version 19.0). *P*-Values $<0.05$ were considered statistically significant.

### Cotton Functionalization Process with AMPs

Firstly the cotton fabric was washed with a standard soap to remove any surface residues that could provide false results regarding the antimicrobial activity. Secondly, the surface of the fabric was oxidized by 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO), which provided the negative net charge at the surface of cotton enabling the adsorption of the cationic AMPs during the functionalization process (Figure 1).

A solution of 0.0125% (w/v) of TEMPO; 0.125% (w/v) of sodium bromide, and 3.2% (v/v) of sodium hypochlorite was prepared and its pH value was adjusted at 10.5. Then, 2 g of fabric samples were submersed in 50 mL of the previous solution and stirred for 60 minutes. Afterward, the pH of the solution was adjusted to 7 and the samples were washed in deionized water. After the activation process, the cotton samples were submitted to the functionalization process by exhaustion.

For the exhaustion functionalization process, solutions of magainin I and LL-37 were diluted to a concentration of 4.17 and 5.00 μg/mL in deionized water, respectively. The concentrations corresponded to the higher MIC values for each antimicrobial peptide and were selected because they have shown the higher antimicrobial effect. Samples, with a total of 4 g, were immersed in 100 mL of the bioactive solution and placed in the containers of the Ahiba IR datacolor dyeing machine from Datacolor, Inc. and functionalized at 45°C for 90 minutes, at 15 rpm.

Afterward, all samples were washed in a 1 g/L of a solution of AATCC 1993 Standard Reference Detergent Without Optical Brightener (WOB), for 5 washing cycles performed at 40°C during 60 minutes, a method adapted from the international standard EN ISO 105-C06:2010.[15] Samples were then dried at room temperature. The soaping procedure was performed over the samples treated with the AMPs prior to further investigation and assessment of antibacterial activity, in order to give evidence of the fastness of the functionalization process.[16]

**Effectiveness of the Functionalization Process on Cotton Color Strength Values (*K/S*).** A color strength test was performed using Coomassie brilliant blue reagent to assess the presence of the antimicrobial peptides, magainin I, and LL-37, in the modified-cotton samples and the *K/S* values were determined. Briefly, the process of staining the treated samples and their controls consisted of immersing the samples in 10 mL of reagent solution Coomassie Brilliant Blue G-250 at room temperature and under constant stirring for 15 minutes. Then, the samples were washed with distilled water to remove unbound peptides out of the fibers.

The color measurement of samples was performed by spectrophotometric readings in the spectrophotometer Spectraflash 110 from Datacolor at 595 nm. The ratio of light absorption to light

scattering at a given wavelength is proportional to the concentration of a dye in the sample.[20] The relationship is derived from the Kubelka–Munk eq. (1)[17]:

$$K/S = \frac{(1-R)^2}{2R} \quad (1)$$

Where $R$ is the reflectance; $K$ = light absorbed, and $S$ = light scattered. Also the relationship between $K/S$ and concentration of the dye is given by the formula[17](2):

$$K/S = kC \quad (2)$$

Where k = constant of proportionality; $C$ = concentration of colorant.[17] An elevated $K/S$ value indicates that more color reagent reacted with the peptides adsorbed in the cotton. Consequently, a high $K/S$ indicates that a higher concentration of AMPs is present in the cotton surface and reacts with the color reagent.

**Fourier Transform Infrared Spectroscopy (FT-IR).** The chemical composition of cotton, magainin I functionalized cotton (MagI-cotton), and LL-37 functionalized fabric (LL-37-cotton), were analyzed on FT-IR. Measurements were done with a Thermo-Nicolet is10 FTIR spectrophotometer. Each sample was scanned 64 times, with a spatial frequency resolution of 4 $cm^{-1}$.

### Efficacy of the Functionalized Material—Antibacterial Properties

The antibacterial effect of functionalized textile samples was tested according with the Japanese Industrial Standard, JIS L Standard 1902:2002.[18] The evaluation of the antimicrobial effect against *Staphylococcus aureus* and *Klebsiella pneumoniae* of the functionalized samples was performed by a suspension quantitative test, in which there was direct contact of the biomaterial with a suspension of bacterial cells. Control samples (without AMPs) and functionalized samples (with AMPs) were tested for its antimicrobial effect.

To test the antibacterial effect of the AMPs, the growth reduction rate was calculated based on the difference between the number of colony forming units on the control fabric and the functionalized fabric. Bacterial inoculums were prepared from an overnight Nutrient Broth suspension, incubated at 110 rpm at 37°C. The bacterial concentration was adjusted to 0.5 McFarland and the working bacterial suspensions were prepared to a final concentration of $1 \pm 0.3 \times 10^5$ colony-forming units/mL (CFU/mL). The square shapes of cotton samples with 0.4 g were placed in a sterile tube and inoculated with 200 $\mu$L working bacterial suspensions. Afterward, half of the samples were incubated for 18 hours at 37°C and the other half was immediately processed and analysed (designated as 0 hour samples). For the release of bacterial cells from the textile samples, before and after the 18 hours incubation period, 20 mL of 0.85% (w/v) NaCl solution with 0.20% (v/v) Tween-80 was added to the tubes and then stirred. The resulting suspensions were used to determine viable counts using serial dilutions prepared in sterile 0.85% (w/v) sodium chloride solution and plated in Nutrient Agar. The plates were incubated at 37°C for 24 hours, and the number of colonies was determined. This procedure was performed in triplicate.

The growth reduction rate of the bacteria was calculated using the eq. (3):

$$\frac{[\text{Control}]-[\text{Functionalized}]}{[\text{Control}]} \times 100\% = \%\ \text{growth reduction} \quad (3)$$

Where, [Control] is the CFU/mL of the control fabric (without the AMPs) and [Functionalized] is the CFU/mL of the functionalized fabric with the AMPs. The growth reduction was calculated for the initial time (0 h) and after 18 h of incubation between textiles and bacterial suspension.

## RESULTS AND DISCUSSION

### Minimal Inhibitory Concentration of the AMPs Against *Klebsiella pneumoniae* and *Staphylococcus aureus*

The minimal inhibitory concentration (MIC) of magainin I (MagI) and LL-37 against *S. aureus* (ATCC 6538) and *K. pneumoniae* (ATCC 11296) was determined using the broth microdilution method, adapted from the National Clinical and Laboratory Standard M7-A6.[13] The MIC against *K. pneumoniae* of magainin I was 4.17 μg/mL and against *S. aureus* was 0.20 μg/mL. The MIC of LL-37 against *K. pneumoniae* was 4.17 μg/mL and against *S. aureus* was 5.00 μg/mL.

In general, the minimum inhibitory concentration of magainins for various micro-organisms were typically in the range of 10–100 μg/mL.[19,20] Consequently, the literature MIC values were higher than those determined in this study.

According to Dürr et al. (2006),[21] LL-37 presented a minimal inhibitory concentration against *K. pneumoniae* of 4.20 μg/mL,[21] which was a very similar value to the one obtained in this study (4.17 μg/mL); and a MIC of > 32 μM[21] (144 μg/mL) against *S. aureus*, which was a much higher value than the one determined here (5.00 μg/mL). As stated by Turner et al. (1998)[22] the minimal inhibitory concentration of LL-37 against *S. aureus* in a standard Mueller–Hinton media was > 64 μg/mL that was also higher that the concentration value determined in this study, but was lower than the MIC by Dürr et al. (2006).[21]

### Cytotoxicity Assay

MTT viability assay was used to determine the AMPs cytotoxicity on normal human dermal fibroblasts (NHDF), as the textile material was intended to be in contact with the human skin. The results represented in Figure 2 are in terms of the viability percentage of the fibroblasts treated with two concentrations of the two AMPs tested, comparatively to the controls that had no contact with the tested agents.

When in contact with LL-37 at concentrations of 4.17 and 5.00 μg/mL, which were the concentrations chosen for the functionalization of the cotton gauzes, there was an increase in cellular viability of 23%, but in contrast a decrease of 6% in fibroblast viability was observed, when in contact with magainin I at concentrations of 0.20 and 4.17 μg/mL. These results mean that none of the magainin I and LL-37 concentrations caused cytotoxic effect in the normal human dermal fibroblasts, since according to Gouveia et al. (2011),[16] only an alteration above 30% in comparison with control is considered cell-toxic.[16] Consequently, these AMPs were considered safe to be applied as antimicrobial agents to contact with the human skin without

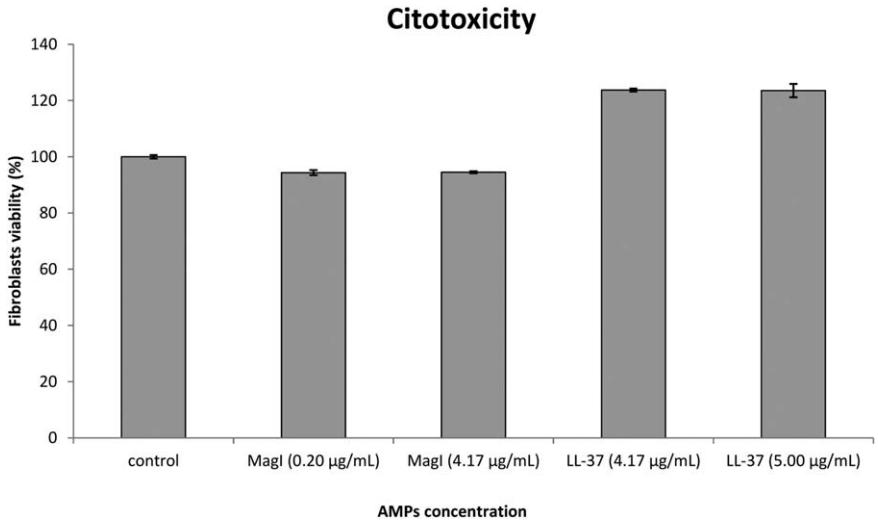


**Figure 2.** Fibroblasts viability percentage (mean ± SEM) when in contact with two concentrations of magainin I (MagI) and LL-37 and the untreated controls.

causing any cutaneous adverse reaction in the tested concentrations. The results were statistically significant for a *P*-value $<0.05$, according to a Student's *t*-test with a 95% confidence interval.

In accordance to this, previous studies reported that higher concentrations are required to cause cytotoxicity. For example, Matsuzaki (1998)[20] stated that more than 1 mg/mL (1000 $\mu$g/mL) was needed to lyse mammalian cells, so as expected no cytotoxic effect of magainin I against normal human dermal fibroblast, at the tested concentrations of 0.20 and 4.17 µg/mL, was observed.

*In vitro* cytotoxicity of LL-37 found concentrations of 13–25 $\mu$M (58.42–112.35 µg/mL) to be sufficient to make human leukocytes and T-cells nonviable.[21] Also, substantial lysis of red blood cells occurred at similar concentrations of LL-37.[21] Moreover, LL-37 can directly act on dermal fibroblasts and may have antifibrotic action during the wound repair process.[23] LL-37 inhibits collagen expression in fibroblasts and was associated and dependent on phosphorylation of extracellular signal-regulated kinase.[23] Murine NIH 3T3 fibroblasts numbers increased in a peptide concentration-dependent manner; the highest activity was achieved at 5 µM (22.47 µg/mL) LL-37.[24] At this peptide concentration the cell number increment was comparable to that induced by 10% fetal cattle serum.[24] LL-37 was ineffective at 10 µM (44.94 µg/mL) possibly due to a significant cytotoxicity.[24]

Additionally in healthy individuals, the antimicrobial function of LL-37 was effective at sites of its epithelial expression at a physiological concentration of approximately 2 µg/mL, which may increase two-to-threefold during infection.[25] This is in accordance with our results whereas no cytotoxic effect was observed at the tested concentrations of 4.17 and 5.00 µg/mL of LL-37. Thus, the concentrations settled at the higher MIC values found against both strains, for both peptides, can be used safely to give cotton gauzes the expected antibacterial effect.

**Effectiveness of the Functionalization Process on Cotton Color Strength Values (*K/S*).** A color strength test was performed using Coomassie brilliant blue reagent to assess the presence of the antimicrobial peptides, magainin I and LL-37, in the modified-cotton samples in order to give evidence of successfully attachment onto cotton fibers. The cotton-modified samples presented *K*/*S* values higher than the one observed in the non-functionalized sample meaning that the peptides are present even after five washing cycles. MagI and LL-37 adsorbed at the surface of the fabric activated with TEMPO-radical and presented a *K*/*S* value of 2.58 and 2.46, respectively, while the *K*/*S* of cotton was 1.78, a much lower value.

In another study, the *K*/*S* values for silk fibers were found to increase as a function of the number of deposited layers of anionic poly(methacrylic acid) capped silver nanoparticles (PMA-capAg) immobilized on silk fibers with cationic poly(diallyldimethylammonium chloride).[26] Comparing with our results, the *K*/*S* value also increased due to the AMP functionalization.

**Fourier Transform Infrared Spectroscopy (FT-IR).** The infrared spectrum of unmodified cotton and the functionalized fabrics are shown in Figure 3. The IR spectrum of unmodified cotton presents the expected characteristic peaks at 3330 $cm^{-1}$ due to O—H stretching vibrations, and at 1335 $cm^{-1}$ associated with O—H deformation vibrations. The peaks associated to the $\beta$(1–4) glycoside bridge came out at 1159 and 897 $cm^{-1}$, and the C—O—C stretching vibration peak in the pyranose ring at 1030 $cm^{-1}$.[27]

Structural changes on cotton after functionalization were also assessed by FT-IR after the 5 washing cycles and 7 months of storage at room temperature. On cotton samples functionalized with LL-37 and Magainin I, the presence of these AMPs was confirmed by the observed peaks of the amides, namely at 1590 $cm^{-1}$ due to N—H bending of primary amines, as well as a peak at 1382 $cm^{-1}$ associated with —$CH_3$ symmetrical angular deformation.[27] LL-37 was used in higher concentration (5 ug/

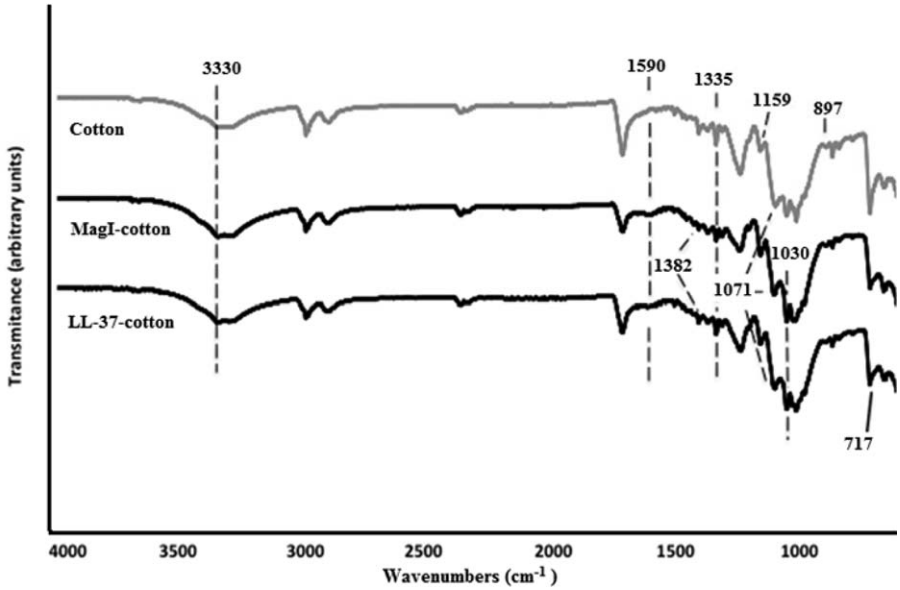


**Figure 3.** IR spectra of unmodified and modified cotton with LL-37 and magainin I (MagI).

mL), and presents higher number of amino acids in its structure (33), higher number of aromatic rings from its Phe amino-acids (4), and higher net charge (+6), when comparing to MagI, which was in lower concentration (4.17 ug/mL) and has a lower number of aminoacids (23), from which only 3 are Phe, as well as lower net charge (+4). Hence, it was possible to confirm increased peaks of LL-37-cotton corresponding to C—N stretching of amines and C—H in plane bending of aromatic rings at 1071 $cm^{-1}$, C—N stretching of aliphatic amines at 1030 $cm^{-1}$, and C—H bending of aliphatic as well as C—H bending of aromatic rings at 717 $cm^{-1}$.[27]

This IR spectrum suggests an explanation for the increased microbial inhibition potency of LL-37 against *K. pneumoniae* when comparing to MagI, based on its amino acid net charge. In addition, since the FT-IR analysis was performed after 7 months of functionalization, one can conclude that this new process is durable and stable to storage, along with efficacy regarding the antibacterial effect.

### Efficacy of the Functionalized Material—Antibacterial Properties

The antibacterial effect of functionalized textile samples was tested by a suspension quantitative test, in which the biomaterial contacted directly with a bacterial suspension. The percentage of bacterial reduction was determined by comparing the functionalized samples (with AMPs) with untreated control samples (without AMPs) as shown in Table I.

At 0 hours of incubation, no reduction of *K. pneumoniae* growth was observed for both magainin I functionalized cotton and LL-37 functionalized fabric, as these biomaterials require time to act against *K. pneumoniae*. MagI-cotton, after 18 hours of incubation with a *K. pneumoniae* suspension revealed a microbial reduction of 15%, while LL-37-cotton presented a bacterial reduction of 89%. Therefore, after 18 hours of incubation, LL-37-cotton presented a much higher reduction percentage of this Gram-negative micro-organism, comparatively to MagI-cotton.

**Table I.** Percentages of Bacterial Reduction at 0 Hours and 18 Hours of Incubation Between Fabric and Bacteria

| | 0 hours of incubation | | | 24 hours of incubation | | |
|---|---|---|---|---|---|---|
| *K. pneumoniae* CFU/mL | Average of CFU/mL | Standard deviation | % of Bacterial reduction | Average of CFU/mL | Standard deviation | % of Bacterial reduction |
| Cotton | $1.55 \times 10^3$ | 0.00 | | $3.30 \times 10^7$ | $1.41 \times 10^6$ | |
| Magainin I | $1.95 \times 10^3$ | $6.36 \times 10^2$ | - | $2.80 \times 10^7$ | $7.07 \times 10^5$ | 15 |
| LL-37 | $2.23 \times 10^3$ | $1.10 \times 10^3$ | - | $3.63 \times 10^6$ | $3.54 \times 10^4$ | 89 |
| *S. aureus* CFU/mL | Average of CFU/mL | Standard deviation | % of Bacterial reduction | Average of CFU/mL | Standard deviation | % of Bacterial reduction |
| Cotton | $9.80 \times 10^3$ | $4.95 \times 10^2$ | | $3.53 \times 10^7$ | $2.93 \times 10^7$ | |
| Magainin I | $8.23 \times 10^3$ | $2.47 \times 10^2$ | 16 | $1.49 \times 10^7$ | $1.20 \times 10^6$ | 58 |
| LL-37 | $8.98 \times 10^3$ | $3.18 \times 10^2$ | 8.0 | $1.46 \times 10^7$ | $1.41 \times 10^6$ | 59 |

As stated by Roy (2009),[28] cationic antimicrobial peptides, like magainin I and LL-37, target bacteria because of the high negative charge of the bacterial cell envelope, which is due, in part, to the elevated content of phospholipids, leading to the formation of pore in the microbial membrane. LL-37 (H-Leu-Leu-Gly-Asp-Phe-Phe-Arg-Lys-Ser-Lys-Glu-Lys-Ile-Gly-Lys-Glu-Phe-Lys-Agr-Ile-Val-Gln-Arg-Ile-Lys-Asp-Phe-Leu-Arg-Asn-Leu-Val-Pro-Arg-Thr-Glu-Ser-OH) is a positively charged molecule (+6 at pH ~7.4) with a high content of basic and hydrophobic amino acids.[25] Since LL-37 has a positive net charge of +6, there might have been a more prominent and effective action against the Gram-negative *K. pneumoniae* as a higher number of electrostatic interactions can occur between the negatively charged membrane of *K. pneumoniae* and the cotton-grafted LL-37 (+6). In addition, according to Smeianov et al., (2000),[29] LL-37 shows a "preference" in its activity toward Gram-negative bacteria.

Magainin I (H-Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Gly-Lys-Phe-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile-Met-Lys-Ser-OH) has a positive net charge (+4) at a neutral pH level and has hydrophobic residues that are essential for its antimicrobial activity.[11] The anti-*K. pneumoniae* activity of MagI-cotton, was much lower in comparison with LL-37-cotton, as MagI has a net charge of +4 and for that reason there might be established fewer electrostatic interactions between the positive charges born by the lysine[30] chains of magainin I and the negatively charged bacterial cells.

At 0 hours of incubation, the reduction percentages against *Staphylococcus aureus* for MagI and LL-37 functionalized fabrics were 16 and 8%, respectively. The *Staphylococcus aureus* reduction percentages of the cotton functionalized with MagI and LL-37, after 18 hours of incubation, were respectively 58 and 59%. At 0 hours of incubation there was a very small percentage of *S. aureus* reduction, which was expected since there was no period of contact between the functional materials and the bacterial suspension. After 18 hours of incubation with *S. aureus*, MagI and LL-37 led to a bacterial reduction of about 59%, which is advantageous for a wound-dressing application, as it decreases the *S. aureus* in the wound bed until the next dressing change. A decrease of about 59% of *S. aureus* is highly desirable for an application as wound-dressing, comparatively to the unmodified cotton, especially since the antibacterial finishing agents (AMPs) showed no evidence of cytotoxicity against NHDF, thus being safe to use.

Dubas et al., (2006)[26] described a layer-by-layer assembly used to immobilize anionic poly(methacrylic acid) capped silver nanoparticles (PMAcapAg) on silk fibers with cationic poly(-diallyldimethylammonium chloride) (PDADMAC), to obtain an antibacterial effect against *S. aureus*.[26] The functionalized silk had a 41% of bacterial reduction for 10 layers of coating.[26] Our findings revealed a higher reduction percentage with LL-37-cotton and MagI-cotton, against *S. aureus* (aprox. 59%) with the extra advantages of using natural antimicrobial peptides at very low concentrations, which proved to be effective against the micro-organisms in study and non-cytotoxic. Moreover, MagI-cotton and LL-37-cotton production posed no environmental constrains that are frequently associated with the disposal of toxic metallic effluents resulting from the textile functionalization process.

## CONCLUSION

With this work, the immobilization of magainin I and LL-37 onto cotton fibers was attempted and their antibacterial effect was evaluated in order to determine the potential application of these biomaterials as wound-dressings.

Magainin I and LL-37 didn't cause any cytotoxic effect in the normal human dermal fibroblasts. Consequently, these AMPs were considered safe to be applied as antimicrobial agents to contact with the human skin without causing any cutaneous adverse reaction at the MIC values.

Regarding the effectiveness of the functionalization process on cotton both MagI and LL-37 peptides were effectively adsorbed at the surface of the fabric activated with TEMPO-radical, as shown by the *K*/*S* values and the FT-IR spectrum, even after five washing cycles and a long storage time.

LL-37-cotton seems to be the best choice for the development of wound-dressings, since it presented a higher bacterial reduction percentage against *K. pneumoniae* and *S. aureus* when compared with MagI-cotton.

The use of AMPs as a novel method to give antibacterial properties to cotton fibers is a novel and successful strategy and allowed to firstly report the use of antimicrobial peptides for cellulosic fibers such as cotton gauzes, an emerging antibiotics universe especially effective against resistant bacteria. In addition, the new processes have shown effectiveness without cytotoxicity, which is a major problem on the design of new antimicrobial finishing processes for textiles and wound-dressings.

## ACKNOWLEDGMENTS

The authors thank Fundação para a Ciência e Tecnologia (FCT) for the funding granted concerning the project - PTDC/EBB-BIO/113671/2009 (FCOMP-01-0124-FEDER-014752) Skin2Tex. Also we thank Fundo Europeu de Desenvolvimento Regional (FEDER) through COMPETE – Programa Operacional Factores de Competitividade (POFC) for the co-funding.

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